# THE INFLUENCE OF ENVIRONMENTAL HYPOXIA IN THE PHYSIOLOGICAL RESPONSES OF LABORATORY RATS AND MICE DURING POSTNATAL LIFE AND ADULTHOOD

Thèse

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# RÉSUMÉ


Il existe chez les différentes espèces de rongeurs une importante variabilité dans les capacités à établir des colonies stables en haute altitude (HA). Par exemple, on trouve des souris (*Mus*) jusqu'à 4000m alors qu'il n'y a pas de rats (*Rattus*).

La capacité des animaux à survivre et réaliser des activités physiques en HA dépend d'adaptations biologiques physiologiques (plasticité phénotypique) et génétiques ou épigénétiques. Des rats Sprague Dawley (SD) maintenus en HA dans des conditions de laboratoire survivent pendant plusieurs générations (La Paz, Bolivia – 3600m) mais présentent des signes de maladaptations physiologiques (érythrocytose excessive, hypertrophie ventriculaire droite – signe d'hypertension artérielle pulmonaire – et altération des structures alvéolaires avec élargissements des espaces pulmonaires). Ces réponses sont principalement liées à une hypersensibilité au niveau d'oxygène ($O_2$) ambient au cours de la période postnatale et élever les rats de HA à une pression d'$O_2$ reproduisant celle du niveau de la mer (NM) au cours de cette période améliore significativement leur adaptation physiologique[1,2]. Actuellement, aucune adaptation génétique n'a été mise en évidence chez des souris (*Mus musculus*) sauvages capturées en HA. Notre hypothèse générale est que les souris possèdent des caractéristiques physiologiques spécifiques qui assurent leur survie en HA.

Pour répondre à cette hypothèse, nous avons réalisé 4 études comparant les réponses physiologiques (ventilation, métabolisme, hématologie, saturation artérielle en $O_2$ et rythme cardiaque) entre des souris FVB et des rats SD élevés au NM (Québec, Canada) ou en HA (La Paz, Bolivie – 3600m).

Nos principaux résultats démontrent que, par rapport aux rats, les souris adultes de HA présentent une surface alvéolaire augmentée associée avec une meilleure extraction d'$O_2$ sans augmentation excessive de l'érythrocytose ni hypertrophie ventriculaire. Au NM, en conditions ambiantes, les deux espèces présentent des réponses physiologiques similaires. Par contre, après 6h d'exposition en hypoxie (12% d'$O_2$), par rapport aux rats, les souris augmentent leur ventilation minute et diminuent leur métabolisme. Les souris augmentent également l'expression de l'hypoxia inducible factor 1$\alpha$ (HIF-1$\alpha$ – molécule principale de régulation des réponses cellulaires en hypoxie) dans le tronc cérébral après 6h d'hypoxie (15% d'$O_2$) ; cet effet n'est pas présent chez les rats.

Au NM, l'hypoxie postnatale induit une augmentation du volume pulmonaire et de la réponse ventilatoire à l'hypoxie chez les souris mais pas chez les rats. Cependant, chez les jeunes rats de HA, l'architecture pulmonaire est préservée comparée aux rats exposés en hypoxie postnatale au NM.

En conclusion, les rats vivant en HA depuis plusieurs générations présentent des stratégies physiologiques pour faire face au manque d'$O_2$ ambient leur permettant de survivre dans des conditions de laboratoire mais qui ne sont pas suffisantes pour assurer leur survie en milieu sauvage. Nos résultats confirment également que les souris possèdent des prédispositions physiologiques permettant la survie en altitude.

# ABSTRACT

Different rodent species present divergent abilities to colonize and establish stable colonies at high altitude (HA). Ecological studies show that mice (*Mus*) can be found at HA (up to 4000m) while rats (*Rattus*) are absent.

The ability of an animal to survive and do physical activities at HA depends upon biological adaptations that can include physiological (phenotypical plasticity) and genetic, or epigenetic modifications. Adult Sprague Dawley (SD) rats can live under laboratory conditions at HA for several generations (La Paz, Bolivia – 3600m), but they display signs of physiological maladaptation such as excessive erythrocytosis, right ventricular hypertrophy (a sign of pulmonary hypertension) and altered alveolar structure with enlarged airspace in the lungs. These responses are mainly linked to an excessive sensibility to the oxygen ($O_2$) ambient level during postnatal life. Indeed, raising the HA rats under sea level (SL) $O_2$ pressure during early postnatal life significantly improved the physiological adaptation[1,2]. Furthermore, in HA wild mice (*Mus musculus*) living at HA, there is no signs of genetic adaptation to this environment. Accordingly, our general hypothesis is that mice possess specific physiological traits ensuring survival at HA.

To assess this hypothesis, we conducted 4 studies to compare physiological responses (including ventilation, metabolic rate, hematology, lung morphology, arterial $O_2$ saturation and heart rate) between FVB mice and SD rats raised at SL (Quebec, Canada) or HA (La Paz, Bolivia – 3600m).

Our main results show that compared with rats, HA adult mice display enhanced alveolar surface area associated with increased $O_2$ extraction, and avoid excessive erythrocytosis and right ventricular hypertrophy. At SL, under ambient conditions, mice and rats display similar physiological variables. However, after 6 hours of sustained hypoxia (12% $O_2$), mice have higher minute ventilation and lower metabolic rate than rats. Mice also had an increased expression of the hypoxia inducible factor 1$\alpha$ (HIF-1$\alpha$ – the principal mediator of the cellular responses in hypoxia) in the brainstem after 6 hours of hypoxia (15% $O_2$), while this response was not observed in rats.

Hypoxic exposure during postnatal life at SL increased the lung volume and the hypoxic ventilatory response in mice but not rats. However, young HA rats preserve their lung architecture compared with young SL rats exposed to postnatal hypoxia.

We conclude that rats living at HA for several generations display physiological strategies to cope with the ambient hypoxia that allow them to survive in laboratory conditions but are not sufficient to establish stables colonies in the wild. Also, our results confirm that mice are predisposed to withstand hypoxic environment.

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| | |
|---|---|
| ATP | adenosine triphosphate |
| CB | carotid bodies |
| CMS | chronic mountain sickness |
| CNS | central nervous system |
| $CO_2$ | carbon dioxide |
| CSN | carotid sinus nerve |
| DTT | dithiothreitol |
| EE | excessive erythrocytosis |
| EPO | erythropoietin |
| $FO_2$ | oxygen fraction |
| fR | respiratory frequency |
| GABI | the great American biotic interchanges |
| HA | high altitude |
| Hb | hemoglobin |
| [Hb] | hemoglobin concentration |
| Hb-$O_2$ | oxyhemoglobin |
| Hct | hematocrit level |
| HD | hypoxic desensitization |
| HIF | hypoxia inducible factor |
| $H_2O$ | water |
| HVA | hypoxic ventilatory acclimatization |
| HVR | hypoxic ventilatory response |
| IBBA | Bolivian Institute of High Altitude Biology |
| $L_m$ | mean linear intercept |
| LV+S | left ventricle + septum |
| Ma | millions of years ago |
| mmHg | millimeters of mercury |
| NM | neuromodulators |
| NT | neurotransmitters |

| | |
|---|---|
| NTS | nucleus tractus solitarus |
| P4,P14,P15 | 4, 14, 15 days postnatally |
| PFA | paraformaldehyde |
| $PCO_2$ | carbon dioxide partial pressure |
| $P_{O2,sat}$ | arterial oxygen saturation |
| $PO_2$ | oxygen partial pressure |
| $PiO_2$ | inspired oxygen partial pressure |
| ROS | reactive oxygen species |
| RV | right ventricle |
| SL | sea level |
| $V_E$ | minute ventilation |
| $\dot{V}_{O_2}$ | oxygen consumption per minute |
| $\dot{V}_{CO_2}$ | carbon dioxide production per minute |
| $V_T$ | tidal volume |

À mon Papé et ma Nanna.

"I have no special talent; I am only passionately curious." Einstein 1952

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# PREFACE

Chapter 2, p69 corresponds to my first article published as first author. The experiments were conducted at the Bolivian Institute of High Altitude Biology (Instituto Boliviano de Biologa de Altura – IBBA) in Bolivia, in the city of La Paz at 3600m of altitude. This paper was published in April 2015 in the *Journal of Experimental Biology*.

I would like to thanks Lic. Gabriella Villalpando, Dr. Marcelino Gonzales and Dr. Ibana Valverde who helped me conducting my experiments in Bolivia and Dr. Rudy Soria who gave me unrestrained access to all the facilities I needed at the IBBA.

My Director, Dr. Vincent Joseph, the director of the physiological department at the IBBA, Dr Rudy Soria, Dr. Marcelino Gonzales and myself developed the concepts and designed the experiments.

Lic. Gabriella Villalpando and Dr. Marcelino Gonzales helped me collecting the physiological datas of the experiments, (plethysmography reccordings, tissue sampling) and Dr. Ibana Valverde included all the samples at her laboratory after manual embedding in paraffin at the IBBA because the IBBA was not equipped for this process. I conducted all the histological processes alone in Quebec City.

Later, I analyzed the data, prepared the figures and wrote the manuscript with the help of my director Dr. Vincent Joseph.

# I. CHAPTER 1

## GENERAL INTRODUCTION

# 1. Introduction

## 1.1. Altitude and atmospheric pressure

### 1.1.1. Definition of altitude

According to the Collins dictionary, altitude can be defined as: "the vertical height of an object above some chosen level, especially above sea level" – SL.

Then, the regions of the earth's surface that are high above SL can be divided into categories depending to the height: low altitude (below 1000m above SL), high altitude (HA – between 1500-3500m (m) above SL), very high altitude (between 3500-5500m above SL) and extreme altitude (above 5500m above SL)[3]. Despite this recent quite thorough altitude division used in medicine, scientists that have studied the biological consequences of altitude for centuries often draw a simpler line that just divide altitude environment into low- and high-altitude. The line was set at 2500m for quite some times now as it was the "limit" where most people experienced "high-altitude effects"[4,5] and this is the line that I am going to use when I speak about low- and high-altitude environment here.

### 1.1.2. Consequences of the earth elevation on the atmospheric pressure

Atmospheric air is composed of several gases that expand in all directions and have a weight, meaning that the air exerts a pressure upon all bodies with which it enters in contact. Because air has a mass, Earth gravity attracts it and gives it weight. Because it has a weight,

and the air molecules constantly bump into things, it exerts pressure. The amount of pressure exerted by atmospheric air upon an object is called the atmospheric pressure. It depends of the amount and the weight of atmospheric air lying above the objects and pressing upon them. As you go up, air pressure goes down because the higher you go, the less air there is pressing down on you. So, the atmospheric air pressure and density diminishes as an object gets closer to the exosphere[1] (upper limit of the atmosphere), and with the elevation of the earth ground, one can observe a diminution of the atmospheric pressure.

We had to wait until the 17$^{th}$ century for the discovery of the existence of the atmospheric pressure. Evangelista Torricelli (1608-1647) an Italian physicist and mathematician was one of the first to discover that air actually had a weight and invented the first mercury barometer in 1643 (Figure I.1.). The first mercury barometer was a device that measured the "weight" of air by displacing a U-shaped column of mercury open to the atmosphere on one side and closed on the other side (the closed side being emptied of air). The first experiment that used the reduction of the height of a column of mercury and associated it with the altitude of the earth (to demonstrate the changes in the atmospheric pressure) was conducted by Blaise Pascal (1623-1662) in 1648. It is called the "Puy-de-Dôme" experiment and Blaise Pascal made his brother-in-law Pascal Perier measure the diminution of pressure between the city of Clermont-Ferrand and the summit of the Puy-de-Dôme (an extinct volcano at 1464m in the "Massif Central" old-mountain range in the center of France)[6].

[1] The exosphere is the highest region of the atmosphere where the air density is so low that a fast moving air particle will have more than 50% chance to escape the atmosphere than hitting other molecules.

**Figure I.1: Torricelli creating the mercury barometer. Camille Flammarion engraving, 1923.**

Then, scientists measured that the weight of the column of air extending from the Earth surface at SL to the exosphere would be equal in weight or pressure to a corresponding column of mercury of 760 millimeters high, and normal SL atmospheric pressure was stated at 760 millimeters of mercury (mmHg)[7].

But what constitutes the atmospheric air? Air is a gas mixture composed of 21% of dioxygen ($O_2$), 79% of Nitrogen, 0,9% of argon, 0,04% of carbon dioxide ($CO_2$), 0,002% of neon, 0,0005% of helium

and 0,0002% of methane. In this gas mixture, the partial pressure of any of the gases represents the percentage of the gas in the atmospheric pressure[7]: if we take the $O_2$ gas for example, at SL, the $O_2$ fraction in the atmospheric air ($FO_2$) is 20,9%. Knowing this allows us to calculate, using the atmospheric pressure, the partial pressure of $O_2$ ($PO_2$) in the air at SL:

Atmospheric $PO_2$ = 21 percent of 760 mmHg = 159,6 mmHg

Then, at 3600m, for instance in the City of La Paz in Bolivia, the atmospheric pressure drop at 490 mmHg (Figure I.2), the $FO_2$ is still 20,9% but the atmospheric $PO_2$ would be 21% of 490 mmHg = 103 mmHg.

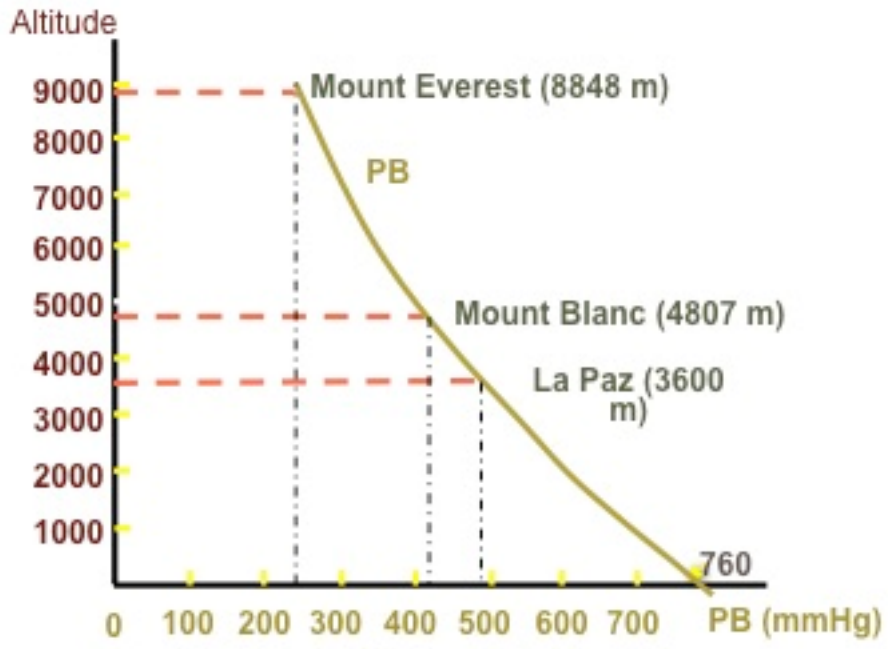


**Figure I.2 Evolution of the barometric pressure (PB) with the altitude elevation of the earth.**

## 1.2. Life at high altitude

Around the earth, HA regions are found in all continents, as examples there is the Himalayas in Asia, the Rockies and the Andes in America, the Alps and Pyrenees in Europe, the Southern Alps in Oceania and the Ethiopian massif in Africa (Figure I.3). Ecological paleontology showed that most of the current HA human and animal species evolve in parallel with the rise of the mountain. Paleo-ecological reports in the 3 HA plateaus that are the Andean, Tibetan and Ethiopian plateaus demonstrate interesting insights of this concomitant evolution.

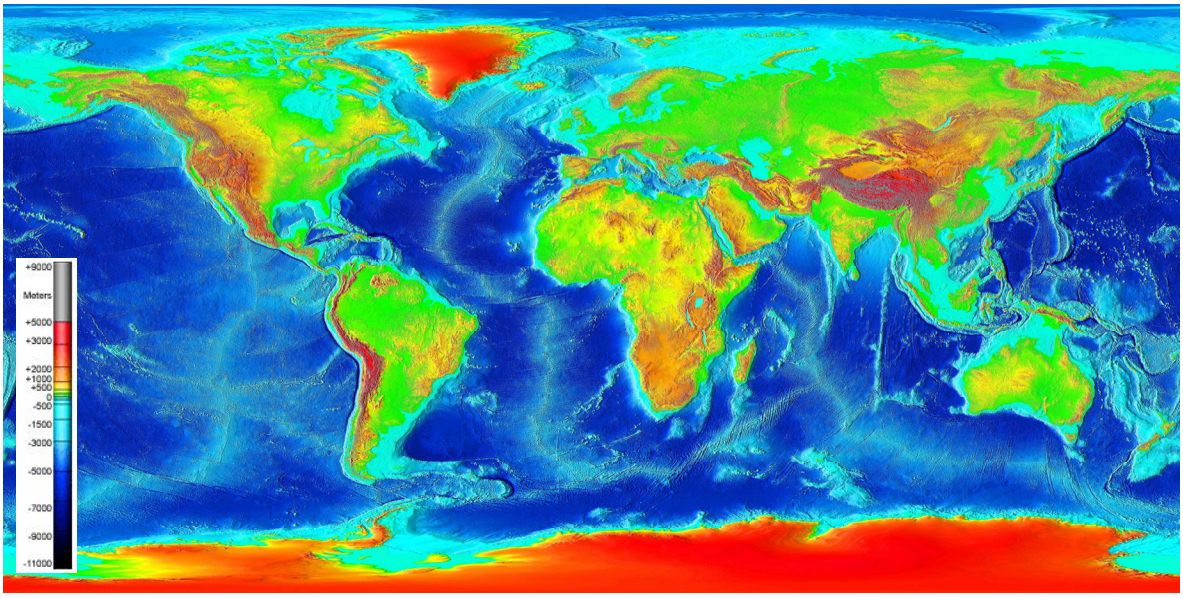


**Figure I.3: Topographical world map representing the global land and undersea elevation.**

*Source: https://commons.wikimedia.org*

### 1.2.1. Paleo-ecological reports of mammals and birds at altitude[2]

Paleoecology, landscape ecology and molecular phylogenies showed that vertebrates have been inhabiting montane[3] regions for more than 20 million years[8].

***The Andean Plateau rise and paleo-ecology***

The Andean Cordillera that extends for over 5000 km along the western coast of South America constitutes the largest mountain chain in direct connection with a tropical rainforest. The uplift of the Andes began at the end of the Cretaceous (65 million years ago – Megaannum, Ma)[9], and took place in episodic bursts (the most intense peaks were during the late middle Miocene ~12 Ma and early Pliocene ~4.5 Ma[10,11]. Molecular phylogeny studies showed that the first vertebrates to colonize HA regions was an ovenbird, belonging to the Tarphonomus Berlepschia clade, 21.8 Ma[12]. As for rodents, they were present even before the high Andes were formed, and fossils of the guinea pig (*Cavia porcellus*) ancestors, caviomorphs, dated from 35 Ma were found in the Tinguiririca region[4] [13]. Later, the accelerated uplift phases during the last 10 Ma resulted into easing the access to HA regions to plants, birds, insects, and some rodents[8]. Paleo-ecological studies of late Pleistocene glacial and fluvial deposits at the Tarija Basin (situated at 1854m in Bolivia) implied that the Bolivian Altiplano sustained an ice cap and that

[2] To ease the understanding of this part, a timescale is presented in Appendix 1.

[3] The montane ecology studies the life system in mountains or other high elevation regions of the earth. Here the montane region refers to elevational zone from 1500-3000m.

[4] The fossils of the "tinguiririca fauna" were entombed in volcanic mudflow and ash layers during the Oligocene and were discovered in the Tinguiririca river situated high in the Andes of central Chile. They give very good information regarding the history of South America endemic fauna before its reunion with the North America.

the faunal composition of the Neogene [5] period results from geographical, ecological and climatic factors. Another important component responsible for the actual HA biotope in the Andes is the extensive migration of the taxa from North America during the Great American Biotic Interchange (GABI)[6] [14] during the late Pliocene ~3 Ma. Alfred Russel Wallace (1823-1913), the father of biogeography, was the first to discuss the occurrence of GABI in 1876 in his books *The Geographical Distribution of Animals. With a Study of the Relations of Living and Extinct Faunas as Elucidating the Past Changes of the Earth's Surface*[15].

### *The Tibetan Plateau rise and paleo-ecology*

The history of the elevation of Tibet, the world's highest plateau, offers ideas concerning the geodynamics of continental collision as it is stated that the Himalayas were created when Eurasia collapsed with India in the middle Cenozoic ~50 Ma. Nevertheless, some studies raised the possibility of an elevated topography prior to continental collision[16] and if records suggest that the southern Tibet has been high since at least 26 Ma[17], estimates from northern Tibet and older rocks are not definitive[18]. During the course of the uplift, the retreat of forests from the interior to the southeastern marge lead to the migration of animals, from the higher to the lower elevations of the mountains causing a faunal composition divergence between the east and the west regions of the plateau. Several studies seem to point out that the major phase of the uplift (~15-8 Ma) coincides with important environmental and climate changes that were responsible for ecosystem modifications and

[5] The Neogene is a geologic timescale extending from 23,03 to 2,58 Ma and subdivided into the early Miocene and the late Pliocene.
[6] During most of the Cenozoic, South America was an island continent with an endemic mammalian fauna. This isolation ceased during the late Neogene after the formation of the Isthmus of Panama, resulting in an event known as the Great American Biotic Interchange (GABI).

ecological diversification in the colonization of the Tibetan plateau[19-22]. About the vertebrate colonization, little is known, nevertheless, studies showed that the pika, a lagomorph that regroups several typically HA species, was living in the north edge of the Qinghai-Tibetan plateau ~37 million years ago suggesting that it evolved in concomitance with the uplift of the mountain.

### *The Ethiopian Plateau rise and paleo-ecology*

The Ethiopian Plateau is situated in the Horn region of the northeast Africa from the central to northern Ethiopia, its northernmost portion reaching the Eritrea. Called the Ethiopian Highlands or sometimes the roof of Africa, the Ethiopian Plateau is a rugged mass of mountains with little surface below 1500m and summits that can reach 4550m. The plateau is located upon tectonic plates and its great height is the result of an intrusion of lava in Tertiary times whereas its net rock uplift has been 2200m since 150 Ma[23]. The rift which splits the Ethiopian plateau into the main western and smaller southeastern sections began to open in the Miocene, 13-12 Ma, and was fully formed by the early Pliocene, 5-4.5 Ma[24]. Yalden and Largen in a review called *the endemic mammals of Ethiopia* regrouped a wide range of discovery of mammals fossils (like equids, bovids but also rodents) dating back to the Pleistocene and the Pliocene times that were discovered at 2000-2300m in the late 70's and early 80's[25]. Later, mammals and plants fossils from the late Oligocene (~23 Ma) were discovered in the Chilga region[7]. These authors pointed out that the indigenous fauna in Ethiopian region at this time might have been the result of a faunal exchange between Afro-Arabia and Eurasia 27 Ma, and that in all mountains regions,

[7] The Chilga region is located in the Ethiopia's northwestern plateau at an elevation of about 1,950 m[26].

vertebrate remains are usually fragmentary and represent medium to large-sized herbivores; a possible reason why small mammals are less found may be due to a diagenesis[8] of the bone before fossilization[26].

It is important to keep in mind that because the uplift responsible for the creation of the very high summits of the mountains in each plateaus is often quite recent (12-4.5 Ma for the Andes, 15-8 Ma for the Tibetan Plateau and 13-4.5 Ma in Ethiopia) the fossils sites dating earlier than 15 Ma currently lying at elevation between 3000-5000m were deposited at significantly lower elevation (Figure I.4)[29]. So, even if mammals or birds were present in mountain region 20 Ma, these animals came from migrations of lowlander species, and, adaptation to the HA (over 4000m), is a quite recent phenomenon that occurred in fact possibly less than 1.9 Ma[14,30].

[8] The diagenesis is the cumulative of physical, chemical and biological environment that will modify an organic object's original chemical and/or structural properties and govern its ultimate fate in terms of preservation or destruction[27;28].

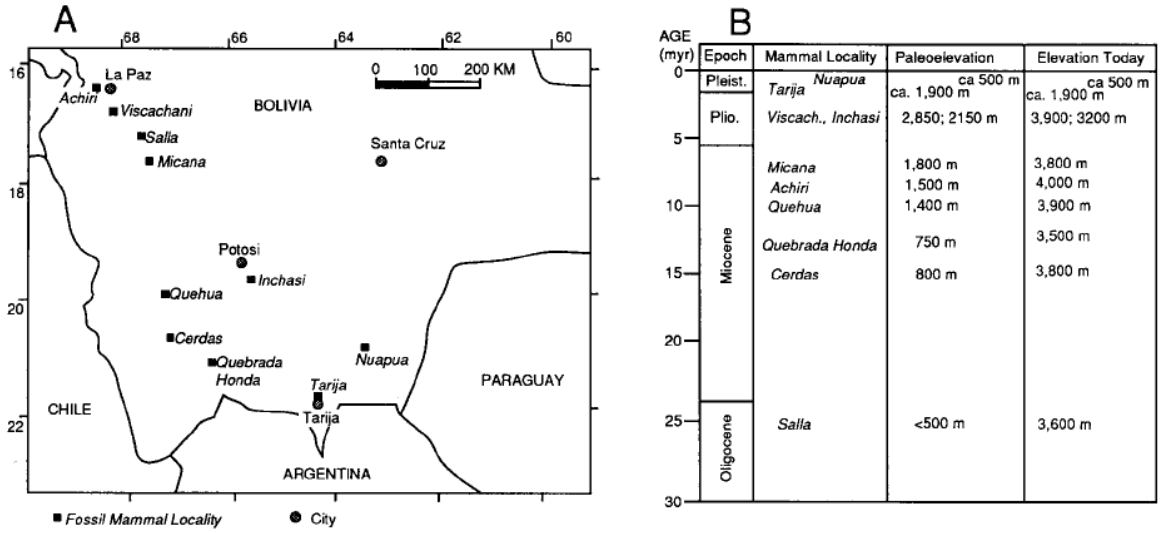


**Figure I.4: (A) Map of Bolivia showing localities where mammals fossils were found. (B) Temporal sequence, calculated paleoelevations at time of deposition and modern elevations of the fossil mammal localities.**
*Adapted from MacFadden et al., South American fossil mammals and carbon isotopes: a 25 million-year sequence from the Bolivian Andes, 1994[29].*

### 1.2.2. Today's ecological report of mammals' distribution at high altitude

It is amazing to notice that today in ecological reports, some of our home pet such as cats (*Felis catus*) or rats (*Rattus norvegicus*) are absolutely absent of HA regions whereas others such as mice (*Mus musculus*), chinchilla (a cross-breeding of the two taxa *Chinchilla laniegera* and *C. brevicaudata*) and dogs (*Canis lupus*) are found at altitude that can reach 5000m[31-35].

Today's ecological fauna at HA regroups land and flying animals. If you decide to climb a mountain, you might encounter rodents, carnivores, birds, camelidae and bovidae. Nevertheless, a distinction needs to be made between endemic species that have been evolving

almost since 2 Ma with the continental drift and the uplift of the Earth, and the species that have migrated more recently, including the ones who followed the different waves of human colonization at HA in North and South America. Some of the species introduced by Europeans stayed intolerant to life at altitude whereas other developed tolerance to hypoxia. It is also curious to notice that some endemic species are alpine obligate (meaning that they depend on mountaintop ecosystem) and that some imported species were not able to colonize HA environment.

### *HA endemic species*

In North America, the deer mice (*Peromyscus maniculatus*) have the greatest altitudinal range as they are continuously distributed from SL to elevation above 4300m and are one of the most studied species to understand HA genetic adaptation and evolution. One lagomorph species, the *Ochotonids* (more commonly known as the pikas), regroups several sub-species, some are typical HA habitants whereas others can be found at a various altitude from SL to HA. *Ochotonids* came from an ancient group of mammals originating from Asia in the Oligocene that migrated to North America through 3 major waves: beginning in the early Miocene, then during the Miocene-Pliocene boundaries and finally in the early Pleistocene. Fossils show that the American pika has been living at altitude since the middle Pleistocene. The distribution of the first pikas was high and low altitude and it is likely that their restriction to montane environment is linked to a climate change (mostly a shift toward warmer and drier conditions) that occurred in the Holocene (10 000 years ago)[36]. Their current geographic diversity is concentrated in Asia where 28 species exist, for only 1 species in Europe, and 2 species

in North America. In the Tibetan Plateau, the plateau pika (*Ochotona curzoniae*) and the ili pika (*Ochotona iliensis*) are alpinate obligate and have a distribution range between 3200-5300m and 2800-3300m respectively whereas in North America, their "cousin" (*Ochotona princeps*) exists from SL to 3000m (http://www.wildlifeinformation.org/). In the Andes, the chinchilla, although almost extinct today in it's wild habitat (because of the extensive hunt for it's fur in the XVIII$^{th}$ century), showed 2 species, one found at low altitude the *Chinchilla laniegera* (SL to 1500m) and one found at HA (3500 to 4500m) the *Chinchilla brevicaudata*[35,37]. Aside from the rodents, in the Andean Cordillera you can also found camelids such as the vicugnas (*Lama vicugna*) or the alpaca (*Lama pacos*), small carnivores such as foxes (*Dusicyon culpaeus* and *D. sechurae*) and larger ones such as the puma (*Felis concolor)*[32]. In the Himalayas and the Tibetan Plateau, bovidae such as the yak (*Bos grunniens* and *B. mutus*) are living between 3000-5500m[38]. As for the birds, more than 20 species are living at HA and some breed at altitudes extending up to 4900 and 6500m in the Andes and Himalayas respectively[39]. A bird worth quoting is the Bar-headed goose (*Anser indicus*) as it is a well-studied species that breed in the Tibetan Plateau and has a road migration that can lead them to fly over the Himalayas at altitude up to 9000m[40,41]. So as you can see, HA ecology is quite diverse and natural HA environment is well furnished in term of species diversity.

### *HA species imported during the Spanish conquest*

In South America, some of the species that are currently inhabiting the Andean Cordillera such as today's chicken (*Gallus gallus*), mice (*Mus musculus*), cattle – such as cows (*Bos taurus*), sheep (*Ovis*

*aries*), goats (*Capra aegagrus hircus*) – or dogs (*Canis lupus familiaris*) are the descendants of introduced domestics animals that were imported either on purpose (for food or work purposes) or as clandestine passengers (mice) on the ships of the Spanish conquistadores. Indeed, in South America, all the bovidae living today in HA environment are descendants of introduced domestic animals[32].

***What about human repartition at HA?***

In the 3 Plateaus, in parallel with animal evolution, humans also evolved and colonized HA environment for millennia. Paleoecology sites often were human occupations sites where butchered animal fossils also laid. In the Ethiopian Plateau, hominid sites dating back to the middle Pleistocene described mammal fossils close to human remains at altitudes above 2300m[25]. In the Andes, before the European arrival, the Incas and pre-Incas civilization had their capital at HA. Then, when immigrant lowland populations arrived in the New-World in the 1500's, they moved their capital from the highland to the coast[42].

During the colonial expansion initiated by the Spanish conquistadores – beginning in 1492 with the arrival of Christopher Columbus (1450-1506) in the Americas – the first conquistadores that visited HA regions reported symptoms like nausea and headaches and designed a disease of altitude that they called soroche (which refers to the disease called today acute mountain sickness) which could, at the time, lead to death. Pioneers first attributed the symptoms of soroche to the cold temperature at HA[43,44]. Nevertheless, father Acosta (1539-1600) a Spanish Jesuit-missionary and naturalist of Latin America was the first to propose in 1590 that HA "thin air" had a role in the

appearance of the symptoms[45]. Later, in colonies established in Potosi (a city at 4000m above SL) it is documented that pregnant ladies were transferred to lower altitude to give birth otherwise babies wouldn't survive most of the times[46].

Today, cities are found at altitude higher than 4000m. The highest town, La Rinconada is situated at an altitude of 5100m in Peru. It is an old gold-mining camp that has grown to a city, and today around 7000 people live there[47]. Nevertheless, low altitude native humans still have trouble acclimatizing to, and inhabiting HA environment.

## 1.3. Oxygen at high altitude

### 1.3.1. Oxygen, "the molecule of life"

The differential repartition of mammalians and humans at HA raised an important question: what makes the altitude environment a challenge for living organisms?

The environmental conditions found in high montane environment are reduced barometric pressure, intense solar radiation and cold, all of theses conditions can be challenging for ecological development. Nevertheless, the reduced barometric pressure represents the most challenging environmental condition for organism living at HA because it leads to a diminution in the partial pressure of all gases constituting the atmosphere, including $O_2$.

In the very early history of science, Leonardo Da Vinci (1452-1519) showed that "where flame cannot live, no animal that draws breath can live"[48]. In 1777, the French chemist Antoine Laurent

Lavoisier (1743-1794) in his book *Sur la combustion en général* proved that the link between combustion and respiration was $O_2$ and that it was essential to both[49].

Today, we know that there is a combustion process that involves $O_2$ at the cellular level in organisms[50]. In eukaryotes[9], each cell contains several organelles such as the nucleus, the Golgi apparatus, the ribosome, vesicles etc. (Figure I.5).

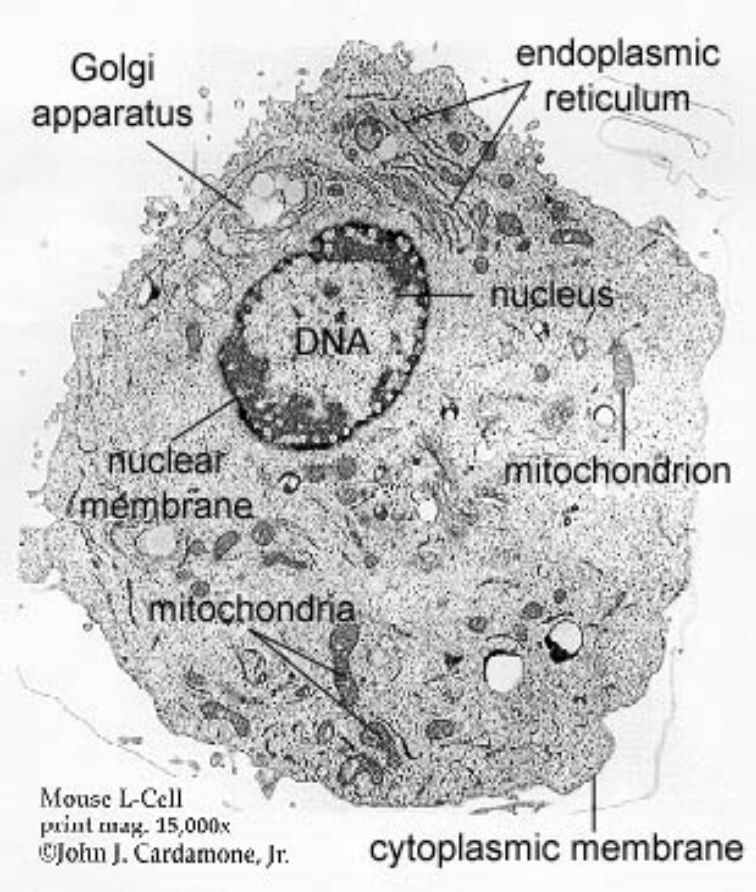


**Figure I.5: Organization of the organelles of the animal cell.**
*Source:*
*http://faculty.ccbcmd.edu/~gkaiser/SoftChalk%20BIOL%20230/Prokaryotic%20Cell%20Anatomy/proeu/proeu/proeu_print.html*

[9] An eukaryote is an organism whose cells nucleus and organelles are enclosed by membranes.

One of them, called the mitochondrion, contains the respiratory chain (Figure I.6), which is an electron transport chain, where a vital part of the metabolism called the oxidative phosphorylation occurs. It consists of an electron transfer from electron donors to electron acceptors in redox reactions. These redox reactions release energy, and $O_2$ is essential to this process as it is the last electron acceptor of the respiratory chain. The reduction of $O_2$ into water ($H_2O$) at the end of the mitochondrial respiratory chain is used to generate adenosine triphosphate molecules (ATP), which are the main energy supplier in cells.

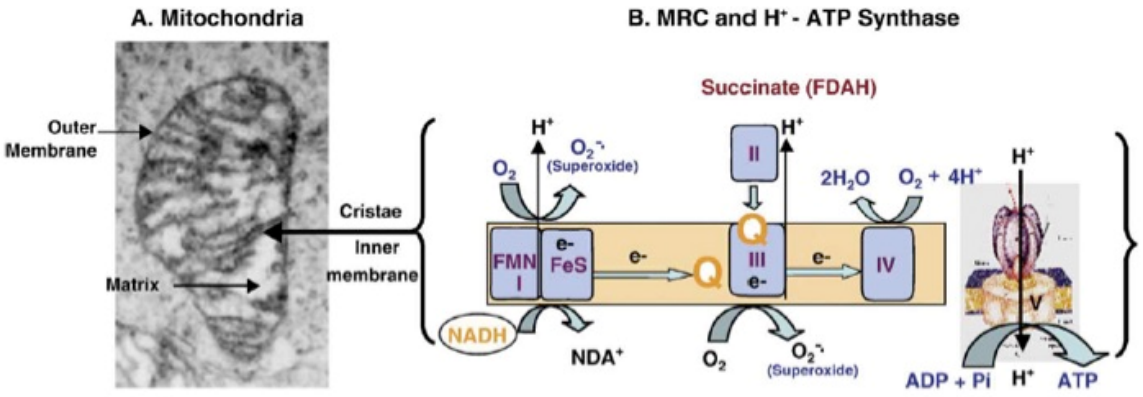


**Figure I.6: Structure of the respiratory chain**
(A) electron microscopy visualization of a mitochondrion. (B) schematic representation of the organization of the respiratory chain and its V complexes. *Adapted from Chen et al., Regulation of mitochondrial respiratory chain structure and function by estrogens/estrogen receptors and potential physiological/pathophysiological implications, 2005*[51].

ATP is used in a wide variety of biological processes including to maintain basal functions but also in energy consuming processes such as muscular exercise[50,52]. Actually, it is well established that the aerobic

metabolism in mammals relies on sufficient $O_2$ availability in the mitochondria. Nevertheless, the total amount of $O_2$ available in aerobic organisms (organisms that need $O_2$ to survive and grow) is low and the quantity stored in the tissue even lower (in human, there is around 1.5 liters of $O_2$ in the body and only 50 ml are stored in the tissues). Then, to maintain the oxidative phosphorylation undisturbed and complete, aerobic organisms require a continuous fresh flow of $O_2$ from the outside air to the tissues almost permanently. The diminution of the $O_2$ partial pressure at HA interferes with this supply of $O_2$ to the tissues and furthermore with the functioning of the mitochondrial respiratory chain, leading to a decrease in the ATP production and/or an increase in the generation of reactive $O_2$ species (ROS)[10].

### 1.3.2. The oxygen cascade

Fick's first law[11] postulates that a molecular flux goes from high concentrated regions to low concentrated regions, with a magnitude that is proportional to the concentration gradient (spatial derivative), without interruption until an equilibrium is reached. It relates the diffusive flux to the concentration under the assumption of a steady state. If we apply the first law to biology:

$$Flux = -P(c2-c1)$$

[10] The ROS describe reactive molecules and free radicals that derive from molecular $O_2$. They are byproducts of the mitochondrial electron transport of the respiratory chain but can also be produced by oxido-reductase enzymes and metal-catalyzed oxidation. They have the potential to cause a number of deleterious events but play an important role in cell signaling such as apoptosis, gene expression, and activation of cell signaling cascades.

[11] Adolf Eugen Fick (1829-1901) was a German physician and physiologist who introduced the laws of diffusion that governs the diffusion of a gas across a fluid membrane. In 1870 he was the first to measure the cardiac output. His law applies both to physics and physiology.

- Where P is the permeability of the membrane (conductance[12]) for a given gas at a given temperature.
- Where c2-c1 is the concentration difference of the given gas across the membrane and depends of the flow direction (here c1 to c2).

The amount of gas that moves across a tissue barrier is also proportional to the area of the barrier and inversely proportional to its thickness, and in a gas mix, each gas is independent and follows its own partial pressure gradient.

In the cells, $O_2$ is used permanently in the mitochondria but its partial pressure is very low, only a few mmHg, whereas in the ambient air, the $O_2$ partial pressure is very high, 160 mmHg at SL (Figure I.7). This difference in the $PO_2$ creates an important gradual partial pressure gradient between the environment and the mitochondria that allow the $O_2$ to travel through the biological barrier by diffusion. The progressive drop of $O_2$ pressure between the atmospheric air and the mitochondria is called the oxygen cascade[53].

[12] The conductance corresponds to the efficiency of a fluid transportation through a medium or a region. In biological tissue, the conductance of a gas depends upon the intrinsic permeability of the tissue to the gas with a define area and thickness.

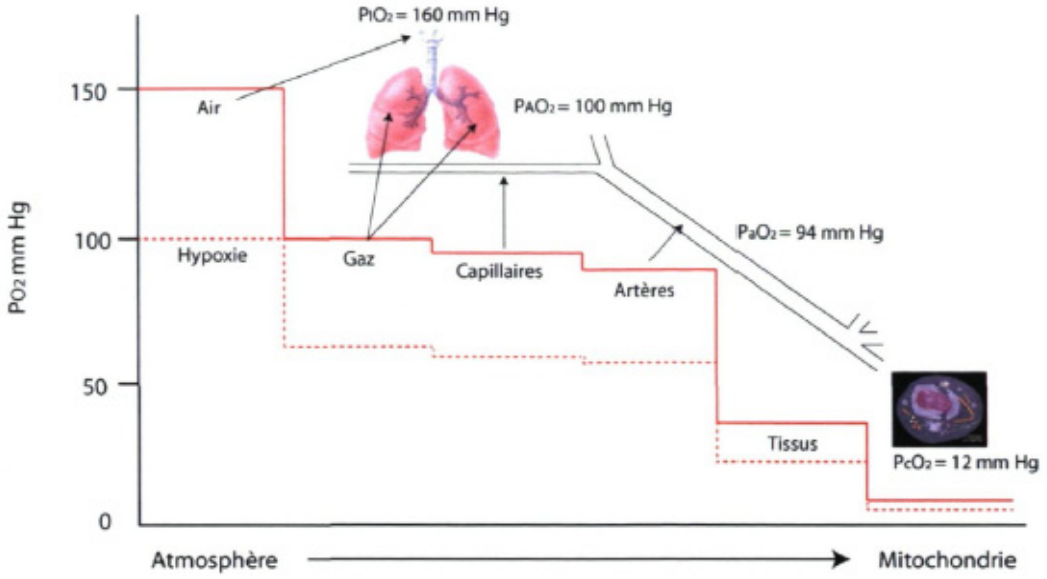


**Figure I.7: Evolution of the $PO_2$ from the atmospheric air to the mitochondrion.**
The plain line represents the sea level condition and the dotted line a hypoxic situation.
*Adapted from John B. West, La Physiologie Respiratoire, 2003*[54]*.*

The $O_2$ cascade path follows the $O_2$ transport path from the atmospheric air to the mitochondria and can be divided into four steps that are similar to, and follow the same road as, the breathing steps (see 1.3.3. p22: ventilation, pulmonary diffusion, circulation and tissue diffusion – Figure I.8).

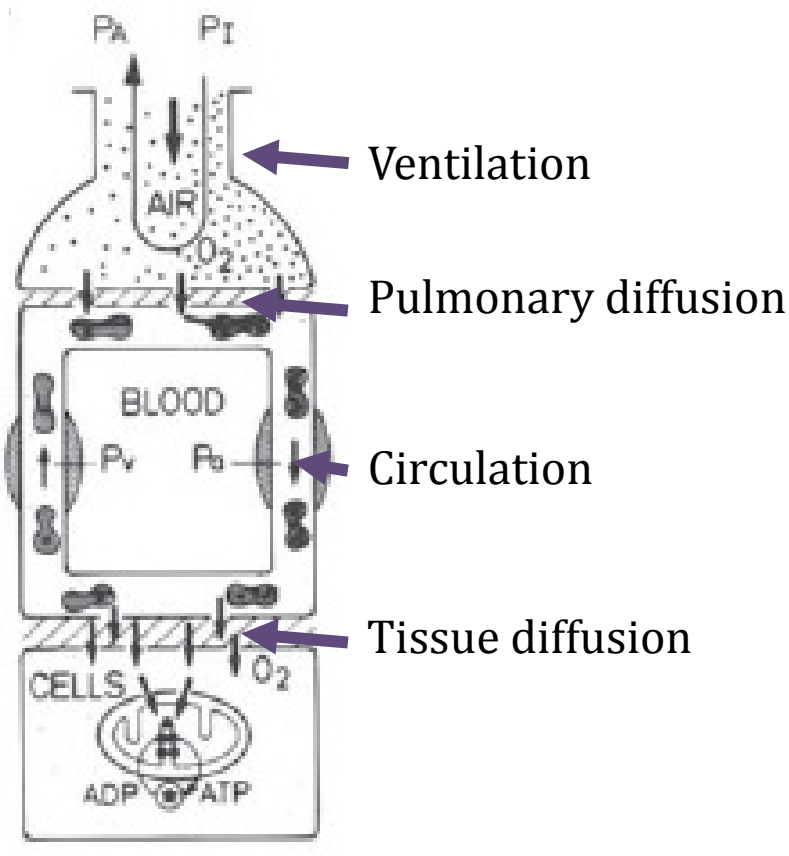


**Figure I.8: The oxygen transport system.**
*Adapted from Weibel and Hoppeler, Respiratory System Adaptation to Hypoxia: Lung to Mitochondria, 1991[55].*

Because of the diminution of the $PO_2$, organisms living at HA experience a "hypobaric hypoxia" which is a physiological situation where the blood $PO_2$ might change. In aerobic vertebrates, the $O_2$ travels from the alveolar lung through the organisms to the different tissues using the blood vessels. At altitude, the $PO_2$ diminution in the atmospheric air leads to a diminution in the $O_2$ partial pressure gradient differences between arterial and venous blood in the organism, which, if the organism is not able to compensate, might lead to a drop in the $O_2$ cascade and a decrease in the body oxygenation (Figure I.7)[56-58].

### 1.3.3. Breathing and gas exchanges

Breathing regroups all the mechanisms that allow an organism to exchange gases with its environment. During inspiration, an air rich in $O_2$ is inhaled into the lungs [13] whereas during expiration, the $CO_2$ produced by the respiratory processes of the body is exhaled from the lungs. The rhythmic movements of breathing maintain the fluid matrix homeostasis because it stabilizes the $O_2$ and $CO_2$ pressures in arteries and tissues as well as the pH in the physiological limits.

Inspiration is an active phenomenon, which requires active movements of muscles, mainly the diaphragm and the intercostal muscles whereas expiration is most of the time passive[59,60].

The breathing steps follow the $O_2$ transport system pathway and is completed in 4 major steps (Figure I.8): pulmonary ventilation, pulmonary diffusion, circulation/perfusion and tissue diffusion that can be described as follow:

- The pulmonary ventilation corresponds to a massive $O_2$ entry by the active convection of atmospheric air from the environment into the lungs, more especially into the alveolar spaces. This active phenomenon is powered by the contraction/relaxation cycles of the respiratory muscles (the pulmonary ventilation also refers to the total volume of gas that is inspired or expired per minute).
- The pulmonary diffusion is a passive diffusion from the alveoli to the capillaries. In the capillary, the $O_2$ goes through the plasma and across the erythrocyte membrane. Inside the

[13] The lungs are the gas exchange organs, their primary function being to allow $O_2$ to move from the air to the venous blood and $CO_2$ to move out. They also plays a role of physical barrier for unwanted material from the circulation, reservoir of blood and can metabolize some molecular aggregates[59].

erythrocyte, $O_2$ will bind to hemoglobin (a specialized protein that transports $O_2$ to the tissues). This passive diffusion is driven by the partial pressure gradient of $O_2$ between the alveoli and the capillaries, the alveolar pressure being superior to the capillary pressure.

- The circulation/perfusion transports $O_2$ using the active convection of the blood from the alveolar capillaries and the left heart through the vascular distribution system to all systemic capillaries and returns to the right heart powered by the contraction/relaxation cycle of the myocardium.
- During the last step, the tissue diffusion, the $O_2$ diffuses passively from the capillary blood across the plasma membrane into the interstitial space, across the cell membrane throughout the cytoplasm, – facilitated by myoglobin[14] (in the cells where it is present) –, and into the mitochondria. Like in the pulmonary diffusion step, the $O_2$ diffusion from the bloodstream to the tissues is driven by a partial pressure gradient for $O_2$ between the capillaries and the mitochondria, the capillary pressure of $O_2$ being superior to the mitochondrial one[50,53,57,61].

At the cellular level is found the cellular respiration. The mechanisms of cellular respiration involve three subdivisions (Figure I.9): glycolysis (where glucose molecules are broken down to form pyruvic acid molecules), the Krebs cycle, and the electron transport system of the mitochondria. Along those 3 steps, $CO_2$ is produced as a waste of these metabolic reactions and will be eliminated, as animals cannot use it, using the same travel road as $O_2$, backward, until being expelled outside of the body by expiration[53].

[14] Myoglobin is the iron- and $O_2$ binding protein found in the muscle tissue of vertebrates.

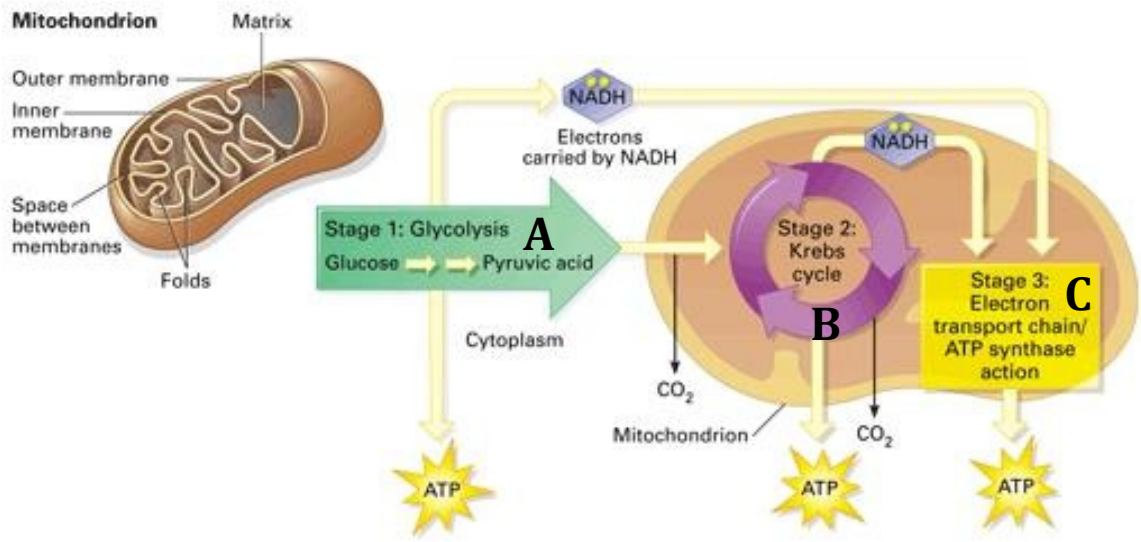


**Figure I.9: The steps of the cellular respiration.**
(A) The glycolysis. (B) The Krebs Cycle. (C) The mitochondrial respiratory chain.
*Source: https://www.studyblue.com*

# 2. Physiological responses to environmental hypoxia

Because most animals rely heavily on $O_2$ to sustain their biological functions, the reduced $O_2$ pressure found in high montane environment is challenging. Nevertheless, many species of birds and mammals successfully exploit HA habitats. Since the beginning of their studies, scientists noticed that, compared with lowland, endemic species as well as humans living at HA displayed specific physiological responses to this particular environment. The main purpose of the physiological adjustments being to improve $O_2$ supply and delivery, to prevent a drop in the $O_2$ cascade of diffusion. The modulation of the responses can occur at several levels (Figure I.10) and among the various possible responses, species living at HA show increased basal ventilation,

modified affinity of the red cells for $O_2$, increased lung exchange surface or reduced metabolism. In the following parts, we will discuss the physiological responses and/or observed phenotypes in hypoxia in humans, mammals and sometimes birds.

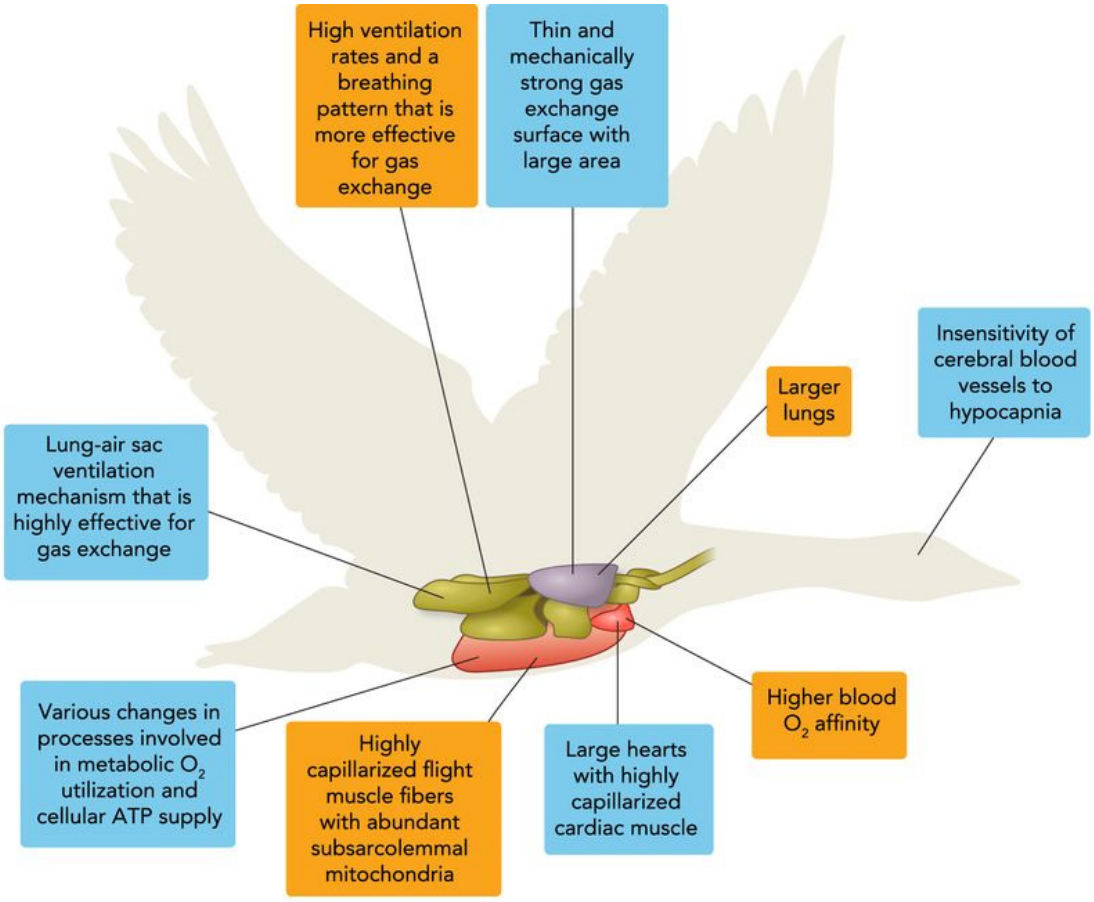


**Figure I.10: The physiological adjustments observed in endemic species from the high altitude environment compared with lowland.**
(Here, the Bar-Headed goose, in orange vs lowland goose in blue).
*Adapted from Scott et al. How Bar-Headed Geese Fly Over the Himalayas, 2015*[62].

The pioneer researches that studied the physiological responses in endemic species living at HA observed that they were different from

lowland and spoke about adaptive traits to the environment that were present in HA natives but not in lowlands. Afterwards, the term adaptation encountered many definition issues and to clarify the situation, a distinction was made between genotypic adaptation and phenotypic adaptation. Bligh in 1976 defined both terms: genotypic adaptation characterizes genetically determined forms and functions of an organism and its organs to the environment in which they occur whereas phenotypic adaptation occurs within the lifetime of individual organisms and are reversible when these circumstances no longer exist[63]. Later, the discovery in the early 2000's that environmental factors can influence gene expression without requiring long-term genetic selection and without becoming hereditary (which refer to the epigenetic mechanisms)[64] lead the scientists to review once again the definitions of adaptations. I think that today, we can postulate that endemic species present genotypic adaptations whereas imported species display phenotypic adaptations[65] and that epigenetic mechanisms can influence both[64,66,67]. However, it is important to keep in mind that the part of the influences of epigenetic factors in the physiological responses to HA either in endemic and/or imported species is still unclear[67].

## 2.1. The ventilatory responses in hypoxia

The $O_2$ molecules enter the body through ventilation and it is well known that only one breath of a hypoxic gas mixture will increase the ventilation. In human, ventilation can be adjusted to a very important extent, as it allows them to reach the top of Mount Everest – where the $PO_2$ is only 1/3 of the SL $PO_2$[58,68]. Maintaining ventilation also plays an

important role in maintaining the adaptive capacity in HA natives (for further information see 2.6. p62).

### 2.1.1. Ventilatory response to acute hypoxia

Acute hypoxia refers to a hypoxic exposure that will last from several minutes to a few hours.

In vertebrate, the ventilatory response to hypoxia is linked to the existence of peripheral and central chemoreceptors. They are "sensing" the content of $O_2$ in the blood and have projection to the central nervous system (CNS) breathing centers, located mainly in the brainstem, and responsible for the adjustment of the ventilation (Figure I.11).

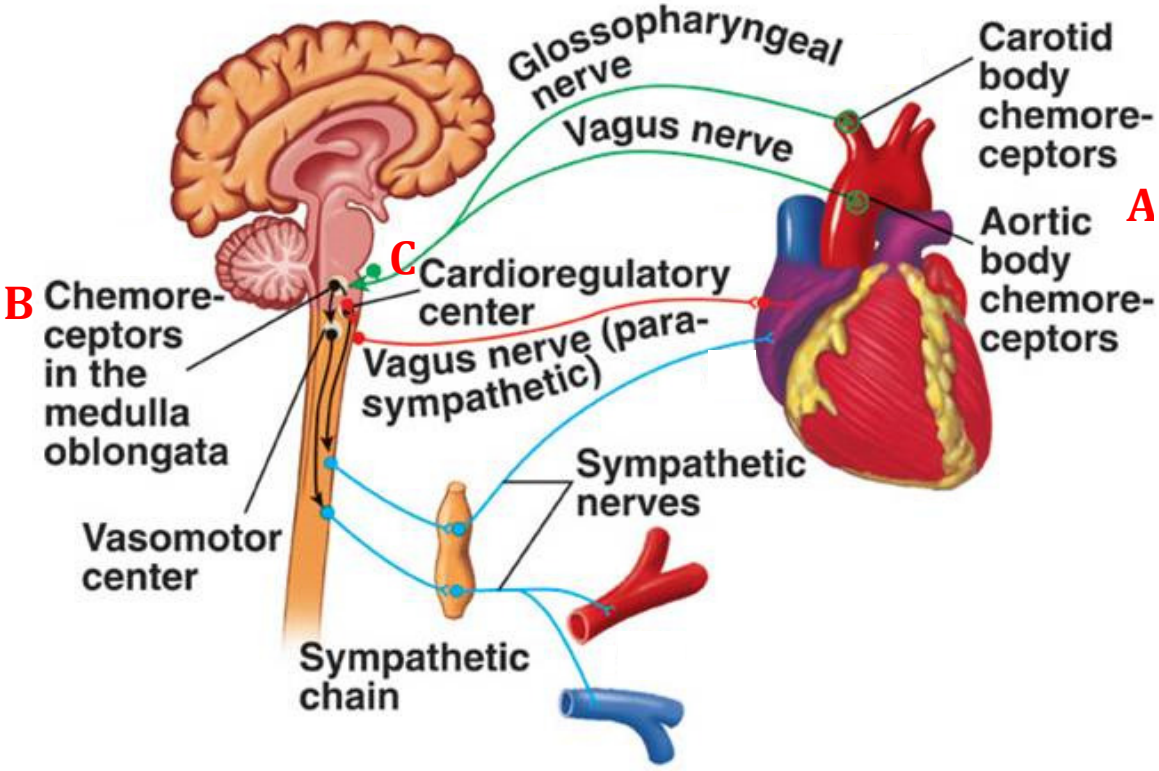


**Figure I.11: Components of the regulation of breathing.**
(A) The peripheral chemoreceptors, (B) the central chemoreceptors, (C) The breathing centers.

### *Chemoreceptors definition, localization and role*

Chemoreceptors or chemosensors can be defined as sensory receptors that transduce chemical signals into action potentials. In humans and animals, they detect chemical stimuli in the arterial blood or the cerebrospinal fluid. There are two types of chemoreceptors responsible for the maintenance of the arterial blood gas: central and peripheral (Figure I.11).

The peripheral chemoreceptors include the carotid and the aortic bodies (Figure I.11). The carotid bodies are located where the common carotid artery bifurcates to form the internal and external carotid arteries and are responsible for the primary sensing of arterial blood $O_2$

but also respond to a wide variety of other respiratory stimuli such as $CO_2$, pH, and temperature as well as non-respiratory stimuli. If the CB cells sense a variation in the arterial $PO_2$, they will trigger action potentials through the afferent fibers of the glossopharyngeal nerve that relays the information to the CNS[69]. The aortic bodies are located along the aortic arch and as well as the CB, measure the changes in blood pressure and the composition of the arterial blood flowing past it. The information is transmitted to the medulla oblongata via the afferent branches of the vagus nerve[69]. The medulla, in turn, regulates the breathing and blood pressure. Nevertheless, it is well admitted since 1970 that the aortic bodies only play a minor role in the respiratory chemoreflex. In rats, it was observed that the nervous impulse linked to the peripheral chemosensibility comes predominantly from the sensory nerves of the CB[70].

The central chemoreceptors are located in the brainstem (Figure I.11), more especially in the medulla[71-73]. They are critical sensors of the arterial $CO_2$ but will also respond to changes in the brain tissue $O_2$ concentration that can result in a modification of the respiratory rhythm and pattern[74,75].

***The hypoxic ventilatory response***

Aerobic metabolism in mammals relies on sufficient $O_2$ availability for metabolic processes. The diminution in the $O_2$ supply that can occur during acute hypoxic exposure has several deleterious physiological consequences (headache, dizziness, pulmonary or brain edema). Animals relying on pulmonary ventilation for their $O_2$ supply can increase their ventilation to improve $O_2$ uptake, leading to a modification

in the observed ventilatory pattern called the hypoxic ventilatory response (HVR), which is a reflex response to a hypoxic stimulation.

The HVR is initiated by the peripheral chemoreceptors in response to a decrease in the arterial blood $PO_2$. Neonates and adult changes their ventilation in a different way; neonates will have only a minor increase in ventilation in response to hypoxia whereas adults will significantly increase their ventilation (with a response that will last several minutes).

During the last two decades, many studies were conducted regarding the ventilatory response to hypoxia in a wide range of mammals including humans. Both endemic and imported large mammals exposed to lifelong environmental hypoxia hyperventilate in response to acute hypoxia (this has been observed in llamas[76], bovidae[77,78], and dogs[79,80]). Some species however such as rats, cats or sheep, demonstrated an attenuated or even absent ventilatory response to experimental hypoxia after acclimatization called blunted ventilatory response[81-84]. In humans, Andean highlanders exhibit a blunted ventilatory response to hypoxia compared with lowlanders[85,86] whereas studies showed that compared with acclimatized Han (Chinese), Tibetans have no blunted response and no hypoventilation in response to hypoxia however, both population demonstrated similar level of effective alveolar ventilation[87]. In HA humans natives however, because of morphological modifications (i.e. increase of the chest cavity and the total alveolar surface area), they might not need to increase their ventilation when exposed to acute hypoxia to improve $O_2$ uptake[65].

In 1991, Leon-Velarde and Monge postulated that the observed difference between mammals including humans would likely be linked to

a displacement of the HVR to a lower arterial $PO_2$ rather than an absolute difference in response[65].

In birds, the issues are all different because they are anatomically predisposed to withstand severe hypoxia[41,62,65]. Birds are flying animals, and flying require important cardiorespiratory performances, so it seems that during evolution, birds managed to seriously improve their cardiovascular and respiratory physiology along the $O_2$ cascade. This peculiar physiology might explain why today they have amplified hypoxia tolerance (Figure I.12). At HA, birds display a pronounced HVR associated with a very low $PCO_2$. This is possible mainly because of the anatomic arrangement of their lung. Birds have a parabronchial lungs. Theoretically, birds arterial $PO_2$ can reach higher levels than the end-tidal $PO_2$ and $PCO_2$[88]. Powell studies demonstrated that the most important consequence of acclimatization in ducks is the decrease in $PaCO_2$ without increasing the $PaO_2$[89-91].

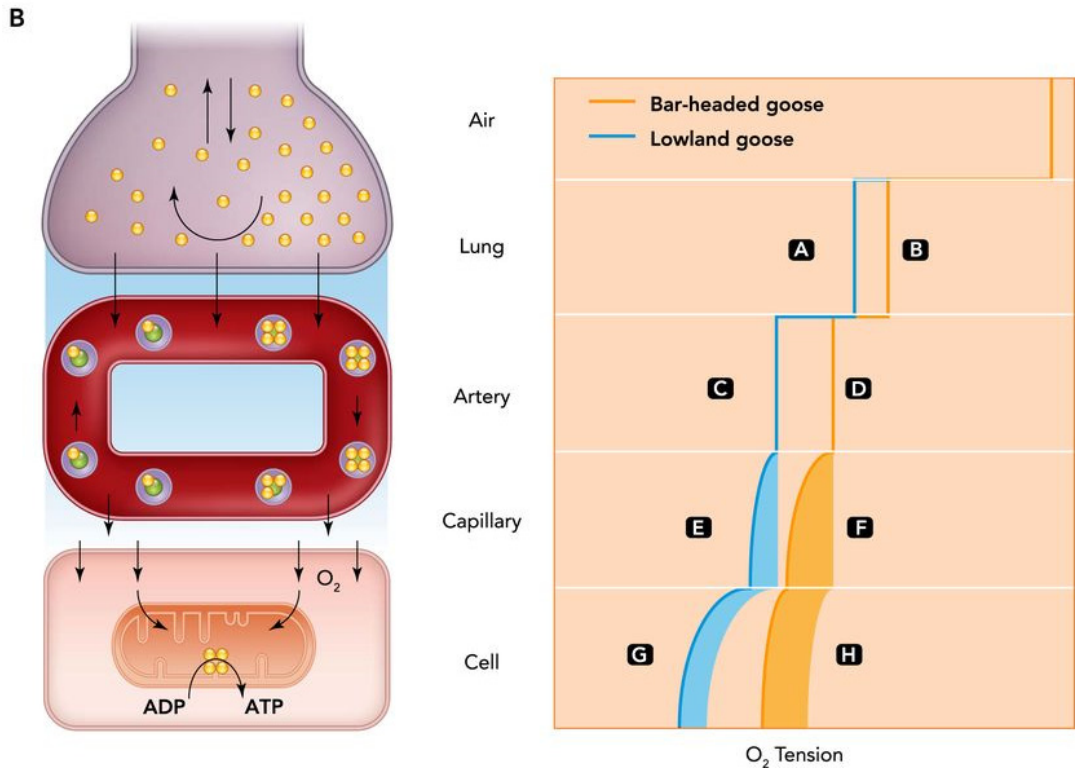


**Figure I.12: How bar-headed goose have improve their cardiovascular and respiratory physiology along the oxygen cascade to cope with chronic environmental hypoxia and flying over the Himalayas.**
*Adapted from Scott et al., How Bar-Headed Geese Fly Over the Himalayas, 2015*[62]*.*

### *The peripheral mechanisms mediating the HVR in mammals*

The CB initiates the HVR. The CB are organized in cluster surrounded by blood vessels, nervous fibers and connective tissue (Figure I.13)[92].

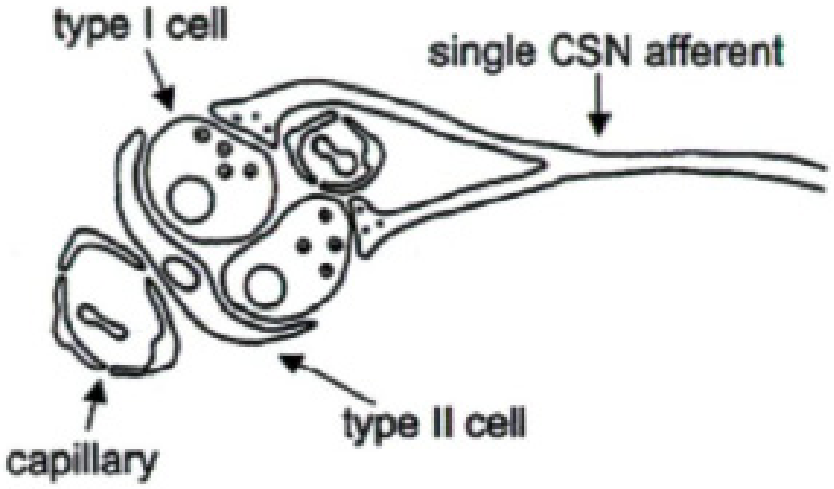


**Figure I.13: Cellular organisation of the carotid body.**
*Adapted from Peers et al., Mechanisms for acute oxygen sensing in the carotid body, 2010*[92]*.*

The two main cell types composing the CB are the type I cells or glomus cells (responsible for the environmental sensing part) and the sustentacular cells or type II cells that have a glial-like appearance (responsible mainly for modulating the transmitted signal). When the CB sense hypoxia, there is an inhibition of the potassium channel activity in the cell membrane inducing the depolarization of the membrane and a massive entry of extracellular calcium in the cytosol of the type I cells which leads to the release of neurotransmitters (NT). The NT will then act on the afferent endings of the carotid sinus nerve (CSN) to increase the impulse traffic of the neurons involved in the control of breathing situated in the brainstem (Figure I.11)[93,94].

The HVR has mainly been attributed to the activity of the peripheral chemoreceptors considered the first line of response for

acclimatization to HA. Aortic chemoreceptors might play a secondary role in HVR in acclimatized animals and have been hypothesized to play a primary role if the CB are removed or non functional[93]. Nevertheless, in the 2000's, my current director, who was in his PhD at the time, used chemo-denervation in rats living permanently at HA to understand the hypoxic ventilatory acclimatization in animals. In fact, in rats that are already fragile at HA, removing the CB asked him to be extra careful and bilateral chemo-denervation had to be done one side at a time in a 2-week period to prevent the death of the animal. This might seem anecdotal but it proves that the CB are critical if not essential to withstand the low $PO_2$ at HA at least in rats[95].

### *The central mechanisms mediating the HVR in mammals*

When the CB sense a drop in the arterial $O_2$, they stimulate sensory discharges in the CSN by releasing excitatory NT (mainly ATP and acetylcholine) from the type I cells[93,94,96]. The type I cells activity depends upon the arterial $PO_2$ but can be modulated by neuromodulators (NM – such as dopamine and adenosine) by an auto- or paracrine action (depending on the origin of the release of NM: type I cell itself or type II cells)[96,97]. The post-synaptic action potential traveling through the CSN transmits the information into the nucleus of the solitary tract (NTS)[98] located in the dorsal medulla. The NTS will integrate the CB chemoreceptive information and deliver it to the respiratory central pattern generator (neurons responsible for the generation of the inspiratory rhythm are located mainly in the pre-Bötzinger complex and neurons responsible for the expiratory rhythm in the retrotrapezoid nucleus)[93,96,99,100], resulting in an increase in the output of the efferent motoneurons innervating the respiratory muscles

that will stimulate the breathing frequency and/or the tidal volume resulting in an increase in the total ventilation (Figure I.14)[101]. The increase in the total ventilation increases the alveolar $PO_2$, reducing the drop in the arterial $PO_2$ and $O_2$ content.

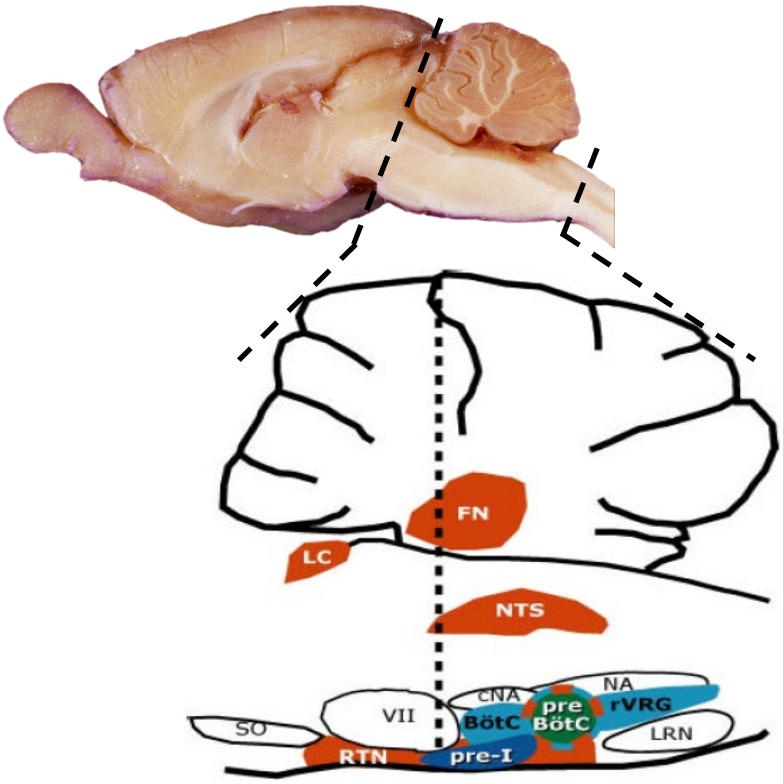


**Figure I.14: Midsagital section of the brain of a rat with representation of the respiratory pre-motor, putative rythmogenic and central chemoreceptors.**
Blues areas are the principal locations of the respiratory bulbospinal premotor neurons that project to phrenic, intercostal, and abdominal motoneurons, which drive muscles of the respiratory pump. Red areas represent regions that play a role in central chemoreception. Abbreviations: FN, fastigial nucleus; LC, locus ceruleus; NTS, nucleus of the solitary tract; VII, facial nucleus; rVRG, rostral ventral respiratory group; NA, nucleus ambiguus; cNA, compact division of the nucleus ambiguus; LRN, lateral reticular nucleus; RTN, retrotrapezoid nucleus; SO, superior olive; BötC, Bötzinger complex; preBötC, preBötzinger Complex.
*Adapted from Feldman et al. Breathing: Rhythmicity, Plasticity, Chemosensitivity, 2003[101].*

### 2.1.2. Ventilatory response to long-term hypoxia in low altitude natives: the ventilatory acclimatization to hypoxia

In low altitude natives, long-term hypoxic exposure results in a profound increase in ventilation and ventilatory sensitivity to hypoxia to maintain adequate $O_2$ distribution to the tissues by increasing the alveolar and arterial $PO_2$[102-105]. This is called the ventilatory acclimatization to hypoxia (VAH).

The apparition of the VAH in time varies between species. Literature has it settled after 7 to 10 days of chronic hypoxia in rats and humans[106-109], whereas, it takes 3 days in mice[110] and only 6 hours in goats[111]. The similar timeline of VAH apparition between rats and human made rats a surrogate to study ventilatory acclimatization to the hypoxic environment[1,2,109]. The VAH has been studied in several animals models (goat[112-115], ponies[116,117], cats[78,118-120], mice[110,121-123], and rats [106,124-127]), as well as in humans[128-138] and result in a time dependent increase in ventilation, a decrease in the arterial $PCO_2$ and an increase in the HVR.

#### *The peripheral mechanisms mediating the VAH in mammals*

Chronic hypoxia induces morphological modification of the CB. In 1972, Arias-Stella and Valcarcel were the first to report that the CB increased in size and weight in Peruvian Andean living in Cero de Pasco (4373m) compared with Peruvian Andean living in Lima (SL)[139,140].

In low altitude natives, the size increase in the CB is reversible, for example, if rats are exposed to hypobaric hypoxia, they will have increased CB size[141,142], but if the animals are returned to SL for 5 weeks, the size increase will be reversed[141]. The hypertrophy of the CB

observed in response to hypoxia is linked to a neovascularization in the vascular bed of the CB and an increased number and size of the type I cell[69,143,144].

CB denervation in many species abolishes the VAH[120,145,146], and CB perfusion with hypoxic blood without hypoxic environmental exposure lead to the apparition of the VAH[111] indicating that the CB are both necessary and sufficient to induce ventilatory acclimatization. Long term hypoxia in the CB induces cellular and molecular modification of the peripheral chemoreceptors leading to an increase in their sensibility to $O_2$ but also an increase in the responsiveness of the central integration sites reached by the afferent inputs of the CB[69,103,120,147]. The increase in the type I cell size is linked to an increase in the number of mitochondria and synaptic vesicles (responsible for the NT liberation into the inter-synaptic space). These modifications are thought to be activated by changes in the ion channel density and NT stored in the type I cells as well as hypertrophy and/or hyperplasia of the cell itself[148].

In highland species the size increase observed in the CB has been linked to an increase in the type I cells mostly and this condition is not reversible whereas in lowland species, the increase in the CB size is the consequence of an increase in the type I cells number associated with a vascular engorgement and is reversible[65]. It is of note that no enlargement of the CB was found in endemic species such as llamas and alpacas, suggesting that the hyperplasia observed in imported species and humans might reflect their absence of genotypic adaptation[65,149]. In the same line of ideas, since the net output of the CB in response to hypoxia integrates both excitatory neurotransmission and inhibitory feedback mechanisms, an increase in the size of the organ will not predict a higher sensitivity.

### *The central mechanisms mediating the VAH in mammals*

The VAH will further increase the responsiveness of the CNS to the CB afferent inputs probably because of changes in the glutamatergic-signaling path in the NTS[126,150]. At some point, living at HA will desensitize the central respiratory control system from the hypoxic environmental stimulus leading to a decrease in the ventilatory rate called the hypoxic desensitization (HD – Figure I.15)[151]. The HD is linked to a central inhibitory action on the respiration induced by hypocapnia and alkalosis[109]. It is of note that the VAH can stay on for months before the HD appears.

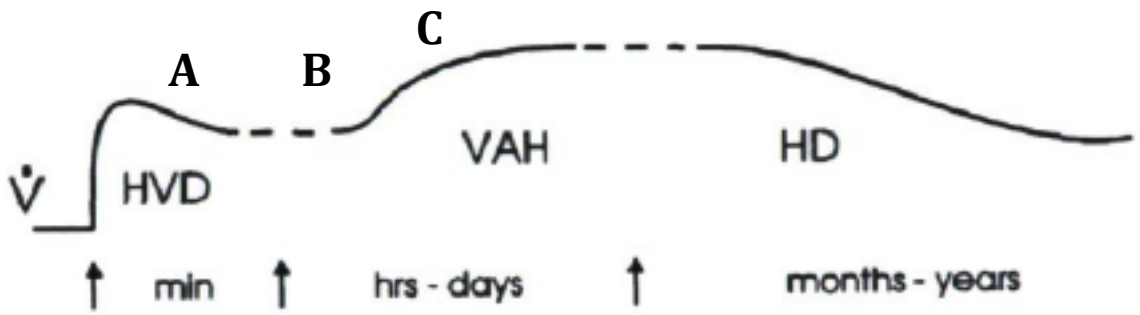


**Figure I.15: Ventilatory responses to (A) acute, (B) sustained and (C) chronic hypoxia.**
(HVD = hypoxic ventilatory decline; VAH = ventilatory acclimatization to hypoxia; HD = hypoxic desensitization)
*Adapted from Powell, et al. Time domain of the hypoxic ventilatory response, 1998[151].*

## 2.1.3. The ventilatory response in HA endemic species including humans

Literature shows numerous differences in the breathing pattern between high and low altitude natives. For instance, compared with rats

raised at low altitudes, the HA plateau pikas breathe using larger tidal volumes and lower breathing frequencies[152]; in humans, HA population from the Tibetan Plateau also breathes with larger tidal volumes compared with lowlanders[153]. These different breathing patterns can have numerous consequences on the physiological responses regarding $O_2$ intake and distribution at HA.

A study comparing two llamas born and raised at HA (3790m) with two llamas of second generation born and raised at SL as an attempt to observe the "blunting" effect in llamas and compare it to humans showed no differences in the HVR between the so-called SL and the HA llamas. Meaning that llamas have an increase in their ventilatory response at HA when exposed to further hypoxic environment[76].

In humans, Tibetan and Andean native have a different ventilatory response to the hypoxic environment. Indeed, Andean HA natives have lower resting ventilation and lower HVR than Tibetan[86,153]. Nevertheless, if lowlanders of the Andes are acclimatized to altitude in childhood or even born at altitude, they demonstrate higher forced vital capacity, higher $VO_2$ max and higher arterial $O_2$ saturation during exercise than lowlanders who migrated at altitude as adults[154] indicating that the blunted ventilation is not limiting for $O_2$ transport. In a recent review Frisancho showed that Andeans extract more $O_2$ despite their lower pulmonary ventilation[155]. Moreover, both population (Andean and Tibetan) demonstrate higher total lung diffusion capacity and greater chest circumference compared with lowland natives acclimatized at HA[156,157], which could compensate for the lower ventilation by increasing the surface area available for gas exchange.

It is of note that we focus on the arterial $O_2$ drop occurring at HA and its consequences on the HVR whereas changes in the arterial $PCO_2$ and pH also affect breathing during HA hypoxia[136]. Indeed, it is possible that changes in the $CO_2$ and/or the pH influence the progressive increase in breathing at HA[158,159]. Nevertheless, because chronic HA hypoxia is associated with an important decrease in the $PCO_2$, and because the secondary increase in ventilation observed in the VAH is not associated with a recovery of pH in the arterial blood or cerebrospinal fluid, changes in $CO_2$ and/or pH in arterial blood and cerebrospinal fluid are not sufficient to explain the time-dependent increase in ventilation during acclimatization[160,161].

## 2.2. The gas-exchange surface in hypoxia

After $O_2$ enters the lungs through ventilation, it diffuses from the alveolar sac to the pulmonary capillaries. Modifications of the lung structure, architecture, volume and/or surface can have notorious consequences in the $O_2$ diffusion capacities of an organism.

The main function of the lung is to provide enough surface through the gas-exchange barrier to supply $O_2$ and eliminate $CO_2$ according to the organism needs. The lungs regroup conducting structures (i.e. the part of the lungs that only serves to conduct air between the alveoli and the upper airways, where no gas exchange takes place, this corresponds to the primary, secondary and tertiary bronchi) and respiratory structures (which contains the bronchioles, alveolar ducts and the alveoli where the gas-exchange takes place – Figure I.16).

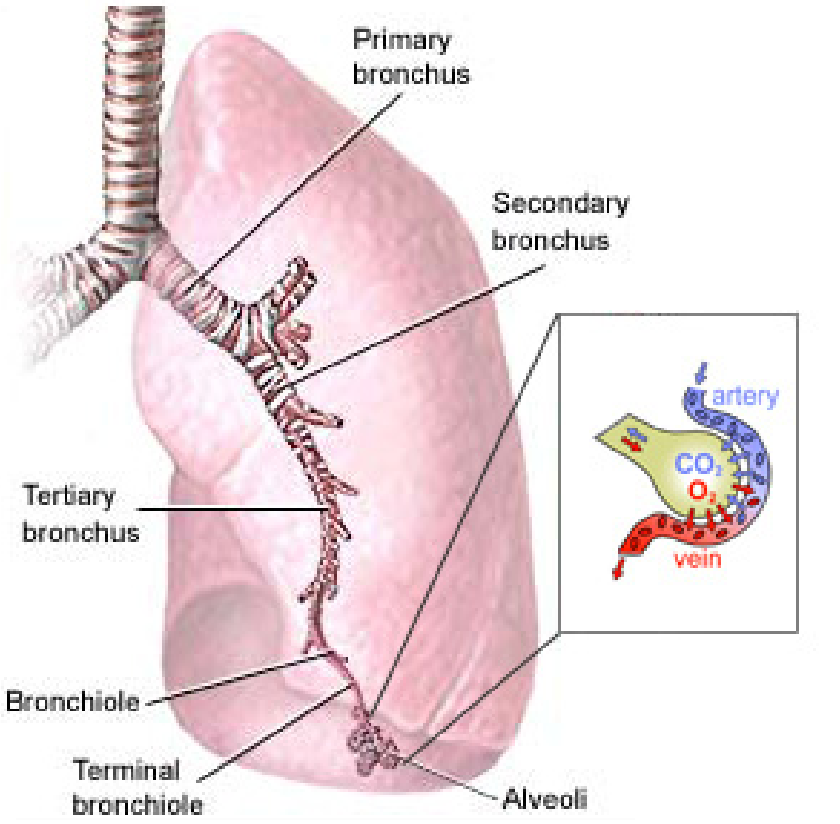


**Figure I.16: Architectural organisation of the lung.**
(A) the conducting zone (regrouping the primary, secondary and tertiary bronchi) and (B) the respiratory structure (regrouping the bronchioles, alveolar duct and the alveoli). (C) represents the gas-exchange occurring in the alveoli. *Adapted from A.D.A.M education.*

When animals became air-breather, the importance of $O_2$ uptake – the "call for oxygen"[162] – lead to the development of a lung architecture that created a direct linear relationship between alveolar surface area and $O_2$ uptake across the entire range of mammalian body mass. Studies showed that this linear relationship is linked to a greater subdivision of the alveolar surface in small organisms compared with larges ones, without a necessary increase in relative lungs volume[163]. At HA, the diminution in the atmospheric pressure, if not properly

regulated, can lead to a drop in the $O_2$ cascade (see 1.3.2 p18). To prevent this drop, HA living organisms often have an increased gas exchange surface, and this is generally associated with an increase in lung volume.

Early studies in human highlanders showed larger lung volume, greater pulmonary diffusing capacity linked to larger and more numerous alveoli, low maximum expiratory flow-rate per lung volume and low upstream conductance compared with lowlanders[164-167]. Until the early 2000, one hypothesis based on old studies from the 60's to the 80's, explained that the conducting zone would be less affected by the hypoxia experienced at HA than the gas-exchange region because the conducting zone develops mainly during gestation in all mammals[168], while the septation (the process by which alveoli are formed) in rats or humans occurs only or mainly after birth[169-172]. To support this hypothesis, studies were conducted using animals in which the septation occurs in utero such as guinea pigs. These studies showed no difference in the alveolar dimensions between high and low altitude natives even after several generations of HA living, they even experimented on one llama living at 4390m that did not show any increase in his area of diffusion either[173]. But in more recent studies, animals (mostly rodents) and humans either living at HA or raised in a hypoxic environment demonstrated higher lung volumes, higher alveolar surface and higher aerobic capacities than lowlanders[155,156,174,175]. Indeed, raising guinea pigs at HA (3800m) between 1 and 6 months induces structural modifications of the lungs including accelerated lung growth resulting in an increased alveolar surface area, reduced diffusive resistance with larger alveolar sac volume and septal crowding associated with a higher volume of alveolar epithelium and interstitial components with smaller alveolar duct volume[174,176]. In the same line of evidence, studies

comparing HA living deer mice with deer mice from SL showed larger lungs in HA animals[175,177].

$O_2$ diffuses to the pulmonary capillaries through the alveolar walls; thus, the thickness of the alveolar walls also plays a role influencing the $O_2$ diffusion capacities. In humans or wild mice (*Phyllotis darwini*), no differences were found in the thickness of the alveolar walls between HA and low altitude natives[178,179] but HA guinea pigs had thinner walls than low altitude animals[178,180] and the plateau pikas are known to have thin-walled pulmonary arterioles characterized by a lower rate of muscular cells in their vessels walls (compared with rats)[181] which would probably also accelerate the diffusion of $O_2$ from the alveoli of the capillary.

## 2.3. The diffusion and circulation of $O_2$ in hypoxia

In hypoxic environment, the increase in ventilation as well as the changes in lung volume and morphology can ameliorate but does not nullify the $O_2$ drop in the blood. If the capacity to take in enough air by virtue of anatomical features, respiration rate and lung morphology is clearly an important aspect of the adaptive strategies observed in HA endemic species, it is also important that the absorption and retention of $O_2$ from the air remains adequate for the need. In May 2007, during a research project called the Caudwell Xtreme Everest, a research team of British doctors and scientists climbed to the summit of Mount Everest and took samples of their blood to measure the $O_2$ content in it. This was the first time that men measured the level of $O_2$ in human blood on the balcony of the Everest mountain and the results relate very low $O_2$ content reaching values that, in a hospital patient, will require intubation with an air enriched with $O_2$. Indeed, if the normal $PaO_2$ is around 90-

110 mmHg, they had reached, after 20 minutes of unassisted breathing at the top of Mount Everest, less than 27 mmHg of $PO_2$ in their arterial blood[182].

### 2.3.1. The lungs diffusive conductance

Diffusion from the alveoli to the pulmonary capillaries is a passive phenomenon, thus, it is dependent upon Fick's first law of diffusion:

$$VO_2 = G_{LO2} (P_AO_2 - PaO_2)$$

- Here $VO_2$ is the body $O_2$ consumption.
- Where $G_{LO2}$ is the diffusive conductance of $O_2$ in the lung.
- Where $P_AO_2$ is the alveolar $PO_2$ and $PaO_2$ the arterial $PO_2$.

In 1957, Roughton and Forster published a paper where they observed that the $O_2$ gradient within the blood (between the content in the red blood cells and the free $O_2$ molecules) may exert an influence on the over-all rate of $O_2$ uptake in the lung and that this needed to be taken into consideration when determining the $O_2$ lungs' diffusing capacity. They developed a resistance equation of gases that can be applied to the lung-blood $O_2$ transfer[183]:

$$1/G_{LO2} = 1/G_{MO2} + 1/G_{BO2}$$

- Where $G_{MO2}$ is the membrane conductance.
- Where $G_{BO2}$ is the blood-kinetic conductance.

Then, combining the Fick's law of diffusion and the resistance equation of Roughton and Forster allows an analysis of the adaptation possibilities of the lung diffusions capacity, its components and the alveolar/arterial differences. Because of the diminution of the atmospheric pressure, the gradient between the alveolar and the arterial $PO_2$ is reduced at HA. Adjustments to counteract the consequences of

the gradient diminution in the $O_2$ diffusion property includes an increased area of diffusion (see above) which logically would be associated with an increase in the capillary number and/or diameter, resulting in a decrease in the resistance to blood flow, facilitating $O_2$ diffusion. Nevertheless, the flow of $O_2$ passing through this area of diffusion remains dependent on the diffusive conductance of the area (pulmonary volume, size and/or alveoli number) and the membrane and blood-kinetics conductance of the components of the area.

Humans either endemic HA natives or Caucasian born at HA have an increased diffusive and membrane conductance associated with an increase in the thoracic blood volume[184-187]. In rats, Weibel and Burri found an increased diffusive conductance after exposure to hypoxia that they attributed to an increase in the alveolar-capillary membrane diffusion capacities rather than a thinning of the walls[188].

A recent study conducted by de Bisschop compared the pulmonary capillary blood volume and membrane conductance in Andeans at HA and Europeans at SL and HA. They found that the pulmonary capillary blood volume is increased in people living at 4000m compared with low altitude natives and that the lung diffusing capacity decreased in low altitude natives after 4 days at HA (La Paz, 3600m) compared with their SL values. They also found an increase in the lung diffusing capacity in HA compared with low altitude natives consistent with an increase in the lung surface area associated with the recruitment of capillaries and a thickening of the capillary blood sheet[189].

### 2.3.2. The pulmonary circulation

Exposure to hypoxia increases pulmonary vascular resistance and induces important structural changes in the pulmonary vascular bed, which, in extreme cases, could lead to pulmonary arterial hypertension.

In the lungs, in response to alveolar hypoxia, pulmonary arteries constrict in an attempt to redirect the blood flow to the alveoli with the higher content of $O_2$. The reduction in the alveolar $O_2$ tension causing this process, is reversible, and known as the hypoxic pulmonary vasoconstriction (Figure I.17)[190].

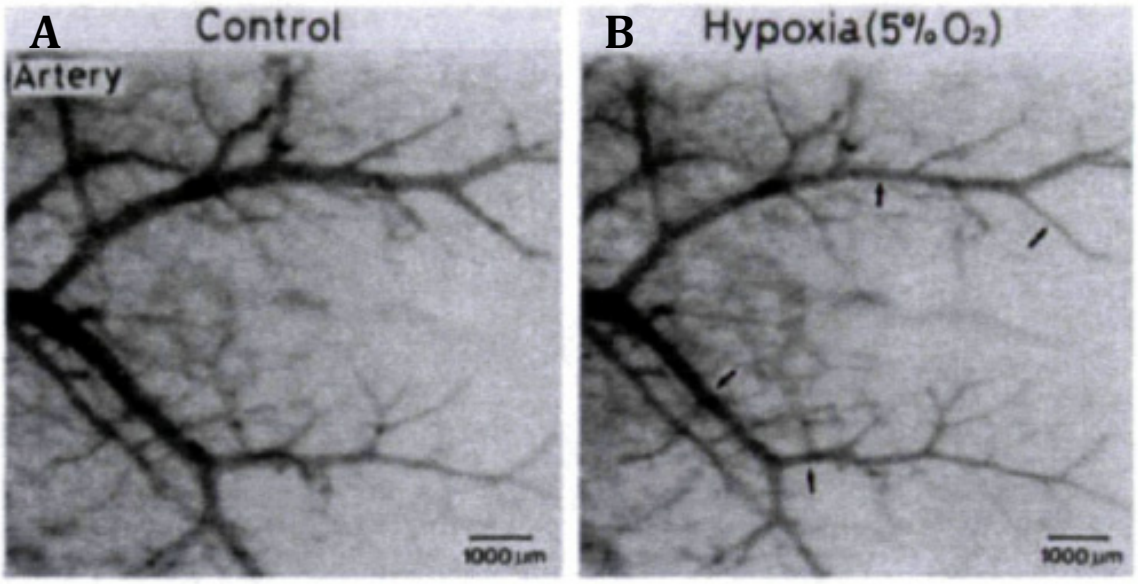


**Figure I.17: Cat left lung inferior lobe arteriogram.**
The cat was anesthetized. (A) normoxia. (B) 5% $O_2$ hypoxia. The arrows represent the pulmonary arterioles vasoconstriction in response to hypoxia.
*Adapted from Shirai et al. Effects of regional alveolar hypoxia and hypercapnia on small pulmonary vessels in cats, 1986[190].*

This process is engaged to improve ventilation-perfusion matching and is dependent upon $O_2$ sensing at the cellular level of the pulmonary

arterial smooth muscle cells[191]. The hypoxic pulmonary vasoconstriction is efficient to improve ventilation/perfusion ratio and arterial oxygenation at SL but can become deleterious in long-term whole-body hypoxic exposure such as HA living (because the entire lung is hypoxic when exposed to environmental hypoxia). At HA, structural changes occur in the pulmonary arteries leading to modifications of the biochemical and functional phenotype of the vascular cells; associated with hypoxic pulmonary vasoconstriction, this can lead to the apparition of pulmonary hypertension.

Chronic hypoxia induces several morphological and/or structural changes in the vessel walls such as neo-muscularization, increased medial thickness, adventitial hypertrophy and deposition of additional matrix components including collagen and elastin[192-197]. All those modifications can lead to a diminution of the lumen of the vessel, thus diminishing its diameter, and participating in the increase in the vascular resistance observed at HA. In the same line of thoughts, chronic hypoxia was linked to a decrease in the total number of blood vessels in the lungs, particularly at the level of capillaries. This rarefaction of the pulmonary blood vessels along with the remodeling of the vessel walls might be responsible for the apparition of chronic hypoxic pulmonary hypertension[197-201]. However, the concept of vascular rarefaction has been challenged by reports demonstrating angiogenesis in the pulmonary capillaries in response to hypoxia, which would counteract the pulmonary hypertension by lowering the resistance in the pulmonary vascular bed[197,202,203]. The magnitude of the changes observed in the vessels structure depends on the species studied, the sex, and the stage at which the hypoxic exposure took place (in utero, as a newborn, during childhood, adulthood, lifetime…).

In 1981, Rabinovitch studied the apparition of pulmonary hypertension after chronic hypoxic exposure in newborn and adult rats and demonstrated that the "pulmonary artery pressure correlates well with right ventricular hypertrophy, which presumably directly reflects a work against resistance"[204]. In domestic animals such as guinea pigs, rabbits, and dogs living at 3000 to 4700m the right ventricular hypertrophy increases over 25% compared with their SL counterpart[205] whereas in cattle (such as sheep and goats) there is a moderate or absent hypertrophy[65,206].

Endemic species at HA have specific adaptations in the structure of their vessels that prevent excessive vascular remodeling in response to hypoxia, and the increase in pulmonary arterial resistance and pressure. Camelids such as llamas, vicuñas and alpacas have thin-walled, elastic and muscular arteries and do not develop neo-muscularization of the pulmonary arterioles[207,208] and studies showed that llamas do not present pulmonary hypertension when exposed to hypoxia[209]. It is also noteworthy to point out that all camelids (including those living at SL) have thin-walled pulmonary arteries corroborating the concept of preadaptation. In rodents, viscachas (*Lagidium viscacia*, a rodent similar to the chinchilla) living at 5000m demonstrate poor muscularization of their pulmonary arteries whereas the Himalayans marmot and the plateau pikas have thin-walled pulmonary arteries and a blunted hypoxic pulmonary vasoconstrictive response[181,210]. Cattle are often characterized by muscularized pulmonary vasculature, however, yak have very thin-walled and non-muscularized pulmonary arterioles[38].

Birds seem to increase their pulmonary arterial pressure at HA but adapted species such as bar-headed geese demonstrate a lower increase when exposed to hypoxia compared with lowland species[211].

To summarize, lowland mammals and birds demonstrate species differences but also intraspecific variations. HA generational selections seem to have favored the elimination of the pulmonary arterial pressure and selected thin-walled pulmonary arteries. Also, prolonged acclimatization to HA hypoxia induces a vasodilatation which would relieve the increased vascular resistances[212].

### 2.3.3. Blood oxygen transport

$O_2$ travels through the blood vessels to the tissues carried out by the Hb proteins contained in the red blood cells (Figure I.18).

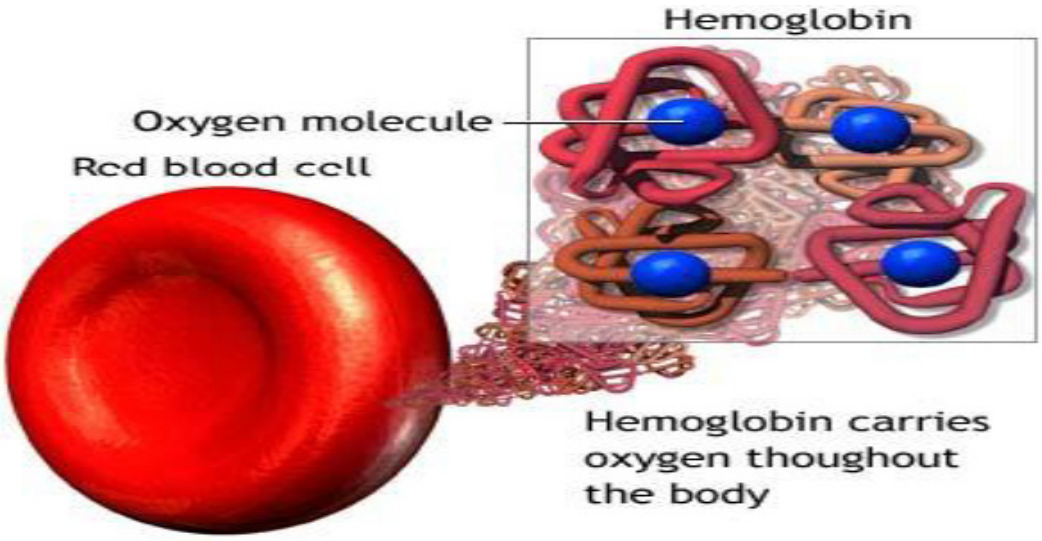


**Figure I.18: $O_2$ molecules are carried out by the Hb proteins inside the red blood cells.**
*Adapted from Clinical skills Coordinator & CNS General Medicine & Respiratory, Adult oxygen therapy. 2012.*

The $O_2$ content in the blood depends upon the $PO_2$, the Hb molecule quantity (or hemoglobin concentration – [Hb]) in the blood and the $O_2$ saturation of the arterial blood, which is dependent upon the affinity of the Hb for the $O_2$.

### *The increase in the red blood cell number*

In 1890, Viault first described a "large increase in the number of red cells in the blood" in humans lowlanders acclimated at HA for 15 and 23 days, humans HA natives, and animals living in the town of Morococha at 4540m (a town located in the Andean Plateau in South America)[65]. His observation triggered the scientific studies of human adaptations to HA hypoxia. This phenomenon was called later polycythemia. Today polycythemia refers to a pathological state where the hematocrit level (Hct) [15] is over 55%.

In hypoxia, the erythropoietin [16] stimulates the erythroid progenitor cells located in the bone marrow increasing the production of red blood cells[213], a process called erythrocytosis. If this was, at first, considered an adequate response to carry more $O_2$ molecules, we now know that if the increase in the Hct level is not properly controlled, it can lead to an increase in the blood viscosity, which can be deleterious for the heart as well as the blood circulatory vessels (because it increases the vascular resistances). Nevertheless, the degree of erythrocytosis is very variable among animals and humans living at HA. Imported species show a variable degree of erythrocytosis whereas endemic species such as camelids (llama, alpaca and vicuna) and

[15] The hematocrit blood test determines the percentage of red blood cells in the blood.
[16] The erythropoietin is a hormone (glycoprotein) that controls erythropoiesis (process which produces the red blood cells).

rodents (chinchilla, viscacha, house mouse, tuco tuco – rodents belonging to the *Ctenomys* genus –, altiplano chinchilla mouse – *Chinchillula shamae*…) show a modest or absent increase in their Hct level[65,76,214-218]. In humans, populations living in the Tibetan and Ethiopian Plateau show lower Hct when compared with the Andeans[219]. However, the Hct reported in Andean HA native is only 10% greater than the SL norms meaning that this increase does not lead to an increase in blood viscosity and vascular resistance but increases the $O_2$-carying capacities of the blood and the tissue oxygenation[155]. Nevertheless, Andean are the only indigenous highlander population to develop a pathological state of erythrocytosis called excessive erythrocytosis (EE) that have been pointed out to play a role in the development of the chronic mountain sickness (see 2.5. p62)[219,220].

Beside the Hct level, the measure of the [Hb] will give information about the $O_2$ carrying capacity of the blood. In the red blood cells, $O_2$ molecules are carried out by Hb. In mammals, Hb correspond to about 96% of the red blood cell dry content and around 35% of the total content[221]. The mammalian Hb protein can carry up to four $O_2$ molecules, which will increase by 70 fold the total blood-$O_2$ capacity compared with the $O_2$ dissolved in blood. Studies showed that endemic mammals and birds considered genotypically adapted to HA have a quite normal blood picture that, if anything, is slightly on the anemic side whereas humans and imported species all showed increased [Hb][65]. In humans, Tibetans have lower [Hb] values than Andeans[65]. In rodents, depending upon the literature, scientists reported variable data, some had increased [Hb] some didn't[65]. Morrison who studied hematological variables in several Peruvian rodents from high and low altitudes in the 60's did not find evidence of differences between SL and HA species. He hypothesized that the variability observed among hematological data

between highlanders and lowlanders might be the result of a continuous genetic mix between lowland and highland[217,218]. Today, his hypothesis is still relevant to explain the differences observed in the adaptation in Tibetan and Andean natives as Tibetans evolved in a more isolated way than Andeans, which expanded with frequent exchanges between high and low altitudes.

### *The affinity of Hb for $O_2$*

The quantity of $O_2$ transported in the blood will depend upon the affinity of the Hb protein for the $O_2$ molecules. This is reflected by the $O_2$ saturation of the arterial blood and can be determined using the oxygen-hemoglobin (or oxyhemoglobin – Hb-$O_2$) dissociation curve. The oxyhemoglobin dissociation curve describes the correlation between the $O_2$ saturation or content in Hb and the $O_2$ tension at equilibrium (the $PO_2$ at which Hb is 50% saturated with $O_2$)[222] in simple words, it is a curve that plots the proportion of Hb saturated with $O_2$ against the $PO_2$ in the blood. This curve is a useful tool to understand how $O_2$ is carried and released in the blood. If the curve is shifted to the left, it indicates an increase in the Hb-$O_2$ affinity and a shift to the right will indicate a decrease in the affinity (this is called the Bohr effect – Figure I.19).

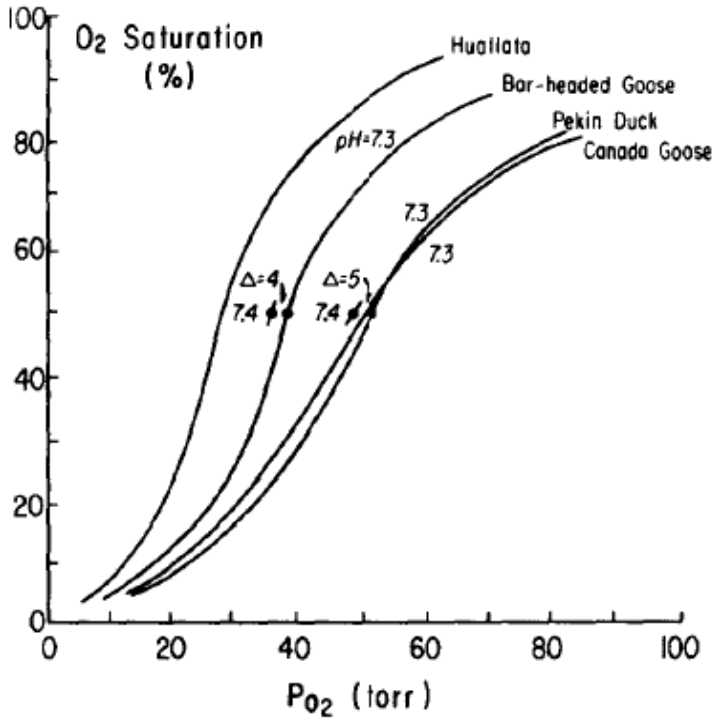


**Figure I.19: Oxyhemoglobin dissociation curve of four avian species.**
It represents the $O_2$ saturation against the arterial $O_2$ pressure. The Δ indicates changes of the P50 in torr (mmHg). The P50 represent the $PaO_2$ at which 50% of the Hb is saturated. The curve of the Bar-headed goose is shifted to the left compared with the Canada goose meaning that the former has increased $O_2$ affinity for Hb.
*Adapted from Black and Tenney, Oxygen transport during progressive hypoxia in high-altitude and sea level waterfowl, 1980[40].*

Many animal natives or acclimatized to hypoxic environment possess left-shifted curves compared with their lowland relatives whereas in HA human natives, the curve shows a shift to the right[223-225,226,57,216]. A left shift will increase the quantity of $O_2$ carried in the blood whereas a right shift will ease the release of the $O_2$ to the tissue[227].

Hb-$O_2$ affinity depends upon the intrinsic $O_2$ affinity of the Hb protein as well as the interaction of Hb with its allosteric effectors[17] (such as organic phosphates, protons and chloride ions) who decrease the Hb-$O_2$ affinity inside the red blood cells[228]. Long term adaptation in the Hb-$O_2$ affinity in endemic species is often linked to molecular changes in the Hb chain structure that can, for instance, be an amino-acid substitution[229]. These changes can alter the intrinsic Hb-$O_2$ affinity but also modify the Hb sensitivity to allosteric effectors. Also, morphological modifications of the Hb that can enhance its affinity for $O_2$ were reported in HA endemic species[230].

In species imported at HA, cattle are known to have multiple Hb systems with different arterial saturation/oxygenation properties. For example, sheep have two Hb types, A and B and sheep carriers of the Hb A have an arterial $O_2$ saturation significantly higher than sheep carriers of the Hb B[231] then one could expect that HA living sheep will express preferably the Hb A type. Other species, such as chickens were able to acquire a low P50 in only 500 years[65].

In endemic HA species, camelids have small elliptical red cells (which increase the surface area available to carry $O_2$) with high Hb concentration, resulting in a more efficient $O_2$ extraction at the tissue level (because the $O_2$ is held at a lower surface tension in the red blood cells, it allows a more easily release to the tissue)[232,233 234]; however, in camelids the high Hb-$O_2$ affinity might not represent an adaptive phenotype for HA environment specifically because it is found in all the family. Yaks do not have an increased Hb-$O_2$ affinity compared with lowland bovines, however, they have higher cooperative binding

[17] Allosteric effectors are molecules that will bind a protein to regulate its activity. The effector bindings often result in conformational changes that can for instance reveal the active site or in the contrary hide it thus enhancing or decreasing the activity of the protein.

properties of the $O_2$ to the Hb in the lungs at low altitudinal ambient tensions[38]. Carnivores such as cats, pumas and foxes, also have left-shifted Hb-$O_2$ dissociation curves[235]. In rodents, the deer mice demonstrate a Hb polymorphism for blood-$O_2$ affinity that is correlated with their altitude of life[236].

It is also interesting to observe that in descendants of HA species such as the laboratory guinea pigs currently used at low altitude, the high Hb-$O_2$ affinity characteristics remains[235].

In humans, the oxyhemoglobin dissociation curve calculated in HA natives was extensively argued in the past. Recently, Winslow et al. reviewed the literature and argued that, despite a right-shifted oxyhemoglobin dissociation curve, human natives from HA regions (either in the Andes or the Himalayans) can increase their $O_2$ affinity. This would be achieved not by changing their Hb structure but because of extreme hyperventilation and alkalosis. Indeed, HA natives hyperventilate which would induce an alkalosis in the blood; then, the net result of the P50 in HA natives is not distinguishable from SL values[237]. However, a recent study using for the first time in-vivo technics to characterize the effect of acute and chronic hypoxia on the oxyhemoglobin dissociation curve in humans HA natives showed that they also have a left-shifted curves compared with SL residents[223].

Leon-Velarde et al. demonstrated that the Hb-$O_2$ affinity characteristics not only separate HA genotypically adapted species from SL non-acclimated species, but it can also separate strains of the same species living at SL from those occupying HA niches. Nevertheless, it seems that, in imported species, it is the increased [Hb] that plays a more major role in the blood $O_2$ transport modulation than the Hb-$O_2$ affinity itself[235].

### *The other factors influencing the $O_2$ transport in the blood*

Ventilation also plays a role in the blood $O_2$ transport. Indeed, in a comparative study of high and low altitude natives mammals, Van Nice et al. observed that the HVR intensity was linked to the Hb-$O_2$ with a negative correlation between the two and Birchard and Tenney confirmed that this relationship was an interspecific property consequence of a natural selection[238,239]. This is of importance because the amount of $O_2$ carried to the tissues will depend upon the Hb-$O_2$ dissociation curve as well as the hemoglobin concentration. Then, the arterial chemoreceptor response is selected to correlate with the P50 (corresponding to the $O_2$ pressure at which 50% of the Hb will be saturated with $O_2$ molecules) so that the arterial saturation will remain protected.

It is of note that the heart also have a role in the transport of $O_2$ in the blood as varying the cardiac output (product of the heart rate and the stroke volume) could modify the blood $O_2$ content and/or the $O_2$ consumption[240)]. Nevertheless, if studies showed that the heart rate increases upon acute hypoxic exposure, it returns to normal values after acclimatization[241].

## 2.4. The diffusion and utilization of $O_2$ in the hypoxic tissues

If we sum up where we are, the $O_2$ came from the ventilation, diffused to the blood through the alveolar-capillary barrier, and is currently travelling in the blood vessels carried out by the Hb in the red blood cells. Now, in order to maintain the energetic needs of organisms living in hypoxic environments, the $O_2$ needs to be delivered to the

tissue. So at the end, the main purpose of all the adaptations that we depicted previously is to minimize the effects of hypoxia and facilitate the transport and delivery of $O_2$ to the tissues.

### 2.4.1. The $O_2$ diffusion and neo-capillarization in muscle at high altitude

To oxygenate the tissue, $O_2$ will diffuse out from the blood capillaries to the interstitial fluid, to the tissue cells and to the mitochondria where it will be consumed in the respiratory chain to produce the energy needed to achieve biological processes. With the earth elevation, there is a decline in $O_2$ tensions that can also reduce aerobic processes by reducing tissue $O_2$ distribution and mitochondrial respiration.

As the $O_2$ diffusion to the tissue is a passive process, Fick's first law of diffusion applies. Pioneer studies using rabbit muscles showed that increasing the environmental level of hypoxia (with the $PaO_2$ maintained over 52,1 mmHg) will increase the $PO_2$ of the venous effluent of muscles to values similar to the average tissue $PO_2$. However, if the level of hypoxia is further increased, the $PO_2$ of the venous effluent of muscles would become greater than the tissue's; the venous $PO_2$ being similar to the tissue $PO_2$ only during normoxia. Authors concluded that the venous $PO_2$ is greater than the tissue $PO_2$ in hypoxia, this difference being the result of the establishment of physicochemical $PO_2$ gradient from red blood cells to tissue cells[242].

In 1919, Krogh demonstrated that to understand $O_2$ delivery and utilization by tissues, it is not only important to know the diffusion constant of the gases, it is necessary to know the rate at which gases

are used in the cells of the tissue in question and the average distance which an $O_2$ molecule has to travel from a capillary into the tissue before entering into the respiratory chain. Then, based on his model, shortening the tissues diffusion distance could raise the tissues $PO_2$ which would be a valuable adaptation for organisms living in low ambient $O_2$ conditions[243]. Shortening the tissues diffusion distance will require increasing the tissues capillary density.

Pioneer studies that measured capillarization in mammal muscle tissue reported controversial results. Some reported a decrease in the skeletal muscle tissue diffusion distance in guinea pigs (Andean guinea pigs and SL guinea pigs exposed to hypoxia)[244-246], rats[247] and dogs (Andean dogs or SL dogs exposed to 435 mmHg for 3 weeks)[248]. Whereas other studies conducted a little later didn't observe any changes in the skeletal muscle capillaries density neither in rats exposed to experimental hypoxia, nor in guinea pigs at high or low altitudes exposed to hypoxia, nor in HA dogs[249-251]. The reasons used at the time to explain the differences in the results were that, because animals with different weight can have different capillary density in normoxia, and because animal exposed to chronic hypoxia often have lighter body weight than SL ones, the differences in capillary density are not necessary linked to an adaptation in the muscle fiber[65].

Recent studies reported that high and extreme altitudes have several negative consequences on muscle tissue (such as loss of muscle mass, decreased muscle oxidative capacity, accumulation of lipid peroxidation products and ROS) but it does not seem to increase capillarity in mammals[252-255]. These findings are counter-intuitive and some studies reported that highland birds have increased muscle capillarity[256-258]. In mammals, Lui et al. studied the muscle capillarity in

deer mice and observed an increase in the capillary and oxidative capacity of the animals that had highland ancestry proposing that ancestry origin might have interactive effect with hypoxia acclimatization[259].

### 2.4.2. The metabolic rate at high altitude

The other means that mammals will use to preserve $O_2$ homeostasis in hypoxia is to optimize the rate of aerobic metabolism. Metabolic processes (understand the biological processes that are required to maintain life) occur at the cellular level in the tissues and require $O_2$ that will be used in the mitochondria to provide the energy needed for optimal body functions (as described in the 1.3.1 p15, last paragraph).

The metabolic rate can be estimated using indirect calorimetry[18] by comparing the volume of $O_2$ consumed to the volume of $CO_2$ produced which give an indication about the energy produced at the cellular level. Furthermore, using the production of $CO_2$/consumption of $O_2$ ratio, we can calculate the respiratory quotient which gives us indications about the substrates used in majority to produce ATP in the cells as well as the $O_2$ requirement of the substrate see Table I.1[260]. Based on Table I.1, you can see that using fat as a fuel source requires more $O_2$ molecules consumption relative to the production of $CO_2$ molecules than using the carbohydrates metabolism.

[18] Indirect calorimetry refers to a method commonly used to estimate animals' energy expenditure based on the rate of $O_2$ consumption and $CO_2$ production.

Table 1. *Typical respiratory quotients and thermal equivalents of metabolic substrates*

| Substrate | RQ | Gas volume produced or consumed ($l\,g^{-1}$ substrate) | | Catabolized energy | | |
|---|---|---|---|---|---|---|
| | | $CO_2$ | $O_2$ | ($kJ\,g^{-1}$ substrate) | ($kJ\,l^{-1}\,CO_2$) | ($kJ\,l^{-1}\,O_2$) |
| Lipid | 0.71 | 1.43[a] | 2.01[b] | 39.7[c] | 27.8 | 19.8 |
| Carbohydrate | 1.00 | 0.80[d] | 0.80[d] | 16.7[c] | 20.9 | 20.9 |
| Protein[e] | 0.74 | 0.70 | 0.95 | 17.8 | 25.4 | 18.7 |

[a]Brouwer (1957); [b]Brody (1945); [c]Kleiber (1961); [d]Average value computed by Gessaman and Nagy (1988); [e]King (1957), for uricotelic species.

**Table I.1: Respiratory quotients and thermal equivalents of metabolic substrates.**
*Adapted from: Walsberg, G. and Wolf, B. Variation in the respiratory quotient of birds and implications for indirect calorimetry using measurements of carbon dioxide production. (1995).*

One way to maintain $O_2$ homeostasis is to decrease the rate of aerobic metabolism, which will reduce $O_2$ demand, and increase ATP production from anaerobic metabolism (glycolysis). In some endemic species adapted to hypoxia such as the naked mole rat (*Heterocephalus glaber*) reducing the metabolic rate preserve the arterial $O_2$ pressure in hypoxia. This is an active process that results from the reduction of the thermoregulatory set point[261-263]. Nevertheless, long-term reduction in the metabolic rate has been pointed out as disadvantageous for growth, reproduction or physical activity[264].

The major hypothesis of metabolic suppression would be a neurogenic origin[265-267], the CNS playing a primary role in down-regulating the metabolism. Studies using the naked mole rats showed that the reduced metabolic rate as well as the reduced core temperature occurring during hypoxia is well regulated. Moreover, the authors did not find significant differences in body temperature in normoxia compared to hypoxia exposure whereas the metabolic rate was significantly decreased in hypoxia. They suggested that the mechanisms

regulating metabolic suppression may be different form those facilitating the body core temperature variation[262]. One hypothesis being that $O_2$ itself influences cellular $O_2$ as a regulatory molecule[268].

Nevertheless, in the past, some researchers recorded the energy consumption in deer mice captured in the field either at high (3800m) or low (between 1460 and 1830m) altitudes. The results showed that the rodents used considerably more energy at HA than at low ones. Their low altitude metabolic rate was in the range of what was reported previously in other small mammals whereas the HA values were almost 50% higher than what one would expect for animals their size. The author explained the differences with differences regarding activity pattern and behavior between low and high altitude deer mice as well as the fact that HA animals had to cope with low ambient temperature and concluded that any increase in aerobic metabolism at HA will be limiting (because these animals $O_2$ consumption under baseline is close to their $VO_2$ max).

### 2.4.3. The role of the mitochondria

The mitochondria can modify the metabolism in organisms; any variation at HA in the total volume of mitochondria in the cells or in their inner membrane area has important consequences regarding the metabolic rate. The inner membrane area of the mitochondria determines the number of respiratory chain complexes that can perform the oxidative phosphorylation and produce the ATP energy molecules[269]. The amount of mitochondria is proportional to the $O_2$ consumption; however, early studies reported no changes in the amount of mitochondria in cells at HA whereas recent studies using mostly human

muscle tissue established that permanent or long-term exposure to severe environmental hypoxia decrease the muscle oxidative capacity, resulting, in the cell, in a decrease in the mitochondrial volume and content[252-254,270].

*At the end, one must keep in mind that all the steps of the oxygen cascade are related and that changes (deleterious or beneficial) in any step cannot be entirely independent from the previous and/or the next step.*

## 2.5. Could adaptation fail in high altitude natives?

In the Andes, in 1925 Carlos Monge described a disease linked to HA hypoxic environment and the role of hypoxia was clearly pointed out as indicated by the subtitle for the paper "Erythremic Syndrome of Altitude"[65,271]. This disease was first described in humans and only affects people who are natives or long-time residents of HA. If the patient moves to lower altitude most of his symptoms will disappear, but will return if the patient comes back to HA. Hence Monge considered it as a "loss of acclimatization". Other, symptoms of the Monge's disease or chronic mountain sickness (CMS) include a progressive hypoventilation resulting in an excessive hypoxemia and polycythemia, headache, excessive erythrocytosis ([Hb] over 21 g/100ml in men and 19 g/100ml in women) and low arterial saturation values (lower than 83% at an altitude of 3600m). In severe cases, arterial pulmonary pressure also rises (leading to pulmonary hypertension)[272]. CMS is found almost exclusively in the Andes natives and affects 10-15% of adult males whereas women are relatively protected before menopause[272,273]

and until today, no effective treatment besides moving to lower altitude level exists.

Today, CMS is defined as a loss of ventilatory acclimatization to HA leading to an arterial hypoxemia and polycythemia. In 2006, Vargas and Spielvoger described a preclinical form of the CMS called the excessive erythrocytosis (EE). They realized a survey of young men with EE over a 4 year period, these men showed a progressive rise in their [Hb] with a gradual development of symptoms that define the CMS[271].

Furthermore, in the 90's the comparative physiology of the loss of adaptation showed that this disease not only affects humans but also domestic animals introduced in the mountain in recent history. More recently, in 2007 Moore suggested that CMS might have a link with the occurrence of a hypoxic episode during pregnancy or newborn life[274,275] pointing out a possible role of the hypoxic environment during perinatal life in the apparition of the disease. These hypotheses were in accordance with our laboratory primary results. The PhD student, who worked in La Paz prior to my arrival, observed that rats raised at HA for several generations present physiological characteristics that could be linked to the CMS and that this phenotype can be reversed if the rats were raised at an $O_2$ percentage mimicking the SL $PO_2$ during 2 weeks after birth[2].

# 3. Research hypotheses

Rats and mice have a very different range of distribution at HA. Indeed, according to ecological reports from different continents, no rats, no matter the strain or species (*Rattus rattus, Rattus norvegicus*), are found in high ecological niches whereas several species of mice

including the house mice (*Mus musculus*) are found living and reproducing in altitudes above 4000m[31,32,276,277]. Nevertheless, despite this very different distribution, early HA studies often used rats to understand physiological responses in hypoxic environment.

Today, scientists know the importance of studying native population in their natural environment and use almost exclusively endemic species (either at HA or SL) to understand the physiology of HA environment. However, rats and mice, because of their easy access (as laboratory animals that can be ordered, received and used for experimental procedure in a 2-week period), and/or their easy genetic manipulations (with, for example, the knock out mice) still have an important place in the understanding of hypoxic responses in organisms. Indeed, rats and mice are often used either as comparative for HA endemic species (to understand HA physiology) or in medical research, to understand diseases caused by, or resulting in, hypoxic episodes (such as the chronic obstructive pulmonary diseases).

In South America, rats and mice were introduced over the last eight to five centuries by human migrations[277,278,279]. The ability of a species to survive and perform aerobic physical activities (such as escaping predators, searching for food, procreate, build a nest…) under extreme altitude (understand extreme altitude as altitude over 4000m) depends on a range of modifications that can be observed at the entire organism level and the ones that occur at the cellular and molecular levels. These adjustments can be achieved either by genetic changes in response to selection (evolution) or by plastic phenotypic adjustments to conditions experienced within the lifetime of individuals (either reversible – phenotypic plasticity – or irreversible)[280]. A study comparing hemoglobin chains in wild mice caught in La Paz (Bolivia, 3600m) with

other specimens at SL (Lima, Peru)[277] observed that HA mice did not display adaptive traits. This suggests that the ability of mice to withstand HA hypoxic environment might be dependent from predisposition and/or plastic phenotypic adjustments. Therefore, it seems relevant to step back and observe what happened at the organism level to try and understand why mice are able to cope with ambient hypoxic conditions in the wild life whereas rats are not. Studying the physiological responses at HA or in response to chronic and acute hypoxia might provide some insight in the matter.

In fact, in the past 30 years, studies regarding the mechanisms underlying physiological adjustments to chronic hypoxia has revealed a fascinating picture of numerous plastic, morphological, biochemical, and functional changes in the organs implicated in the hypoxic responses both during neonatal development and in adulthood. Immediately after birth and within the period of postnatal maturation, there is a critical period called the "critical time window" where environment can modulate structural and functional properties of the biological systems. For example, neonatal chronic hypoxia will affect or delay the maturation of the central respiratory neuronal network, induce delays in the maturation of the CB and reduce the HVR[94]. Furthermore, in mammalian species not naturally residing at HA, perinatal exposure to hypoxia is known to reduce birth weight and slows lung development. These changes can be permanent and may be irreversible in adulthood[1].

The main purpose of the work performed during these 4 years was to understand how species with different physiological characteristics respond to the hypoxic challenge present at HA. We questioned the role of physiological plasticity in successful colonization of hypoxic

environment by comparing physiological responses of the organs involved in gas-exchange and circulation between common laboratory rats and mice. We choose to use common laboratory rats and mice as we had the very unique opportunity to access laboratory animals that had been bred at HA for more than 20 years and 30 generations in Bolivia, in the city of La Paz at 3600m (inspired $PO_2$ – $PiO_2$ – of ≈100 mmHg). Rats and mice originally came from IFFA CREDO France, and the rats were imported in Bolivia by my Director Dr. Joseph when he was at the dawn of his scientific life. The specific hypothesis leading each of the studies described in this thesis are exposed hereafter.

1- Ecological studies showed that rats and mice have a different range of distribution at altitude but no data were available to explain this discrepancy and no systematic comparison of the physiological responses to environmental hypoxia between rats and mice existed. Our first hypothesis was that mice and rats that have been raised at HA for several generations would display different physiological responses to the environment. To assess this hypothesis, we compared physiological responses between adult rats (*Rattus norvegicus,* Sprague-Dawley strain) and mice (*Mus musculus,* FVB strain) that have been living for more than 30 generations at HA. We compared ventilatory and metabolic responses to ambient air and graded level of hypoxia (18, 15 and 12% $O_2$ for 10 min each) but also in response to 32% $O_2$ for 15 minutes (in La Paz, at 3600m, 32% $O_2$ correspond to a $PiO_2$ of 21% at SL – ≈160 mmHg). We assessed the lung architecture, right ventricular hypertrophy (a sign of arterial pulmonary hypertension) and Hct and Hb levels. As today, no previous studies had compared basic physiological responses to hypoxic environment between rats and mice that had been living under chronic hypoxia for several generations. This first study was

successfully published and an integrated version of this paper is presented in chapter 2.

*The results of this study showed that HA mice had a phenotype well suited for life under hypoxic conditions. There results are in line with the ecological reports demonstrating that mice, but not rats, are found in the wild life at HA. From this study, we postulated three hypotheses to explain the divergent physiological responses between HA rats and mice:*

2- We hypothesized that mice present signs of physiological predispositions to withstand hypoxic environmental conditions that are not present in rats. To this aim, we compared the lung morphology, hematological and cardiac variable in SL animals. We recorded the arterial $O_2$ saturation and heart rate in response to graded levels of hypoxia (18, 15, 12 and 9% $O_2$ for 10 min each). Also, because HIF expression in the brain tissue is at a maximum level after 4-6 hours of exposure to hypoxia, we exposed rats and mice to different levels of hypoxia (15 and 12% $O_2$ – 6h each – sustained hypoxia), or room air (21% $O_2$) to address their ventilatory and metabolic responses and expression of HIF 1$\alpha$ in the brainstem. The study was performed in adult male Sprague-Dawley rats and FVB mice raised at SL (Quebec, Canada). The manuscript in preparation for publication is presented in chapter 3.

3- During postnatal life, environment can have numerous consequences on a wide variety of biological systems. Based on the previous work achieved at the laboratory by Dr Lumbroso, we knew that most of the phenotype observed at HA in the adult rat can be improved if rats are raised at a SL $PO_2$ during the first two postnatal weeks of their life[1,2]. In rodents, the alveolarization phase of the lungs maturation takes place between the 4$^{th}$ and the 14$^{th}$ day after birth[169,170]. Thus, we

hypothesized that phenotypical plasticity occurring in response to hypoxia during the postnatal life, might explain part of the physiological differences we observed between rats and mice living at HA. To test this hypothesis, we used newborn FVB mice and Sprague-Dawley rats raised between the postnatal days 4-14 (P4-P14) under normoxia or at a $PiO_2$ that would be similar to the ambient conditions in La Paz (13,5% $O_2$ corresponding to a $PiO_2$ of ≈100 mmHg). The manuscript in preparation for publication is presented in chapter 4.

4- Finally, when we compared the lung architecture between adult rats living at SL and HA we observed that rats raised at HA for 35 generations had similar lung volume and alveolar surface area than SL rats whereas, raising SL rat in postnatal hypoxia had deleterious consequences regarding the lung architecture. Thus, our last hypothesis was that raising rats under chronic hypoxia for several generations lead to a certain form of adaptation (that might depend upon epigenetic mechanisms) that preserve the lung architecture. To test this hypothesis, we used newborn Sprague-Dawley rats raised at HA (La Paz, Bolivia) or SL (Quebec, Canada) between P4-P14. In Canada, the animals were exposed to normoxia or to a $PiO_2$ that would be similar to the ambient conditions in La Paz ($PiO_2$ ≈100 mmHg $O_2$ or 13.5 % $O_2$ in inspired air at SL). In Bolivia, the animals were exposed to ambient air or to a $PiO_2$ that would be similar to the SL ambient condition ($PiO_2$ ≈160 mmHg or 32% $O_2$ in inspired air in La Paz, 3600m above SL). Prior to, and, at the end of, the postnatal hypoxic exposure, we recorded the arterial $O_2$ saturation in 4 and 14 days old SL and HA laboratory rats. Then, we compared right ventricular hypertrophy (a sign of pulmonary hypertension) and lung architecture in P15 rats living at SL or HA.

# II. CHAPTER 2

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## DIVERGENT PHYSIOLOGICAL RESPONSES IN LABORATORY RATS AND MICE RAISED AT HIGH ALTITUDE

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# 1. Abstract

Ecological studies show that mice can be found at high altitude (HA - up to 4000m) while rats are absent at these altitudes, and there are no data to explain this discrepancy. We used adult laboratory rats and mice that have been raised for more than 30 generations in La Paz, Bolivia (3600m), and compared their hematocrit levels, right ventricular hypertrophy (index of pulmonary hypertension), and alveolar surface area in the lungs. We also used whole body plethysmography, indirect calorimetry, and pulse oximetry to measure ventilation, metabolic rate ($O_2$ consumption and $CO_2$ production), heart rate and pulse oximetry $O_2$ saturation ($P_{O2,sat}$) under ambient conditions, and in response to exposure to sea level $PO_2$ (32% $O_2$ = 160 mmHg for 10 minutes), and hypoxia (18 and 15% $O_2$ = 90 and 75 mmHg for 10 minute each). The variables used for comparisons between species were corrected for body mass using standard allometric equations, and are termed mass-corrected variables. Under baseline, compared with rats, adult mice had similar levels of $P_{O2,sat}$, but lower hematocrit and hemoglobin levels, reduced right ventricular hypertrophy, and higher mass-corrected alveolar surface area, tidal volume and metabolic rate. In response to sea level $PO_2$ and hypoxia, mice and rats had similar changes of ventilation, but metabolic rate decreased much more in hypoxia in mice, while $P_{O2,sat}$ remained higher in mice. We conclude that laboratory mice and rats that have been raised at HA for > 30 generations have different physiological responses to altitude. These differences might explain the different altitude distribution observed in wild rats and mice.

# 2. Introduction

Physiological changes at high altitude (HA) are required to optimize the diffusion, transport, and cellular utilization of $O_2$ and counteract the ambient low $O_2$ partial pressure ($PO_2$). In endemic species that have been living for million of years at altitude, physiological adaptations are thought to be linked to genetic selection, and are mostly characterized by a low or absent pulmonary hypertension[281], elevated affinity of hemoglobin (Hb) for $O_2$[282,283], higher density of micro-vessels and mitochondria surface area in the heart[284], left-ventricle hypertrophy that could lead to increased stroke volume[285], and hematocrit (Hct) level that remains within the sea level (SL) range[65,286]. Contrastingly, some species originating from SL have raised Hct level, drastic pulmonary hypertension, or hypertrophy of the heart when exposed to HA[281], illustrating the fact that these species might have a genetic pre-disposition that would interfere with survival at HA, and present a "genetic barrier" for colonization of HA regions.

Interestingly, ecological reports from South America and New Zealand indicate that mice (*Mus musculus*) are present up to an altitude of 4000m[277], while rats (*Rattus norvegicus*) are notably absent at HA[31,32,287]. In these two regions, rats and mice have been introduced over the last eight to five centuries by human migrations[278,277]; accordingly, comparative studies between rats and mice might be useful to further understand the physiological responses at altitude in species that are not "high altitude native", but might nonetheless have different success for life at altitude.

Over the past 20 years, we have been able to raise laboratory rats (Sprague-Dawley) for more than 30 generations, in laboratory conditions at an altitude of 3600m above SL, in La Paz, Bolivia. These

animals present an elevated Hct and Hb levels, a right ventricular hypertrophy (a sign of pulmonary hypertension), an altered alveolar structure with enlarged airspaces in the lungs, and impaired respiratory control[2]. Considering that elevated Hct and pulmonary hypertension impair survival and lead to right heart failure[2,286,288], this might explain why rats have not been able to establish stable colonies in HA regions under natural conditions.

In the present study, we asked whether laboratory mice with a similar history of life at HA have different physiological responses than rats. To this aim we compared arterial oxygen saturation, hematological, cardiac, ventilatory, metabolic, and lung characteristics in adult mice and rats from a population that have been born and raised at an altitude of 3600m (La Paz, Bolivia) since 1992. The study was performed in males and females to address whether sex-specific physiological differences would be present in mice and rats at HA.

# 3. Materials and methods

## 3.1. Animals

We used adult (2-3 month old) male and female rats (6 animals of each sex) and mice (10 animals of each sex) from different breed. Mice were obtained from the Instituto Nacional de Laboratorios de Salud (INLASA, La Paz, Bolivia). These mice are descendants of a lineage of animals that were originally imported from France (IFFA-CREDO) 20-25 years ago. Because the genetic background of the mice was not available, we performed a complete genetic analysis on a sample of DNA extracted from the lungs of an adult male mouse, by using the background characterization offered by Charles-River (St Constant,

Québec, Canada). This analysis compares allele variations of 384 single-nucleotide-polymorphisms of the unknown DNA to the DNA of 40 common inbred and F1 hybrid strains. The analysis indicated that the mice scored as 73.11 % FVB, and that they are either a mix of FVB and 2 other strains, or an outbred strain.

Rats were Sprague-Dawley from the Instituto Boliviano de Biologa de Altura (IBBA, La Paz, Bolivia). These animals were originally imported from France (IFFA-CREDO) in 1992, and constantly bred at the IBBA (for the present study, we used rats from the 30$^{th}$ generation).

Animals were housed under standard conditions, had access to food and water ad-libitum, and were exposed to a 12h:12h light/dark cycle. After they were transported from INLASA to the IBBA, mice were left undisturbed for 1 week before starting the experiments. All protocols were reviewed and approved by the scientific committee of IBBA in Bolivia and are in concordance with the guidelines of the Canadian Council of Animal Care.

## 3.2. Recording of ventilatory parameters, arterial oxygen saturation, and heart rate in unrestrained, unanesthetized rats and mice at high altitude

The animals were placed in a whole-body, flow-through, plethysmograph chamber for mice or rats (Emka Technologies, Paris, France) that was constantly flushed with fresh room air and previously calibrated by injecting a known volume of air (0.5 ml for mice, 5 ml for rats). The respiratory flow trace was recorded using a differential pressure transducer (ML141, ADInstruments, Colorado Springs, CO,

USA). The flow of air through the chamber was set and continuously monitored at 200 ml/min (mice), or 1500 ml/min (rats) using a pump and gas flow restrictor/monitor (G265, Qubit systems, Kingston, ON, Canada). Inlet and outlet gases were alternately subsampled, directed toward a water pressure analyzer (RH-300, Sable Systems, Las Vegas, NV, USA), dried and directed to an oxygen/carbon dioxide analyzer (ML206 Gas analyzer, ADInstruments) for respiratory gas analysis. All signals were directed toward a PowerLab acquisition interface for analog-to-digital conversion and storage on a computer running the LabChart 5 software (ADInstruments).

Before each experiment, rectal temperature was measured, and the animal was weighed, and then fitted with a limb sensor for continuous recordings of pulse oximetry capillary oxygen saturation ($P_{O2,sat}$) and heart rate (MouseSTAT – Kent Scientific, Torrington, CT, USA). The animal was then placed in the chamber for a period of tranquilization (10-15 min), and baseline recordings were initiated for 20 min, followed by acute exposure to 32% $O_2$ – corresponding to the SL $PO_2$ – for 10 min. The animal was then exposed to graded levels of hypoxia (18, 15, 12% $O_2$ for 10 min each). The mean barometric pressure in La Paz being around 490 mmHg, these percentage $O_2$ values correspond, respectively, to a partial pressure of inspired $O_2$ ($PiO_2$) of 90, 75, and 60 mmHg. At SL (barometric pressure=760 mmHg), these $PiO_2$ would be equivalent to 12, 10, and 8% $O_2$ in inspired air, respectively. Rectal temperature was measured immediately at the end of the last hypoxic exposure. Tidal volume ($V_T$) was calculated from the integrated flow trace as previously described[1,2], by using standard equations[289]. All values were obtained while the animal had a stable breathing pattern during baseline recordings and within the last 3 minutes of each condition.

$O_2$ consumption ( $\dot{V}_{O_2}$ ) and $CO_2$ production ( $\dot{V}_{CO_2}$ ) rates were calculated using the following equations [290]:

$$O_2 consumption = \frac{flow \times \left[ \left( O_{2_{in}} - O_{2_{out}} \right) - O_{2_{out}} \times \left( CO_{2_{out}} - CO_{2_{in}} \right) \right]}{\left( 1 - O_{2_{out}} \right)}$$

$$CO_2 production = flow \times \frac{\left[ \left( CO_{2_{out}} - CO_{2_{in}} \right) - CO_{2_{out}} \times \left( O_{2_{in}} - O_{2_{out}} \right) \right]}{\left( 1 - CO_{2_{out}} \right)}$$

where "flow" is the flow of air measured before entry into the chamber, "$O_{2,in}$" and "$CO_{2,in}$" are the gas fractions in the inflowing air (considered at 20.9% and 0.038%, respectively), and "$O_{2,out}$" and "$CO_{2,out}$" are the gas fractions measured in the outflowing line. The respiratory exchange ratio was calculated as $CO_2$ production / $O_2$ consumption.

## 3.3. Hematological parameters, dissection of hearts and lungs

A sample of blood was drawn from the tail. The hematocrit (Hct) was measured by microcentrifugation (Micro-MB centrifuge – International Equipment Company, USA) for 15-20 minutes, and hemoglobin concentration (Hb) was determined by using a Hemocue field spectrophotometer (Agelholm, Sweden). All samples were processed in duplicate for rats, and for mice if enough blood was obtained.

Following blood sampling, animals were anesthetized by an intraperitoneal injection (0.1 ml/100g of body mass) of ketamine (87.5 mg/ml) and xylazine (12.5 mg/ml) then perfused through the left ventricle with ice-cold PBS (pH 7.2) at a constant pressure of 24 $cmH_2O$ for mice and 35 $cmH_2O$ for rats. The heart was quickly dissected and

weighed. The atria were separated from the ventricles, then the right ventricle (RV) was cut off from the left ventricle (LV, which was left with the cardiac septum, S)[28]. We weighed the ventricles separately (RV and LV+S) and these values were used to measure the ratio of RV/(LV+S), an index of right ventricular hypertrophy and pulmonary hypertension. In 4 males and 4 females of each species, after cardiac perfusion with PBS, a catheter was fixed in the trachea and the lungs were inflated with 4% PFA for 30 minutes at a constant pressure of 24 $cmH_2O$, then dissected. The total volume of the inflated lungs was measured by liquid displacement, and they were kept in 4% PFA for 24h at room temperature. The next day, the lungs were separated into left and right lung (for mice) and 5 lobes (for rats), which were dehydrated in alcohol (1h in 65% alcohol solution, then 2h in each graded alcohol solution: 75%, 85%, 95% and 100%); alcohol was then replaced by xylol (two baths, 1h each), and paraffin (two baths, 1h each and a final overnight bath). The samples were included in paraffin and shipped to Quebec City where they were processed to determine lung histology.

## 3.4. Lung histology

Paraffin-embedded lungs were cut at a thickness of 5 µm using a microtome (Jung RM2065 Leica instruments GmbH, Germany). Sections were then mounted on glass slides and dried for 24h in room air. Slices were deparaffinized in toluene baths (2 x 10 min), and re-hydrated by successive immersions in alcohol 100% (x2), 95%, 70%, 50%, and in water before being colored with Harris hematoxylin solution (VWR international) for 3 min, rinsed in water for 1 min, exposed in acid-alcohol solution (five successive immersions in 1% HCl, 70% ethanol), washed with water for 1 min, dipped in Bluing Reactive RTU (VWR

international) and then in water for 1 min. The slides were mounted in water-based mounting medium Liquid Coverglass SHUR/Mount™ (EMS, Hatfield, PA, USA). The images were captured using a Nikon eclipse E600 digital imaging system at a magnification of x100.

## 3.5. Lung morphology

We randomly selected 3 non-overlapping images from each slide using 3 slides per animal and 8 animals per group (4 males and 4 females). The Mean Linear Intercept ($L_m$) was determined by overlapping a grid of 20 horizontal and vertical lines (189 µm each) on each image and by counting the number of intersections with alveolar walls[291]. When a line crossed a vessel wall rather than an alveolar wall, it was counted as 0.5 intersection. $L_m$ was calculated by using the following equation: $L_m=(Nd)/m$, with $N$ being the number of line (20), $d$ the length of each line (189 µm), and $m$ the number of intersections with alveolar walls. From $L_m$ values, we calculated the relative alveolar surface area as $S(m^2/cm^3)=4V/L_m$, with $V$ being the volume of one image[291]. An estimation of the total alveolar surface area was calculated as the product of the relative alveolar surface area and lung volume (measured by water displacement after fixation).

## 3.6. Allometric scaling in rats and mice

To compare physiological and morphological values between control mice and rats, we used allometric scaling, a standard approach to compare animals of different size[292,293]. The allometric scaling variables ($b$ in the equation below) were obtained by calculating the

slope of a regression line fitted through a log-log plot of a parameter ($x$) as a function of body mass ($M$). This allowed us to obtain an equation of the following form: $x=aM^b$. From this equation, we expressed and compared reported mass-specific variables with $M^b$. For the respiratory variables we used the scaling variable calculated by Stahl[292], which are: lung mass and lung volume ($b$=1), minute ventilation ($b$=0.8), $V_T$ ($b$=1.04), respiratory frequency and heart rate ($b$=-0.25), $O_2$ consumption and $CO_2$ production ($b$=0.76). For relative and total alveolar surface area we respectively used ($b$=-0.13) and ($b$=0.88) as reported for mammals by Maina[293]. In the text, data corrected for the allometric scaling variables are referred to as mass corrected whereas data compared for body mass are referred to as mass specific.

## 3.7. Sea level values

Expected SL values for a selected series of variables are presented in Table II.1. These values were either selected from the literature or were obtained from our colony of SL Sprague-Dawley rats and FVB mice (see chapter 3 p103). Values of lung morphology were obtained by using the approach described above. These values are mostly informative, and we have not made statistical analysis to compare HA vs. SL values.

## 3.8. Statistical analysis

We used GraphPad Prism 6.0c (for 2-way ANOVA and *post-hoc* analysis) and JMP 11.0 (for 2-way-ANOVA with repeated measures) for

statistical analysis. All values are reported as means±s.e.m., and the significant *P*-value was set a 0.05.

For the Hct, right ventricular hypertrophy, and lung morphology data, we first performed 2-way ANOVAs with species and sex as grouping variables. When significant effects, or a significant interaction between species and sex appeared, a *post-hoc* analysis was performed (Fisher's LSD).

For variables measured at different levels of $PiO_2$ we used a MANOVA model (in JMP) with species and sex as grouping variables and $PiO_2$ as the repeated term. When no significant effect of sex, or significant interactions between sex and species, or sex and $PiO_2$ appeared for these values, data from males and females were pooled. When significant species effects appeared, a *post-hoc* analysis was performed for each $PiO_2$ level (Fisher's LSD) to determine the effects of species, or for each group to determine the effects of $PiO_2$ level. *P*-values are reported in the figures with the following general pattern: **P*<0.05, ***P*<0.01, ****P*<0.001, and *****P*<0.0001, respectively.

# 4. Results

## 4.1. Physiological parameters, hematology, and lung architecture in high altitude rats and mice

### 4.1.1. Arterial oxygen saturation, heart rate, and hematology

Under baseline conditions, rats and mice had $O_2$ saturation ($P_{O2,sat}$) values around 80%, without significant effect of species or sex (Figure II.1A). Mice had a lower right ventricular hypertrophy compared with

rats ($P$<0.0001 for species, Figure II.1B). Heart rate was higher in mice than in rats ($P$<0.0001 for species, Figure II.1C), but when the difference in body mass was taken into account by applying the allometric scaling factor (heart rate in beats/min/mass in $g^{-0.25}$ – see materials and methods), mice had a lower mass-corrected heart rate than rats ($P$<0,0001 for species, Figure II.1D), and female rats had a lower mass-corrected heart rate than male rats (Figure II.1D).

Compared with rats, HA mice showed lower Hb concentration ($P$<0.0001 for species, Figure II.1E), and Hct ($P$<0.0001 for species, Figure II.1F) values. Interestingly, the Hb value in mice was below the normal range of the SL value (see Table II.1) while Hct was slightly higher than the normal SL range (Table II.1). In rats, both Hct and Hb values were above the normal SL range (Table II.1). Compared with males, females of both species had lower Hct ($P$<0.0001 for sex, Figure II.1E), and female rats had lower Hb values than male rats (Figure II.1F).

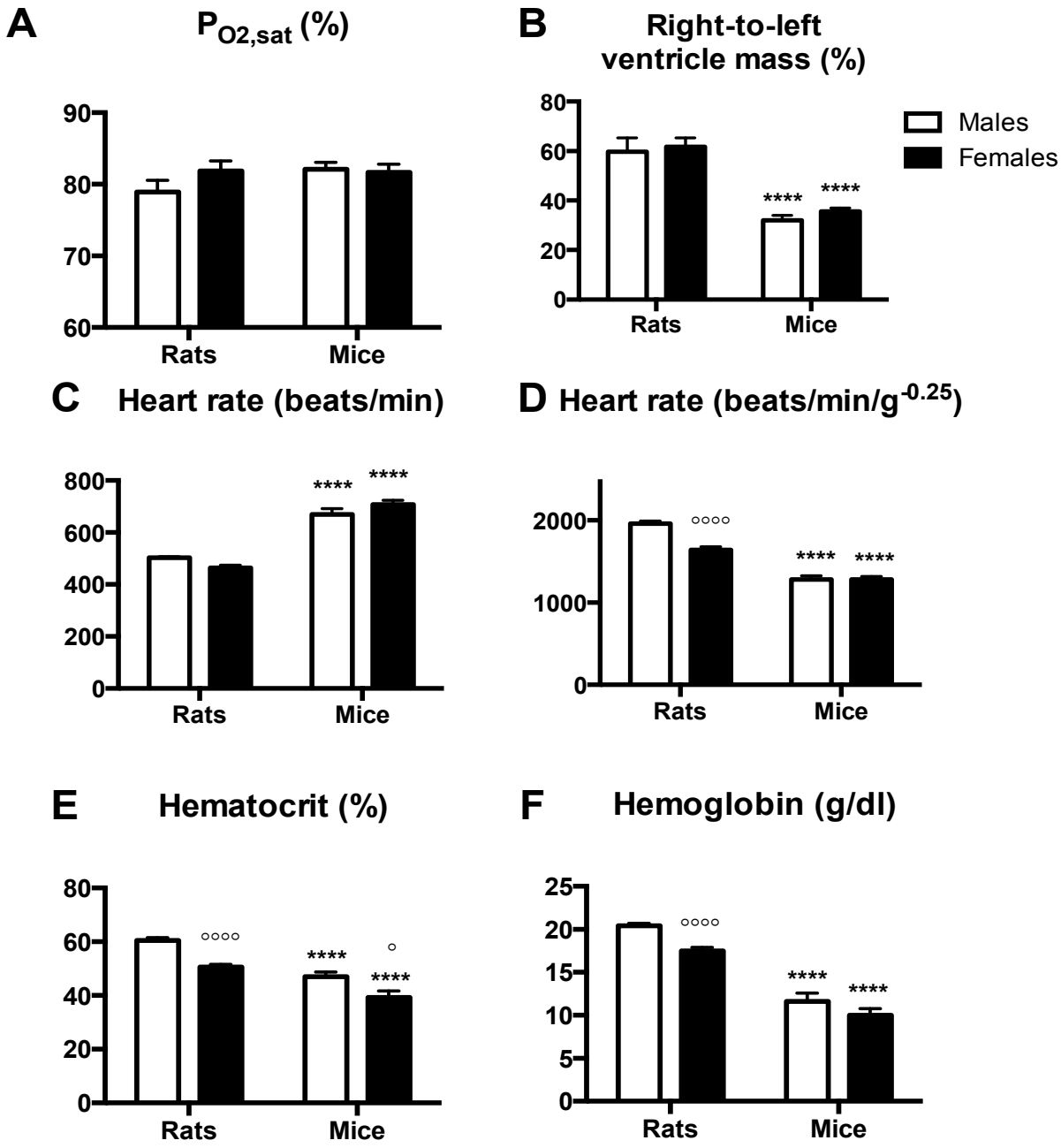


**Figure II.1: Hematological variables, right ventricular hypertrophy, heart rate and arterial saturation in high altitude rats and mice.**
(A) arterial $O_2$ saturation ($P_{O2,sat}$ – %), (B) right-to-left ventricle ratio (%), (C) heart rate (beats/min), (D) mass-corrected heart rate (beats/min/$g^{-0,25}$), (E) hematocrit (Hct – %), and (F) hemoglobin (Hb – g/dl), in 2-month-old male and female rats and mice living at high HA for 30 generations. Means+s.e.m. ****$P$<0.0001 mice versus rats. °$P$<0.05 and °°°°$P$<0.0001 females versus males.

### 4.1.2. Minute ventilation, tidal volume, and respiratory frequency

Compared with rats, mice had a higher mass-specific minute ventilation ($V_E$), tidal volume ($V_T$), and respiratory frequency (fR – $P<0.0001$ for species, Figure II.2 A-C). Female mice had a higher mass-specific $V_E$ ($P=0.04$ for sex) and $V_T$ ($P=0.04$ for sex) than male mice (Figure II.2 A-B). When corrected for body mass using standard allometric corrections, there was a significant effect of species ($P<0.0001$) and sex ($P=0.04$) for $V_E$: male mice had a similar mass-corrected $V_E$ and fR than male rats, but higher mass-corrected $V_T$ (Figure II.2 D-F). Female mice had a higher mass-corrected $V_E$ and $V_T$ compared with male mice (Figure II.2 D-E). Mass-corrected fR was lower in female rats compared with male rats (Figure II.2F), but there was no significant effect of species.

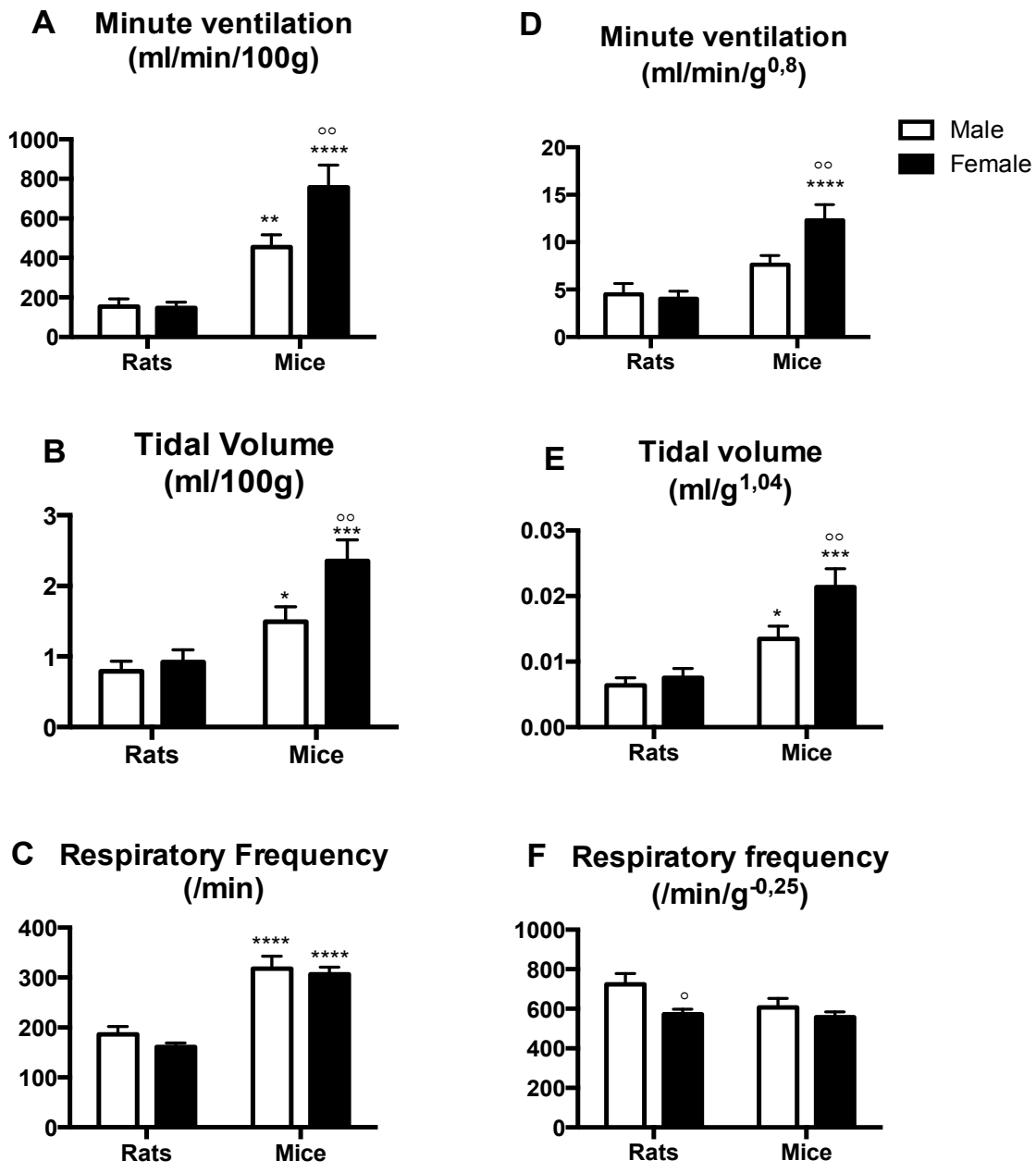


**Figure II.2: Ventilatory variables under baseline conditions (21% $O_2$) in high altitude rats and mice.**

(A,D) minute ventilation ($V_E$ – ml/min/100g and ml/min/$g^{0,8}$), (B,E) tidal volume ($V_T$ – ml/100g and ml/$g^{1,04}$), (C,F) respiratory frequency (fR – breaths/min and breaths/min/$g^{-0,25}$), in 2-month-old rats and mice. A and B are mass-specific values, and D-F are mass-corrected values; means+s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 mice versus rats. °*P*<0.05 and °°*P*<0.01 females versus males.

### 4.1.3. Metabolic rate, respiratory exchange ratio and body temperature

Compared with rats, mice had a higher mass-specific (Figure II.3 A,B) or mass-corrected (Figure II.3 C,D) $O_2$ consumption ( $\dot{V}_{O_2}$ ) and $CO_2$ production ( $\dot{V}_{CO_2}$ ) (*P*<0.0001). The respiratory exchange ratio ( $\dot{V}_{CO_2}/\dot{V}_{O_2}$ ) was higher in rats than in mice (*P*<0,0002 for species, Figure II.3E). Body temperature measured before the onset of respiratory and metabolic recordings was similar in rats (35.2±0.2°C) and mice (35.2±0.2°C – Figure II.9C). There was no effect of sex for metabolic variables.

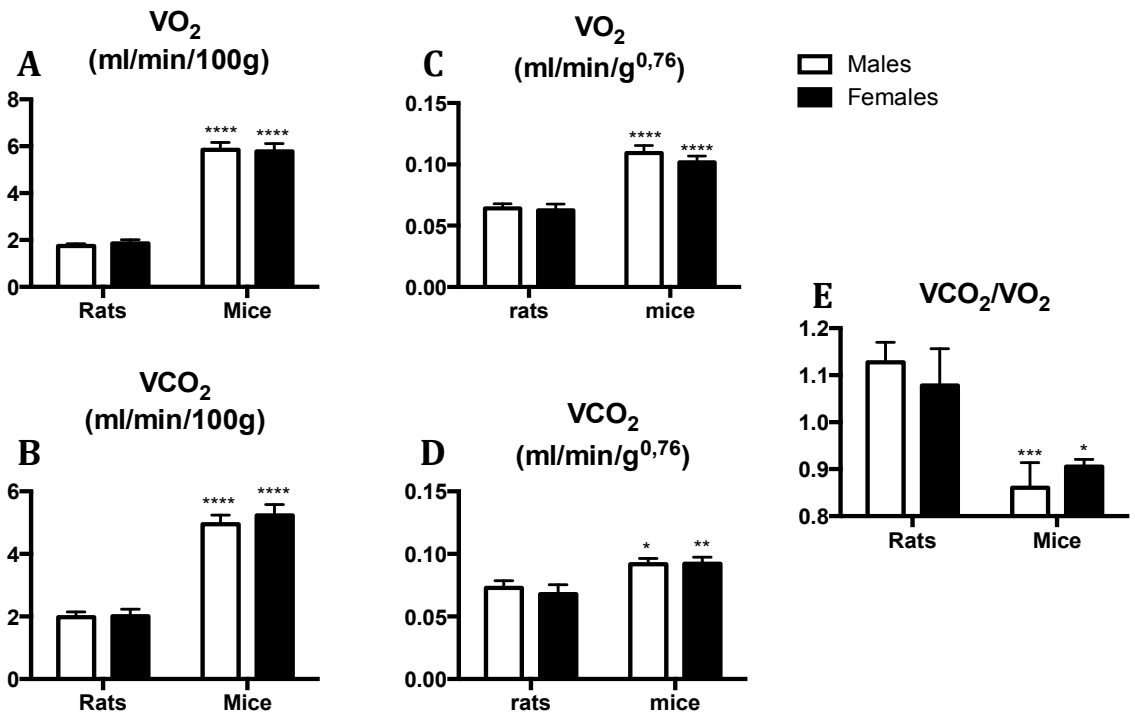


**Figure II.3: Metabolic variables under baseline condition (21% $O_2$) in high altitude rats and mice.**

(A,C) $O_2$ consumption ( $\dot{V}_{O_2}$ – ml/min/100g and ml/min/$g^{0,76}$), (B,D) $CO_2$ production rate ( $\dot{V}_{CO_2}$ – ml/min/100g and ml/min/$g^{0,76}$) in 2-month-old rats and mice. A and B are mass-specific values, C and D are mass-corrected values. (E) Respiratory exchange ratio ( $\dot{V}_{CO_2} / \dot{V}_{O_2}$ ). Means+s.e.m.

**P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 mice versus rats.

### 4.1.4. Lung volume, lung mass, and lung architecture

Compared with rats, mice had a higher mass-specific lung volume and lung mass (*P*<0.0001 for species, Figure II.4 A-B). Because the allometric scaling parameter is 1 for lung volume and lung mass (see materials and methods), mass-specific values allow direct comparison between species of different body mass. Representative lung images are presented in Figure II.5A (for rats) and Figure II.5B (for mice): note the

enlarged air spaces in rats compared to mice. The mean linear intercept ($L_m$) was lower in mice than in rats ($P<0.0001$ for species, Figure II.5C), which was reflected in higher mass-corrected relative and total alveolar surface area in mice ($P<0.0001$ for species, Figure II.5 C-D). There was no effect of sex for these variables.

Because alveolar surface area is directly proportional to $O_2$ consumption[163], we also calculated the ratio of the total alveolar surface area to the $O_2$ consumption ($m^2$/ml $O_2$ consumed in 1 minute). There was no significant effect of sex ($P=0.58$ for sex) or a significant sex x species interaction. The mean value was higher in mice than in rats (Figure II.6), and both values were higher than the expected sea level values (Table II.1).

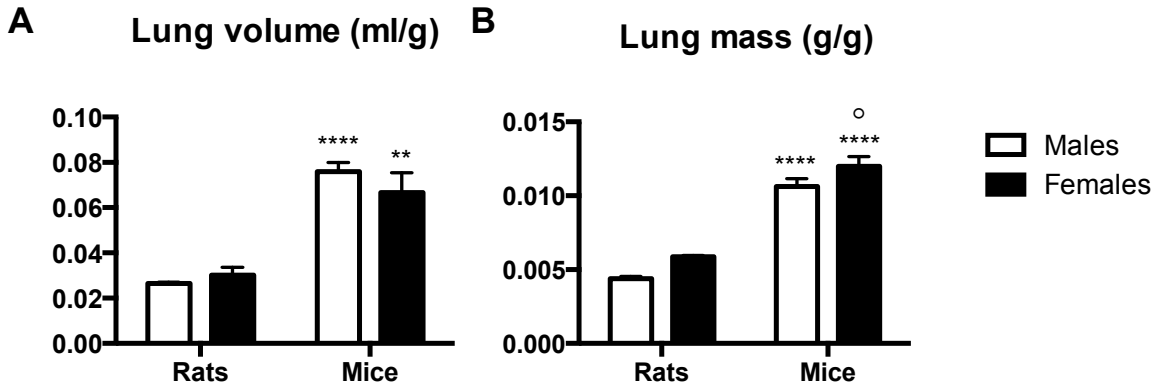


**Figure II.4: Lung morphology variables in high altitude rats and mice.**
(A) mass-specific lung volume (ml/g), and (B) mass-specific lung mass (g/g) in 2-month-old rats and mice. Means+s.e.m.
**$P<0.01$, and ****$P<0.0001$ mice versus rats. °$P<0.05$ females versus males.

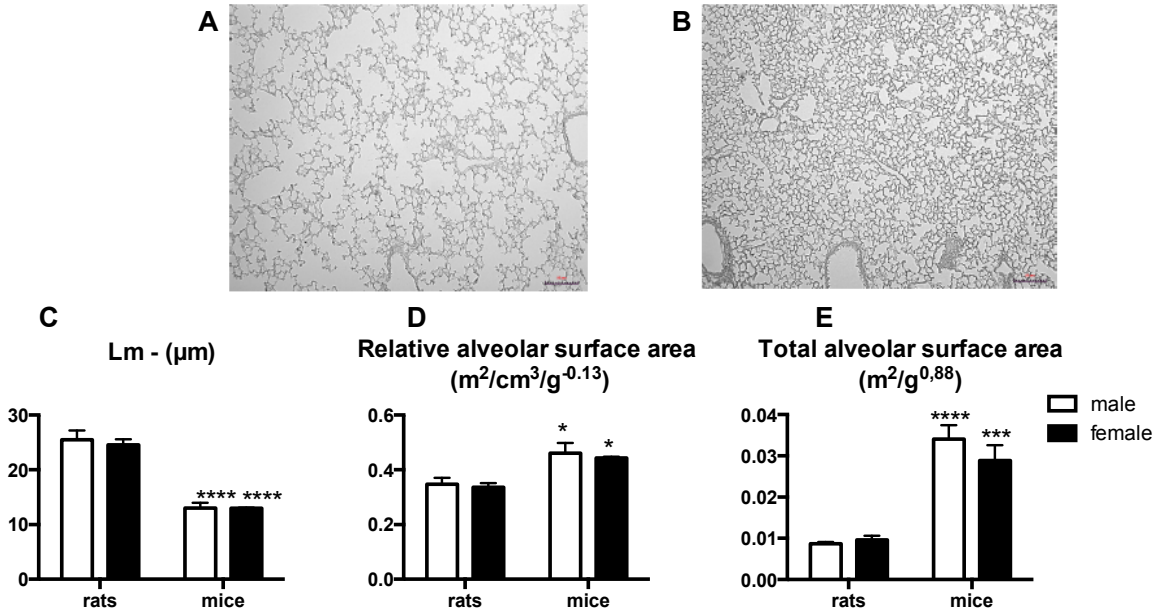


**Figure II.5: Lung architecture variables in high altitude rats and mice.**
Typical image of the lung architecture obtained for rats (A) and mice (B). (C) mean linear intercept ($L_m$ – µm), (D) mass-corrected relative alveolar surface area ($m^2/cm^3/g^{-0.13}$) and (E) mass-corrected total alveolar surface area ($m^2/g^{0.88}$) in 2-month-old rats and mice. Means+s.e.m.
Scale bars in A and B, 50 µm.
*$P$<0.05, ***$P$<0.001 and ****$P$<0.0001 mice versus rats.

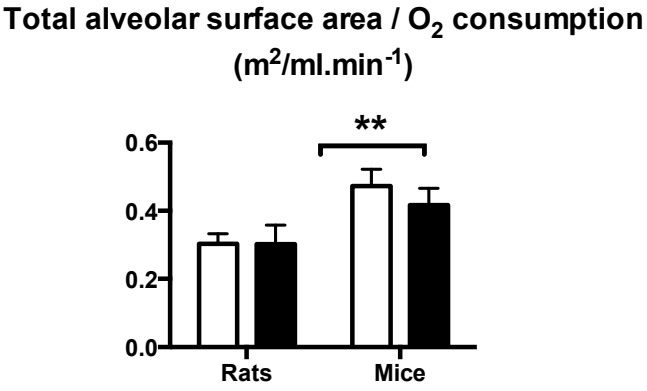


**Figure II.6: Total alveolar surface area to $O_2$ consumption ratio ($m^2$/ml/min) in high altitude rats and mice.**
Means+s.e.m.
**: $P$<0.01 mice versus rats.

## 4.2. Respiratory, metabolic and heart rate responses to changes in inspired $PO_2$ in high altitude rats and mice

For the responses that were normalized to baseline values, there was no significant effect of sex; accordingly, males and females were pooled. At 12% $O_2$ (corresponding to a partial pressure of inspired $O_2$ ($PiO_2$) of 60 mmHg – see materials and methods), four rats (three males and one female) had signs of distress, prolonged apneas, and gasps within the first minute of exposure. The experiments were therefore stopped and data recorded at 12% (for rats and mice) are not presented – with the exception of rectal temperature. All mice supported exposure to 12% $O_2$ without signs of distress, apneas or gasps.

### 4.2.1. Arterial oxygen saturation and heart rate

$P_{O2,sat}$ was higher in mice than in rats upon exposure to SL $PiO_2$ (32% $O_2$) and in moderate hypoxia (18% $O_2$, Figure II.7A). Heart rate declined in rats when exposed to SL $PiO_2$, but remained unchanged upon hypoxic exposure (Figure II.7B). In mice, heart rate remained unchanged when exposed to SL $PiO_2$, slightly declined upon hypoxic exposure to 18% $O_2$, and returned to baseline levels at 15% $O_2$ (Figure II.7B).

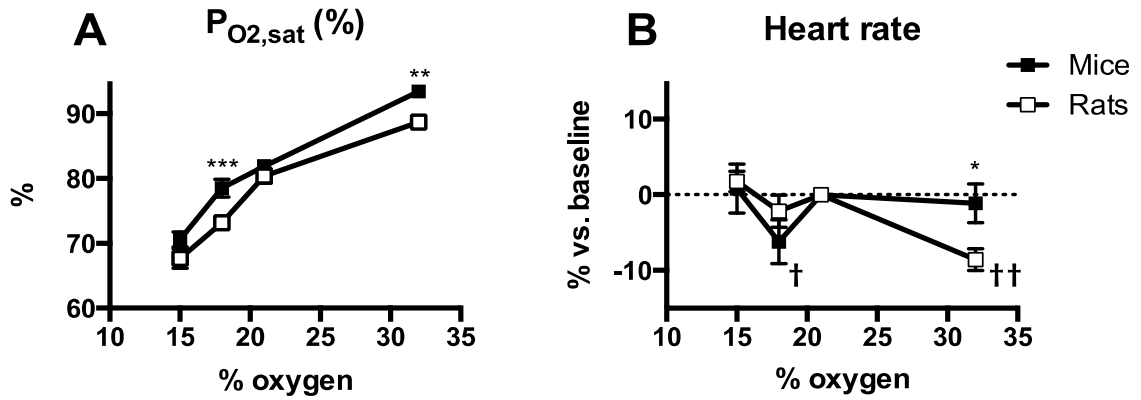


**Figure II.7: Response to changes of inspired $O_2$ in high altitude rats and mice.**
Means±s.e.m.
**P*<0.05, ***P*<0.01, ****P*<0.001 mice versus rats. †*P*<0.05 and ††*P*<0.01 versus baseline (21% $O_2$).

### 4.2.2. $V_E$, $V_T$, fR, metabolic rate and body temperature

Exposure to SL $PiO_2$ reduced $V_E$ to a similar extent in mice and rats (Figure II.8A); however, rats had a more pronounced decline of $V_T$ than mice (Figure II.8B), and contrastingly mice had a more marked decline of fR than rats (Figure II.8C). Under moderate hypoxia (18%) $V_T$ was higher in mice than in rats (the apparent drop at 18% $O_2$ in rats is not significant compared with that at 21% $O_2$).

In response to SL $PiO_2$, there was no change of metabolic rate in either species (Figure II.9), but upon hypoxic exposure metabolic rate decreased in mice but not in rats: at 15% $O_2$, $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ had fallen by more than 30% in mice (Figure II.9A and Figure II.9B). Similarly, body temperature fell by about 2°C in mice (from 35.2±0.2°C to 33.1±0.2°C), but remained unchanged in rats (Figure II.9C).

Under hypoxic conditions, rats did not increase $V_E$ and maintain their metabolic rate at the baseline level, while in mice metabolic rate fell during hypoxic exposure. To find out whether this pattern in rats was achieved by increasing $O_2$ extraction from pulmonary gas, we calculated the percentage of $O_2$ extraction ($O_2$ consumption / $V_E$x$FiO_2$ where $FiO_2$ is the inspired oxygen fraction – Figure II.10 A,B). There was a significant effect of species for this parameter (*P*=0.05) and a significant species x hypoxia interaction (*P*=0.0009). In male rats, $O_2$ extraction increased in hypoxia (18 and 15% $O_2$) compared with 32% $O_2$, while it remained unchanged in male mice (Figure II.10A). Female mice had a lower $O_2$ extraction value in hypoxia than female rats (Figure II.10B), because mass-specific $V_E$ (Figure II.10 C,D; ANOVA *P*=0,002 for sex, *P*=0,004 for sex x species) and $V_T$ (not shown) were higher in female mice than in males throughout the hypoxic exposure, while there was no effect of sex for mass-specific $\dot{V}_{O_2}$ (Figure II.10 E,F).

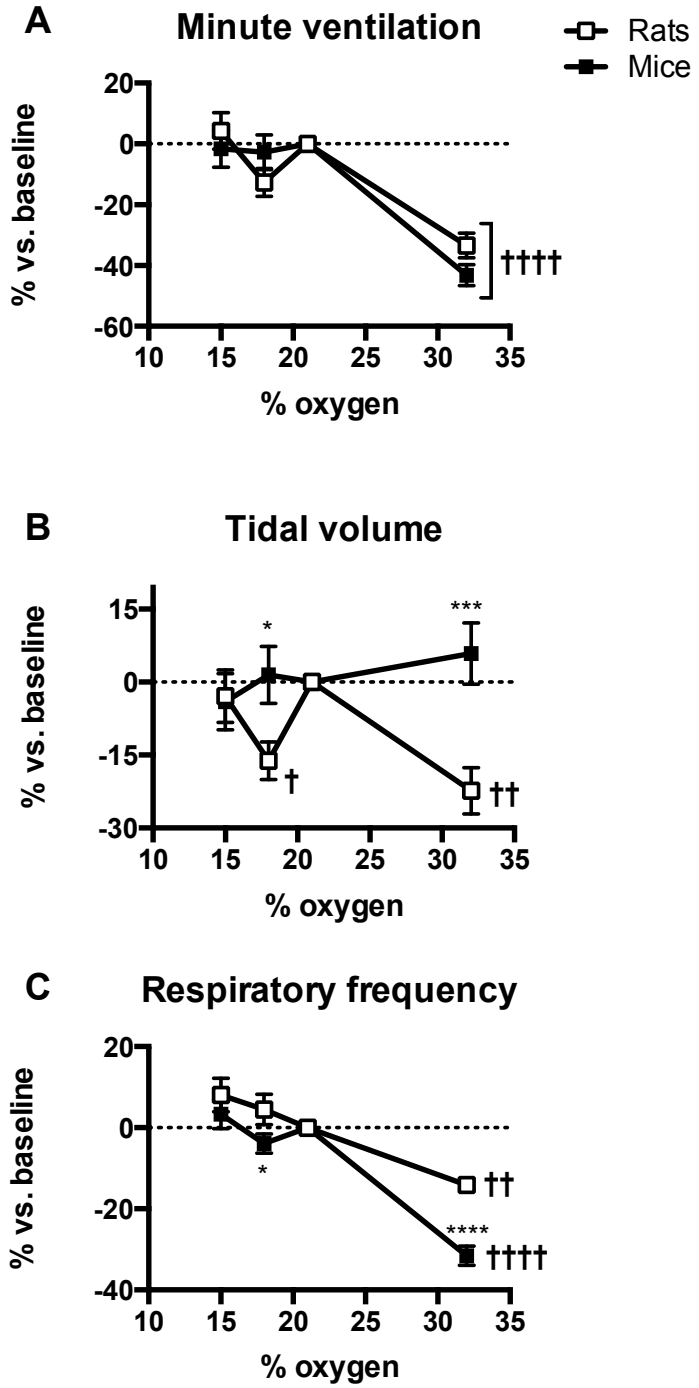


**Figure II.8: Ventilatory responses to changes of inspired $O_2$ in high altitude rats and mice.**

(A) minute ventilation ($V_E$), (B) tidal volume ($V_T$) and (C) respiratory frequency (fR) in 2-month-old rats and mice. Means±s.e.m.

*$P$<0.05, ***$P$<0.001 and ****$P$<0.0001 mice versus rats. †$P$<0,05, ††$P$<0.01 and ††††$P$<0.0001 versus baseline (21% $O_2$).

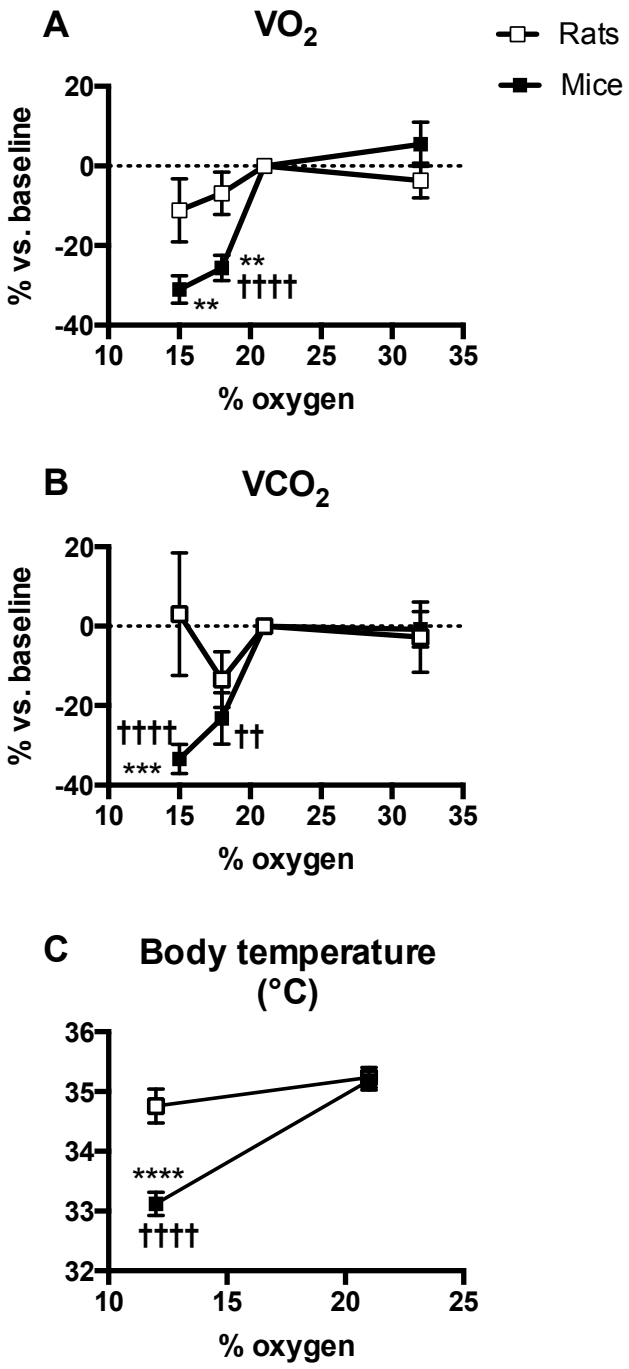


**Figure II.9: Metabolic responses to changes of inspired $O_2$ in high altitude rats and mice.**

(A) $O_2$ consumption rate ( $\dot{V}_{O_2}$ ), (B) $CO_2$ production rate ( $\dot{V}_{CO_2}$ ), and (C) body temperature in 2-month-old rats and mice. Means±s.e.m.
***P*<0.01, ****P*<0.001 and *****P*<0.0001 mice versus rats. ††*P*<0.01 and ††††*P*<0.0001 versus baseline (21% $O_2$).

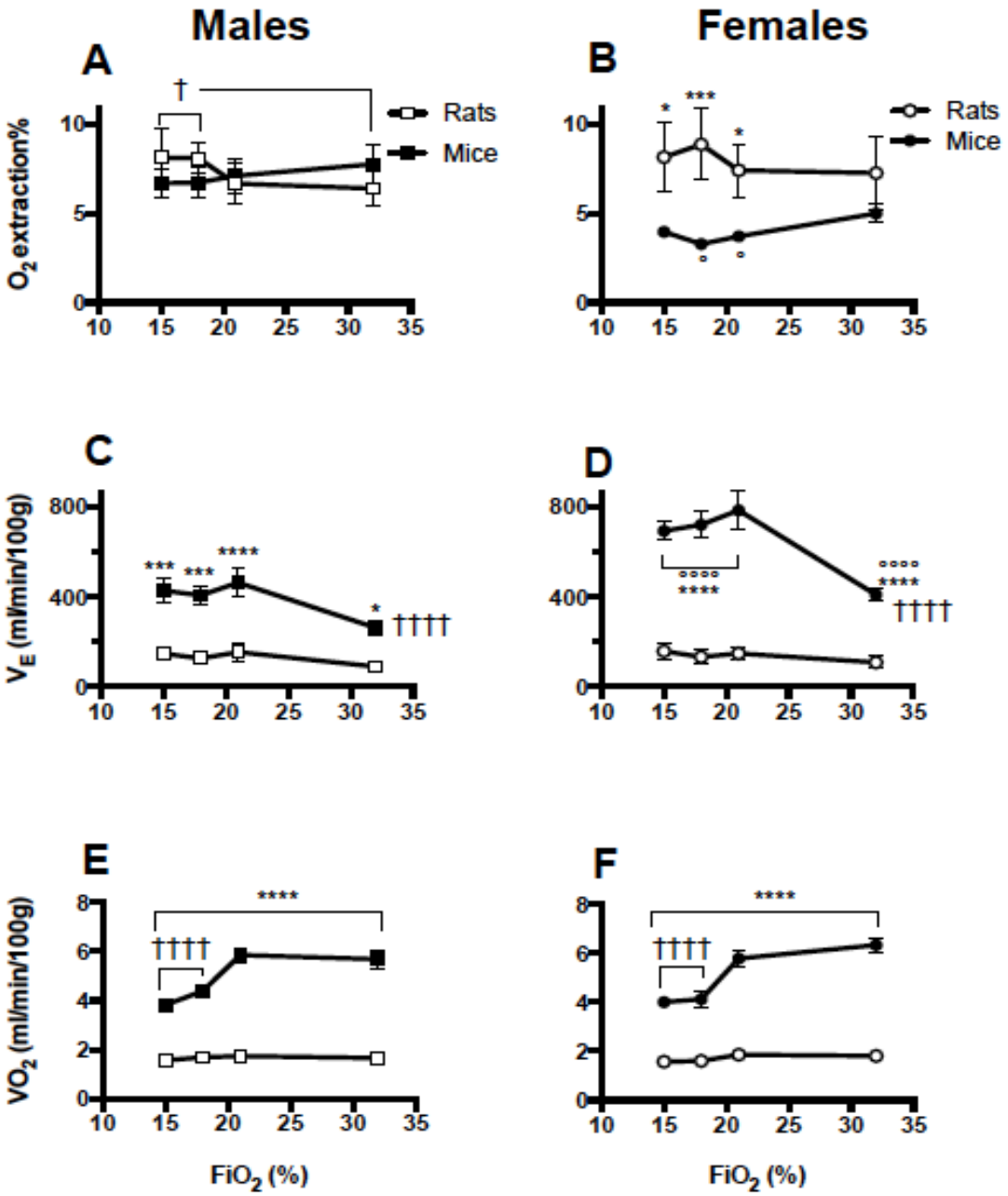


**Figure II.10: Responses to changes of inspired $O_2$ in high altitude rats and mice.**

(A,B) percentage $O_2$ extraction, (C,D) minute ventilation $V_E$, (E,F) mass-specific $O_2$ consumption rate ( $\dot{V}_{O_2}$ – ml/min/100g) in 2-month-old, male and female rats and mice. Means ± s.e.m.

°$P$<0.05 and °°°°$P$<0.0001 females versus males. *$P$<0.05, ***P<0.001, and ****$P$<0.0001 mice versus rats. †$P$<0.05, and ††††$P$<0.0001 versus baseline (21% $O_2$); in A †P<0.05 versus 32% $O_2$.

Table 1. Comparison between values obtained at sea level and high altitude for selected variables in adult (3 months old) male rats or mice

| | Rats | | Mice | |
|---|---|---|---|---|
| | Sea level | High altitude | Sea level | High altitude |
| Body mass (g) | 333±21 | 232±9 | 27.3±0.8 | 13.7±0.9 |
| Rectal temperature (°C) | 36.8±0.2 | 35.2±0.2 | 35.6±0.3 | 35.2±0.2 |
| Hematocrit (%) | 41.2–47.3[a] | 60.5±1.1 | 39.0–42.5[b] | 47.0±1.7 |
| Hemoglobin (g $ml^{-1}$) | 14.4–16.0[a] | 20.4±0.3 | 14.3–15.2[b] | 11.6±1.0 |
| RV/(LV+S) (%) | 27.9±0.6[c] | 59.7±5.6 | ~20[d] | 32.0±2.0 |
| Heart rate (beats $min^{-1}$) | 250–400[e] | 503±4 | 500–700[f] | 669±23 |
| Lung volume (×$10^2$ ml $g^{-1}$) | 2.4±0.2 | 2.6±0.1 | 2.7±0.2 | 7.6±0.4 |
| Lung mass (×$10^2$ g $g^{-1}$) | 0.29±0.01 | 0.44±0.02 | 0.49±0.01 | 1.06±0.05 |
| Total alveolar surface area (×$10^2$ $m^2$ $g^{-0.88}$) | 0.99±0.05 | 0.87±0.04 | 1.04±0.13 | 3.41±0.34 |
| $O_2$ consumption rate (ml $min^{-1}$ $g^{-0.76}$) | 0.144±0.003 | 0.064±0.001 | 0.149±0.01 | 0.109±0.006 |
| Total alveolar surface area to $O_2$ consumption ratio ($m^2$ $ml^{-1}$ $min^{-1}$) | ~0.10 | 0.30±0.03 | ~0.11 | 0.47±0.05 |

**Table II.1: Comparison between values obtained at sea level and high altitude for selected variables in adult (3 months old) male rats or mice.**
Values are ranges (minimum – maximum) or means±s.e.m. All HA values are those obtained in the present study. Sea level values are unpublished data from our colony of SD rats and FVB mice or are from the following references: (a)[294], (b) http://www.informatics.jax.org/mgihome/other/mouse_facts1.shtml, (c)[1], (d)[295], (e)[296], (f)[297].

# 5. Discussion

We compared physiological responses in laboratory rats and mice that have been raised at 3600m above SL for about 30 generations. The key differences between these two species include higher erythrocytosis and elevated right ventricular hypertrophy (a sign of higher pulmonary hypertension) in rats compared with mice, and lower mass-corrected lung volume, alveolar surface area, $V_T$ and $\dot{V}_{O_2}$ in rats compared with mice. However rats and mice have similar levels of $P_{O2,sat}$.

The relevance of performing comparative studies between two species as an approach to understand adaptive processes to a given environmental condition has been questioned[298]. In line with the limitations of this approach, this study was not designed to draw conclusions on genetic adaptation to altitude, but rather to illustrate

physiological differences at altitude between two species that are known to have a different altitudinal range of distribution. As such, our results provide a relevant model of divergent physiological responses at HA, and might help to explain ecological reports suggesting that mice are more easily found under natural conditions at HA than rats[32,277,287].

To illustrate how rats and mice might differ in their responses to HA hypoxia, the Table II.1 presents a series of data obtained from the current HA study and from other studies at SL, including some data obtained in our SL laboratory in Sprague-Dawley rats and FVB mice (all adult males of similar age to the HA animals).

One should, however, keep in mind that we have not been able to precisely determine the identity of the mouse species from the HA colony. However, as they are descendants from a provider of standard laboratory mice, they should be *Mus musculus domesticus*[299], but we cannot exclude the possibility that they have been hybridized with local species such as *Mus musculus castaneus,* which – with *Mus musculus domesticus* – have been identified among wild-caught mice in La Paz[277]. Nevertheless, it remains unclear from the literature whether these groups of mice should be considered as distinct species or subspecies of *Mus musculus*[31].

## 5.1. Right ventricular hypertrophy, excessive erythropoiesis and elevated heart rate are present in high altitude rats but not in mice

In mice, the right-to-left ventricle ratio, an index of arterial pulmonary hypertension, was around 35%. While this is higher than values normally reported at SL (around 20%)[295], it is much lower than

the values reported in rats in our present and past studies[2]. Protection against elevated pulmonary hypertension is common in species adapted to HA, which demonstrate thinner pulmonary vessel walls with a reduced number of muscular cells[281]. We did not perform specific analysis of the arterial wall structure in mice, but it is likely that they would also present this typical characteristic.

There was also a pronounced difference of Hct and Hb values between rats and mice, and similar $P_{O2,sat}$ levels. The enhanced Hct value in rats might be due to a more sensitive hypoxia-sensing system in the kidneys, which could include stabilization of the Hypoxic-Inducible-Factor (HIF), expression of HIF regulatory proteins, and/or synthesis of erythropoietin (Epo)[300]. In addition, it is worth mentioning that the synthesis of Epo in the kidney is regulated by the glomerular filtration rate [301] because the consumption of $O_2$ in the kidneys (a major determinant of local $PO_2$) is tightly dependent on sodium reabsorption and glomerular filtration rate[302]. Accordingly, a lower glomerular filtration rate and/or sodium reabsorption in mice compared with rats could contribute to lower Epo production and reduced Hct.

In male rats raised at altitude, heart rate was around 500 beats/min, whereas normal SL values of heart rate are around 250-300 beats/min during daytime, and 350-400 beats/min during nighttime[303]. However, it is not possible from our data to elucidate whether the elevated heart rate effectively increases cardiac output or compensates for a reduced stroke volume. By comparisons, while the heart rate reported in mice under baseline conditions (670±20 beats/min in males) is higher than normal SL values at rest (500-600 beats/min daytime), it

remains within the normal range of values recorded during the active phase (nighttime 600-700 beats/min)[297].

## 5.2. Reduced metabolic rate and elevated respiratory exchange ratio in high altitude rats versus mice

Compared with mice, rats had a reduced $\dot{V}_{O_2}$ (either mass-specific or mass-corrected), which might help them to maintain the $P_{O2,sat}$ value despite the reduced mass-corrected alveolar surface area of the lungs. In several vertebrate species, reduced $O_2$ consumption rate is a key strategy of adaptation to hypoxia[264]. Subterranean species, such as naked mole rats that live in burrows under severe hypoxic conditions, have mechanisms of tolerance to hypoxia including low metabolic rate and reduced core body temperature[262]. Accordingly, rats raised under chronic hypoxia for several generations might have developed cellular mechanisms to reduce $O_2$ consumption and therefore help maintains elevated values of arterial $P_{O2,sat}$. However, it should be emphasized that in rats this strategy is apparently not successful and does not allow them to overcome the drastic elevation of right ventricular hypertrophy and pulmonary hypertension.

Rats showed a higher respiratory exchange ratio ($\dot{V}_{CO_2}/\dot{V}_{O_2}$) than mice. We previously reported elevated values in HA rats[2]. High values of the respiratory exchange ratio indicate that energy production is mostly accomplished by oxidation of glucose molecules, which is an effective way to optimize synthesis of ATP under low $O_2$ availability[304] as glucose oxidation produces the highest ratio of ATP synthesized for each molecules of $O_2$ consumed compared with other metabolites.

Interestingly, deer mice (considered as being genetically adapted to altitude) maintain low values of $\dot{V}_{CO_2}/\dot{V}_{O_2}$ and high levels of fatty acid oxidation under combined exposure to cold and hypoxic stress[305]. This pattern probably helps to maintain elevated glycogen stores that can be used for bursts of intense exercise ("fight or flight" stress response). As fatty acid oxidation requires more $O_2$ than glycolytic pathways, it should be supported by physiological adjustments of the $O_2$ transport system.

## 5.3. Reduced lung volume and alveolar exchange surface area in high altitude rats versus mice

Compared with mice, rats had enlarged airspaces leading to lower mass-corrected relative alveolar exchange surface area. Combined with a reduced mass-corrected lung volume, this leads to a drastic difference of the estimated mass-corrected total lung exchange surface area between rats and mice. In mammals – with a range of body mass from 10 g (bat) to > 1000 kg (whale) – lung alveolar surface area in $m^2$ is directly proportional to $O_2$ consumption in ml/min, with a scaling variable of 1[163]. Therefore, the ratio of alveolar surface to $O_2$ consumption should be similar between 2 species, which is not the case when comparing rats and mice at altitude (see Figure II.6). However, if we compare the values obtained at HA with an estimation of expected SL values (see Table II.1), it is clear that, relative to $O_2$ consumption, the total alveolar surface area is enhanced in rats and mice living at HA. Yet, while in mice this was achieved by increasing the volume of the lungs and the total alveolar surface area, in rats this is possible only because $\dot{V}_{O_2}$ is much lower at HA than at SL. This striking difference might be due to an ability of the lungs of mice to respond to hypoxia by

increasing the gas exchange surface area, while these responses would not be present in rats, which therefore have reached a fragile equilibrium at altitude by reducing $\dot{V}_{O_2}$. Of note, in HA rats compared with SL rats (see Table II.1) there is an increase in the mass of the lungs but not in lung volume, probably as a consequence of neo-angiogenesis and hypertrophy of lung vessels.

## 5.4. Respiratory and metabolic responses to hypoxia in high altitude rats and mice

Chemoreflex function helps maintain adequate ventilation under chronic hypoxia, and is an important contributor to efficient adaptation to hypoxia[133,306]. At HA, a relevant approach to evaluate the basal activity of the peripheral chemoreceptors is to relieve the chronic hypoxic stimulus, which in the present study was achieved by exposure to SL $PO_2$. Rats and mice showed a similar decrease of $V_E$ in response to SL $PO_2$ suggesting similar sensibility of peripheral chemoreceptors to hypoxia, but this was achieved by a different pattern of response with a higher decrease of fR being observed in mice compared with rats, and a decrease of $V_T$ in rats but not in mice. This is a striking difference if we take into account that under baseline conditions the $V_T$ of rats (either mass-specific or mass-corrected) is already smaller compared with that of mice. Hence, the chemoreflex drive in rats increases the $V_T$, which probably helps to maintain an elevated $P_{O2,sat}$ by optimizing ventilation of the lungs. Such differences between rats and mice probably rely on different neurochemical processes within the central respiratory pathways leading to differential translation of the peripheral chemoreceptors inputs into phrenic nerve activity. It is intriguing to report that HIF and HIF target genes appear to be key elements in the

plasticity of the respiratory control system under chronic hypoxia[307-311], accordingly, species differences might be related to differential control of HIF or HIF-regulating proteins in rats compared with mice.

It is also noteworthy that mice are able to reduce metabolic rate and rectal temperature in response to hypoxia, while this response is not present in rats. This response is typically seen as being protective and contributes to the preservation of arterial $O_2$ pressure in hypoxia. This is an active process resulting from a reduction of the thermoregulatory set point, regulated within the preoptic hypothalamic nucleus[261,263]. It is possible to ask whether the drop in rectal temperature accounted for the fall in metabolic rate or whether is there evidence of metabolic suppression beyond that due to a resetting of the body temperature set point[261]. We used a web-based calculator (www.physiologyweb.com/calculators/q10_calculator.html) and calculated that in mice, $O_2$ consumption would drop from 5.82 to 4.62 ml/min/100g with a drop of rectal temperature from 35.2 to 33.1°C and a Q10 of 3. As $O_2$ consumption was below these values, there is indeed an evidence of a metabolic suppression beyond that due to a resetting of the body temperature set point. The excess metabolic suppression is 1.37 ml $O_2$/min/100g – almost 1/4th of the basal $O_2$ consumption of mice. The fact that this response is present in mice but not in rats indicates that mice are able to display protective responses to counteract further reduction of $O_2$ level, which is clearly another advantage at HA.

The high heart rate observed in rats might also explain the fact that several rats were not able to withstand hypoxic exposure at 12% $O_2$. At the altitude of La Paz, this $O_2$ level corresponds to a $PiO_2$ of 60 mmHg (or 7.8% $O_2$ in inspired air at SL), a severe level of hypoxia that could be close to the minimum $O_2$ level necessary to maintain the function of the heart under an already challenging condition.

## 5.5. Sex specific effects in high altitude rats and mice

Females of both species showed lower Hct values than males, probably reflecting the inhibitory effect of estradiol on Epo synthesis[312]. However, sex-specific effects on respiratory parameters were mostly present in mice, with female mice showing higher $V_T$ and $V_E$ than male mice (either mass-specific or mass-corrected data). In rats, females had lower mass-corrected fR than males, but $V_T$ and $V_E$ were similar (either for mass-specific or mass-corrected data). Higher values of $V_E$ and $V_T$ in females compared with males likely reflect the respiratory stimulant effect of ovarian steroids[313], and have been reported in our previous studies[2]. The fact that sex-specific effects were not reported in the present study in rats might be related to the reduced sample size (6 males, 6 females), while we compared 17 males to 16 females in our previous study[2].

We conclude that adult laboratory rats and mice that have been raised for a similar period of time under conditions of chronic hypoxia at HA display divergent physiological responses. Interestingly, a previous genetic study showed that there is no adaptive modification of the Hb

function in wild *Mus musculus* caught in La Paz compared with other specimens at SL (Lima, Peru)[277]; therefore, it is tempting to speculate that the physiological responses observed in mice that have been bred in La Paz might explain the ability of this species to successfully withstand the HA hypoxic environment.

# III. CHAPTER 3

## DIVERGENT PHYSIOLOGICAL AND MOLECULAR RESPONSES TO HYPOXIA IN LABORATORY RATS AND MICE AT SEA LEVEL

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# 1. Abstract

We previously observed that laboratory rats and mice that have been raised for more than 30 generations in La Paz, Bolivia (3600m), display divergent physiological responses. Compared with rats, mice have numerous signs of adequate responses to environmental HA. In the present study we asked whether similar traits are present in mice and rats born and raised at sea level (Quebec City, Canada). We compared the lung morphology, hematological and cardiac variable in FVB mice and Sprague Dawley rats and recorded the arterial $O_2$ saturation and heart rate in response to graded level of hypoxia (18, 15, 12, and 9% $O_2$ for 10 min each). Because the expression of the Hypoxia Inducible Factor 1 alpha (HIF-1$\alpha$ – main mediator of the responses to hypoxic environment in the cells) in the brain tissue is at a maximum level after 4-6 hours of exposure to hypoxia, we exposed rats and mice to different levels of hypoxia (15 and 12% $O_2$ – 6h each – sustained hypoxia), or room air (21% $O_2$) to address the expression of HIF 1$\alpha$ in the brainstem and their ventilatory and metabolic responses. SL adult rats and mice display similar cardiac, hematological and lung characteristics, but had divergent ventilatory and metabolic responses to sustained hypoxia. Compared with rats, mice had higher minute ventilation and lower metabolic rate in response to 6h at 12% $O_2$. Theses physiological responses in the ventilatory and metabolic responses to hypoxia were correlated with an increase in the expression of HIF-1$\alpha$ in the brainstem of mice.

# 2. Introduction

In the introduction, I showed you that different species have been able to colonize and adjust at different altitude and that several species of mice are commonly found up to an altitude of 5000m, whereas rats (no matter what the species or the strain is) are notably absent in high montane environment[31,32,276,277]. In the precedent chapter, we used laboratory Sprague-Dawley rats (originally imported from Charles Rivers France) that have been breed and raised at the IBBA (La Paz, 3600m above sea level – SL) for more than 20 years and over 30 generations and compared the physiological responses in the high altitude (HA) rats with HA laboratory mice (FVB strain) raised in the same conditions. We concluded from our experiments that compared with rats, mice have numerous signs of adequate responses to environmental HA such as low hematocrit and hemoglobin levels, reduced right ventricular hypertrophy (a sign of reduced pulmonary hypertension), increased alveolar surface area with smaller units in the lungs, and improved respiratory control (see chapter 2 p69).

Key element for survival and adaptation at HA is to enhance $O_2$ uptake and distribution, which can be achieved by increasing alveolar surface area and pulmonary ventilation. The increased ventilatory response observed in the HA mice reflects increased ventilation and tidal volume which might be linked to differences in the $O_2$ sensing abilities of the respiratory control system to maintain pulmonary ventilation under hypoxic conditions. The differences we observed, between laboratory rats and mice living at HA, raised the hypothesis that mice might display traits of predisposition to survive in a hypoxic environment without requiring long-term selection and genetic adaptations. In line with this hypothesis, it is worth mentioning that in wild mice living at HA, there

are no signs of genetic adaptation (at least in the sequence of the gene coding for hemoglobin as found in other species of rodents adapted to HA)[277,283,314]. Moreover, as mentioned in the discussion of the precedent chapter p99, the hypoxia inducible factor (HIF) and its target genes might play a key role in the plasticity of the respiratory control system under chronic hypoxia[307-310,315], thus it is possible that the species differences between rats and mice at HA are related to a differential control of HIF expression.

To address the potential role of the predetermined traits in the HA mice phenotype, we compared the lung morphology, hematological and cardiac variable in SL animals. We recorded the arterial $O_2$ saturation and heart rate in response to graded level of hypoxia (18, 15, 12, and 9% $O_2$ – 10 min each). Also, because HIF expression in the brain tissue is at a maximum level after 4-6 hours of exposure to hypoxia[309], we exposed rats and mice to different levels of hypoxia (15 and 12% $O_2$ – 6h each – sustained hypoxia), or room air (21% $O_2$) to address the expression of HIF 1$\alpha$ and asked whether it correlates with ventilatory or metabolic responses. The study was performed in adult male Sprague-Dawley rats and FVB mice raised at SL (Quebec, Canada).

# 3. Materials and methods

## 3.1. Animals and experimental groups

We used Sprague-Dawley rats and FVB mice from Charles-River (St Constant, Quebec, Canada) or raised in our colony (see below). Before the experiments, all the animals were housed for at least 7 days under standard conditions, had access to food and water ad-libitum, and were exposed to a 12h:12h light/dark cycle. All protocols have been

reviewed and approved by the local committee of animal care and use of Laval University and are in concordance with the guidelines of the Canadian Council of Animal Care.

Data for lung morphology, hematology, cardiac and arterial saturation were obtained from 6 adult male rats (2-3 months) born from 2 primiparous female rats (Sprague-Dawley, Charles-River – St Constant, Quebec, Canada) from our local colony, and from 10 adult male mice (2-3 months) ordered from Charles-River, St Constant, Quebec, Canada.

Ventilatory, metabolic and molecular (dosage of the expression of the Hypoxia Inducible Factor 1 alpha – HIF-1$\alpha$) measurements in response to 6hof hypoxic exposure (sustained hypoxia) were performed in 17 adult Sprague-Dawley male rats and 21 adult FVB male mice (2-3 months) ordered from Charles-River, St Constant, Quebec, Canada. Adult rats and mice were divided into three groups, a control group was exposed for 6 hours (6h) under room air conditions (6 rats and 6 mice), and two hypoxic groups were exposed for 6h at 15 (5 rats and 7 mice) and 12% $O_2$ (6 rats and 8 mice).

## 3.2. Hematological variable

A sample of blood was drawn from the tail and the hematocrit (Hct) was measured by microcentrifugation (Micro-MB centrifuge – International Equipment Company, USA). Samples were centrifuged 15 minutes. All samples were processed in duplicates when possible.

## 3.3. Lung architecture

### 3.3.1. Dissection of heart and lung

Prior to lung and heart sampling, all animals were deeply anesthetized by an intraperitoneal injection (0.1 ml/100g of body mass) of ketamine (87.5 mg/ml) and xylazine (12.5 mg/ml). The perfusion was performed through the left ventricle with ice-cold PBS (pH 7.2) at a constant pressure of 24 $cmH_2O$ in adult mice, and using a pump in adult rats. The heart was quickly dissected and used to measure the ratio of RV/(LV+S), an index of right ventricular hypertrophy and pulmonary hypertension as previously described in chapter 2 p75.

After cardiac perfusion with PBS, a catheter was fixed in the trachea of the animals used for lung histology assessments, the lungs were inflated with 4% PFA for 30 minutes at a constant pressure of 24 $cmH_2O$, then dissected. The total volume of the inflated lungs was measured by liquid displacement, after that, the lungs were kept in 4% PFA for 24 hours at room temperature (as previously described in chapter 2 p75). The next day, the lungs were separated into left and right lung for the mice and into 5 lobes for the rats and embedded in paraffin using the Tissue-Tek VIP (Miles scientific, USA). 24 hours later, the samples were included in paraffin and stored until they were processed to determine lung histology (described in chapter 2 p76).

### 3.3.2. Lung histology and morphology

All the experimental procedures for the measurements of lung histology and morphology have been extensively described in chapter 2 p76-77. Paraffin embedded lungs were cut at a thickness of 5 µm.

Sections were then deparaffinized, colored with Harris hematoxylin solution and mounted as previously described. The images were captured using an optical microscope at a magnification of x100. We randomly selected 3 non-overlapping images from each slide using 3 slides per animal and 4 animals per group (4 rats and 4 mice). The Mean Linear Intercept ($L_m$) was determined as previously described and from $L_m$ values, we calculated the relative alveolar surface area as $S(m^2/cm^3)=4V/L_m$, with $V$ being the volume of one image. An estimation of the total alveolar surface area was calculated as the product of the relative alveolar surface area and lung volume (measured by water displacement after fixation see chapter 2p77 for detailed descriptions).

## 3.4. Recording of arterial oxygen saturation and heart rate in unrestrained, unanesthetized rats and mice at sea level.

Arterial $O_2$ saturation and heart rate were recorded using a whole body plethysmograph chamber for adult mice or rats (Emka Technologies, Paris, France) flushed constantly with fresh room air. The airflow through the chamber was constantly recorded and maintained at 200 ml/min for the adult mice and 1.5 L/min for the adult rats.

Rectal temperature was taken at the beginning and immediately at the end of the recordings of graded levels of hypoxia. Before starting the experiments, animals were weighed, and then fitted with a neck collar of the Mouse OX® STARR (Life Sciences Corp, USA) pulse oximeter for continuous recording of pulse oximetry capillary $O_2$ saturation ($P_{O2,sat}$) and heart rate. Animals were then placed in the chamber for a period of tranquilization (10-30 min), and baseline recordings were initiated for 20 min. The acute exposure to graded levels of hypoxia was

done by switching the inflowing tube to a nitrogen gas line calibrated to obtain the desired percentage of $O_2$ (18, 15, 12, and 9% $O_2$ for 10 min each). For analysis, we selected a period of at least 30s with a stable heart rate during the last 3 minutes of each condition.

## 3.5. Recording of the ventilatory and metabolic variables

Ventilatory variables were recorded using a whole body plethysmograph chamber for adult mice or rats (Emka Technologies, Paris, France) flushed constantly with fresh room air. The chamber was calibrated with a known volume of air (0.5 ml for the adult mice and 5 ml for the adult rats) and the airflow through the chamber was constantly recorded and maintained at 200ml/min for the adult mice and 1,5 L/min for the adult rats. Animals were left undisturbed for only 10 minutes prior to start the baseline recording as it lasted for 6 hours. The sustained hypoxia was obtained same as the graded levels of hypoxia by switching the inflowing tube to a nitrogen gas line calibrated to obtain the desired percentage of $O_2$. Once the $O_2$ level stabilized, the animal was placed inside the chamber for 6h. Tidal volume ($V_T$) was calculated from the integrated flow trace as previously described[1,2] by using standard equation[289]. $O_2$ consumption ($\dot{V}_{O_2}$) and $CO_2$ production ($\dot{V}_{CO_2}$) rates were calculated as described in chapter 2 p73. Ventilatory and metabolic recordings were calculated every hour over the 6h recording by selecting periods of stable breathing patterns without movements. Only the average of the last ($6^{th}$) hour is presented in the present chapter.

During all recordings, subsampling of inlet and outlet gas were

dried and directed toward an oxygen analyzer and a carbon dioxide analyzer (S3A and CD3A analyzers; AEI Technologies – previously calibrated with a certified gas tank), the airflow through the chamber was recorded continuously by a mass flowmeter (TSI series 4140 mass flowmeter; TSI, Shoreview, MN). All signals (from the plethysmograph, gas analyzers and flowmeter) were directed toward a computer for storage and analysis using the Spike 2 software (Cambridge Electronic Design, Cambridge, UK).

## 3.6. Dosage of HIF-1$\alpha$ in the brainstem

### 3.6.1. Dissection of the brainstem

Animals were deeply anesthetized with 2-3 % of isoflurane flushed inside the plethysmograph chamber under normoxic and hypoxic conditions. After anesthesia, rectal temperature was measured followed by cardiac puncture. Then immediately the animals were sacrificed by decapitation and the brainstem was collected, immediately frozen using dry ice and kept at -80°C until further assessments.

### 3.6.2. Extraction of the nuclear fraction

We used the nuclear protein extraction kit (Item No: 10009277) from Cayman Chemical Company, USA. Rat and mice brainstem were used to separate the nuclear from the cytosolic protein fraction. The summarized protocol was as follows:

The whole Brainstem was put into pre-chilled vial containing ice-cold hypotonic buffer 1X supplemented with dithiothreitol (DTT) and

Nonidet P-40. The sample was homogenized on ice for 15 min then centrifuged at 300 g for 10 min at 4°C to separate the first part of the cytosolic fraction. The pellet obtained after centrifugation was gently re-suspended in an additional volume of 1x hypotonic buffer to complete the lysis of the cells followed by 15 min additional incubation on ice. After incubation, Nonidet P-40 was added, mixed well and centrifuged at 14,000g for 30 seconds (pulse spin) at 4°C to separate second part of the cytosolic fraction. The pellet was then re-suspended in ice-cold extraction buffer 1X (with protease and phosphatase inhibitors). Each vial was vortexed for 15 seconds at the highest setting and then rocked gently on ice for 15 minutes using a shaking platform. Samples were vortexed for an additional 30 seconds at the highest setting and rocked gently for an additional 15 minutes. The sample was then centrifuged at 14,000g for 10 minutes at 4°C and the supernatant was the nuclear fraction. Aliquoted fraction were stored at -80°C and used further for transcription factor assay.

### 3.6.3. Dosage of HIF-1α expression in the nuclear fraction of the brainstem cells

We used the HIF-1α transcription factor assay kit (Item No: 10006910) from Cayman Chemical Company, USA. Rat and mice brainstem nuclear fraction were used to detect HIF-1α. The summarized protocol was as follows:

Ready to use 96 well ELISA plate were used. A known volume of complete transcription factor binding assay buffer (CFTB), a competitor to the dsDNA, positive control and nuclear fraction of each sample were added in the appropriate wells. The ELISA plate was incubated at 4°C overnight. After incubation all the wells were washed five times with 1X

wash buffer and the HIF-1$\alpha$ antibody was added in each well except the blank followed by 1h incubation at room temperature. After incubation wells were washed properly with 1X wash buffer and the secondary antibody was added in each well except the blank and incubated for 1h at room temperature. Each well was washed once more with 1X wash buffer, and the developing solution was added in each well and incubated for 15 to 45 min. After the final incubation, the stop solution was added in each well and the absorbance measured at 450 nm.

## 3.7. Allometric scaling in rats and mice

As described in chapter 2 p77, to compare physiological and morphological variables between mice and rats, we used allometric scaling (each specific variable *A* is reported to body mass (in grams) exponent *b*: $A/g^b$). For the respiratory variables we used the scaling variable calculated by Stahl[292], which are: $b$=1 for lung mass and lung volume, and $b$=-0.25 for heart rate, $b$=0.76 for $O_2$ consumption and $CO_2$ production. For relative and total alveolar surface area, we respectively used $b$=-0.13 and $b$=0.88 as reported for mammals by Maina[293]. In the text, data corrected for the allometric scaling variables are referred to as mass corrected whereas data compared for body mass are referred to as mass specific.

## 3.8. Statistical analysis

We used GraphPad Prism 6.0c for 2-way ANOVA, unpaired t-test and *post-hoc* analysis. All values are reported as means±s.e.m., and the significant *P*-value was set a 0.05. *P*-values are reported in the figures

with the following general pattern: *$P$< 0.05, **$P$<0.01, ***$P$<0.001, and ****$P$<0.0001, respectively.

For the variables measured at different levels of $O_2$ percentage, we first performed 2-way ANOVAs with species and $O_2$ percentage (hypoxia) as grouping variables. When significant effects of species or hypoxia, or a significant interaction between species and hypoxia appeared, a *post-hoc* analysis was performed (Fisher's LSD).

# 4. Results

## 4.1. Arterial oxygen saturation, heart rate, hematology and lung architecture in baseline conditions in sea level rats and mice

### 4.1.1. Arterial oxygen saturation, heart rate, and hematology

Under baseline conditions, rats and mice had similar values of $P_{O2,sat}$ (around 95%), right-to-left ventricle ratio and Hct level without significant effect of species (Figure III.1A and B). Heart rate was higher in mice compared with rats ($P$<0.0001 for species, Figure III.1C) but when the difference in body mass was taken into account by applying the allometric scaling factor (heart rate in beats/min/mass in $g^{-0.25}$ – see materials and methods), rats and mice had similar values of heart rate without significant effect of species (Figure III.1D).

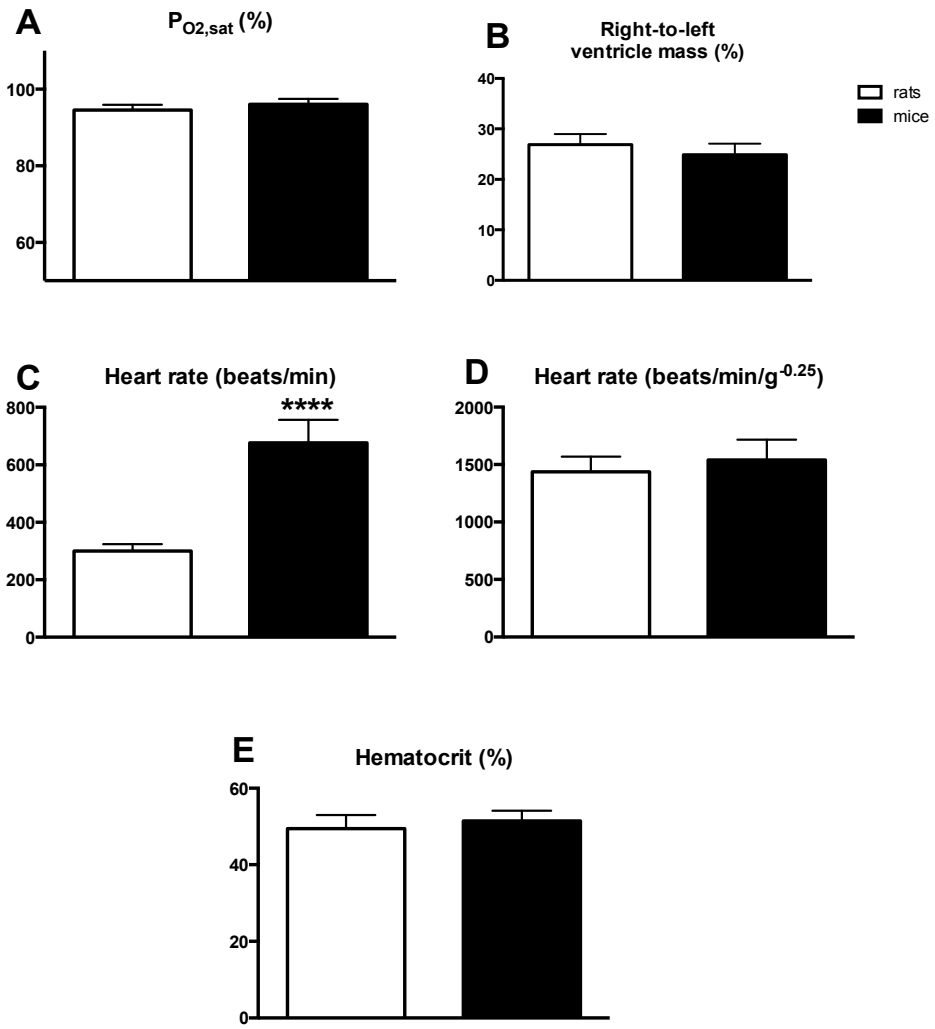


**Figure III.1: Hematological variables, right ventricular mass ratio, heart rate and arterial $O_2$ saturation in sea level rats and mice.**
(A) arterial $O_2$ saturation ($P_{O2,sat}$ – %), (B) right-to-left ventricle mass (%), (C) heart rate (beats/min), (D) mass-corrected heart rate (beats/min/$g^{-0,25}$), and (E) hematocrit (Hct – %), in 2-month-old males rats and mice. Means+s.e.m. ****$P$<0.0001 mice versus rats.

### 4.1.2. Lung volume, lung mass and lung architecture

Rats and mice had similar values of lung volume without significant effect of species (Figure III.2A). Mass-specific lung mass was

higher in mice compared with rats ($P$<0.0001 for species, Figure III.2B). Representative lung images are presented in Figure III.3A (for rats) and Figure III.3B (for mice). The mean linear intercept ($L_m$) was lower in mice compared with rats ($P$<0.05 for species Figure III.3C) however; this was not reflected in mass-corrected relative and total alveolar surface areas between rats and mice (Figure III.3 D,E).

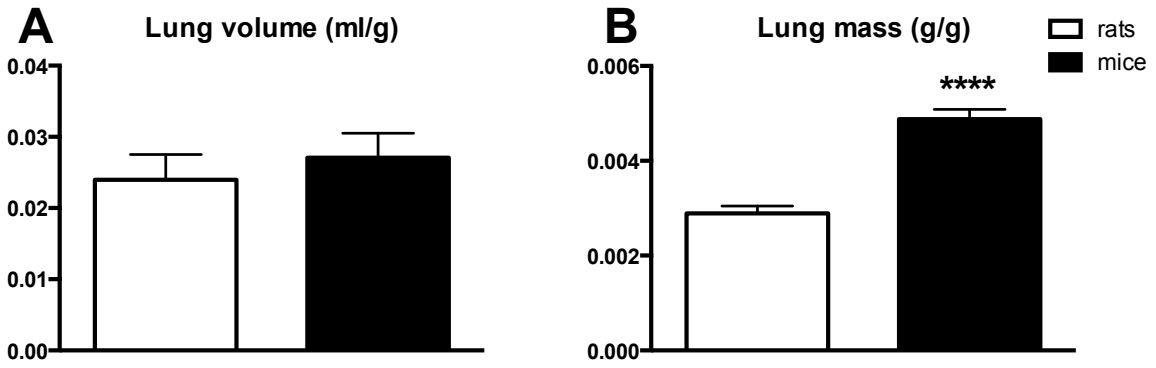


**Figure III.2: Variables of lung morphology in sea level rats and mice.**
(A) mass-specific lung volume (ml/g), and (B) mass-specific lung mass (g/g) in 2-month-old rats and mice. Means+s.e.m.
****$P$<0.0001 mice versus rats.

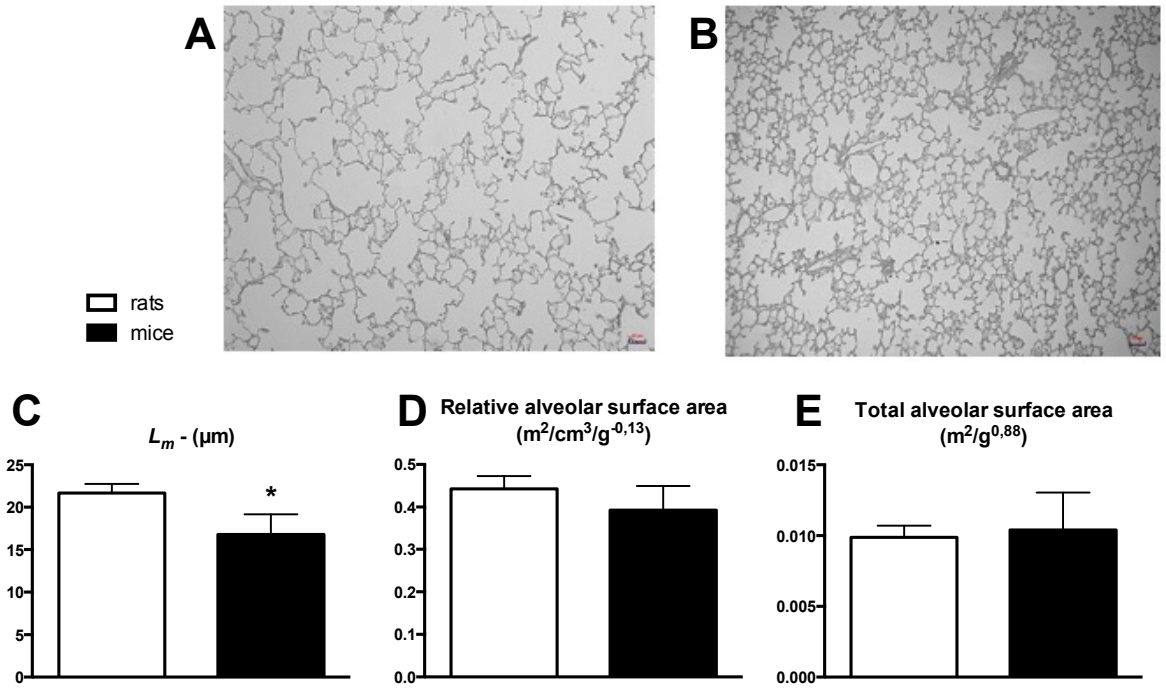


**Figure III.3: Variables of lung architecture in sea level rats and mice.**
Typical image of the architectural lungs obtained for rats (A) and mice (B). (C) mean linear intercept ($L_m$ – µm), (D) mass-corrected relative alveolar surface area ($m^2/cm^3/g^{-0.13}$), (E) mass-corrected total alveolar surface area ($m^2/g^{0.88}$) in 2-month-old rats and mice. Means+s.e.m.
Scale bars in A and B, 20 µm.
*$P$<0.05 mice versus rats.

## 4.2. Arterial oxygen saturation, heart rate and body temperature in responses to acute hypoxia in sea level rats and mice

### 4.2.1. Arterial oxygen saturation and heart rate

There were no differences between rats and mice arterial $O_2$ saturation in response to acute moderate (18, 15, 12% $O_2$) and severe (9% $O_2$) hypoxia. Heart rate increased in rats upon hypoxic exposure to 12 and 9% $O_2$. In mice, heart rate increased upon hypoxic exposure to

15 and 12% $O_2$, and returned to baseline levels at 9% $O_2$ (Figure III.4B).

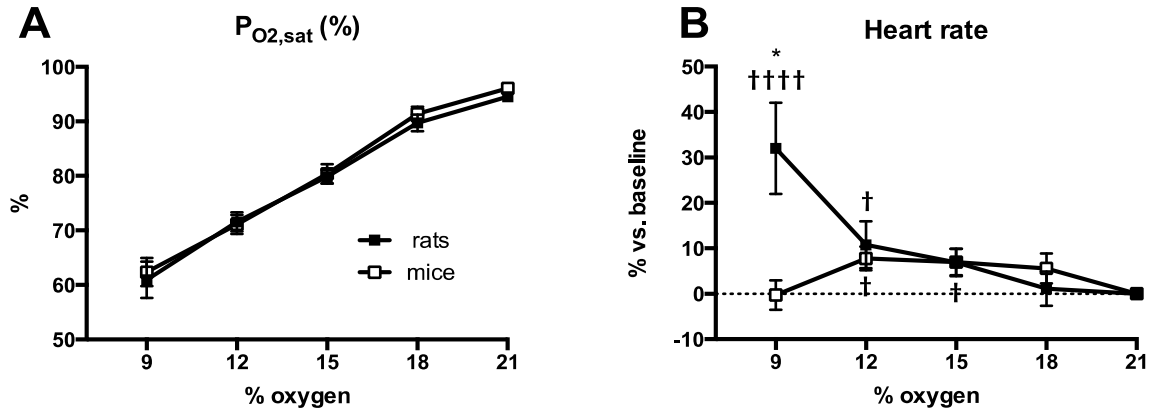


**Figure III.4: Response to changes of inspired $O_2$ in sea level rats and mice.**
(A) Arterial oxygen saturation ($P_{O2,sat}$ – %), (B) heart rate (% vs. baseline) in 2-month-old rats and mice. Means± s.e.m.
*$P$<0.05 mice versus rats. †$P$<0.05 and ††††$P$<0.0001 versus baseline (21% $O_2$).

### 4.2.2. Body temperature

Mice had higher body temperature compare with rats prior to the hypoxic exposure and significantly decreases their temperature after the hypoxic exposure. Furthermore, mice had a greater drop in their body temperature in response to hypoxia than rats (Figure III.5).

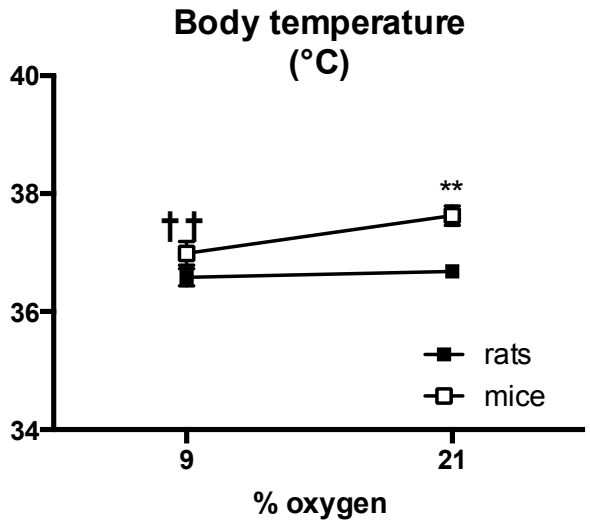


**Figure III.5: Body temperature before and after exposure to acute graded levels of hypoxia.**
(°C) in 2-month-old rats and mice. Means±s.e.m.
***P*<0.01 mice versus rats. ††*P*<0.01 versus baseline (21% $O_2$).

## 4.3. Minute ventilation, tidal volume, respiratory frequency, metabolic rate, and rectal temperature in response to sustained hypoxia in sea level rats and mice

### 4.3.1. Minute ventilation, tidal volume and respiratory frequency

Exposure to sustained hypoxia (15 and 12% $O_2$) for 6h globally increased the ventilation in mice. Compared with rats, mice had a higher mass-specific and mass-corrected minute ventilation ($V_E$ – *P*<0.0001 for mass-specific and *P*<0.05 for mass-corrected species, Figure III.6 C,F), tidal volume ($V_T$ – *P*<0.01 for mass-specific and *P*<0.001 for mass-corrected species, Figure III.6 B,E), and respiratory frequency (fR – P<0.0001 for species, Figure III.6 A,D). Exposure to hypoxia also significantly influences the ventilatory variables ($V_E$: *P*<0.0001; VT:

*P*<0.01; fR: *P*<0.0001 for hypoxia in mass specific and mass-corrected values) without hypoxia x species mass-corrected data interaction and with mass-specific $V_E$ and fR hypoxia x species interaction (*P*<0.05 and *P*<0.0001 respectively).

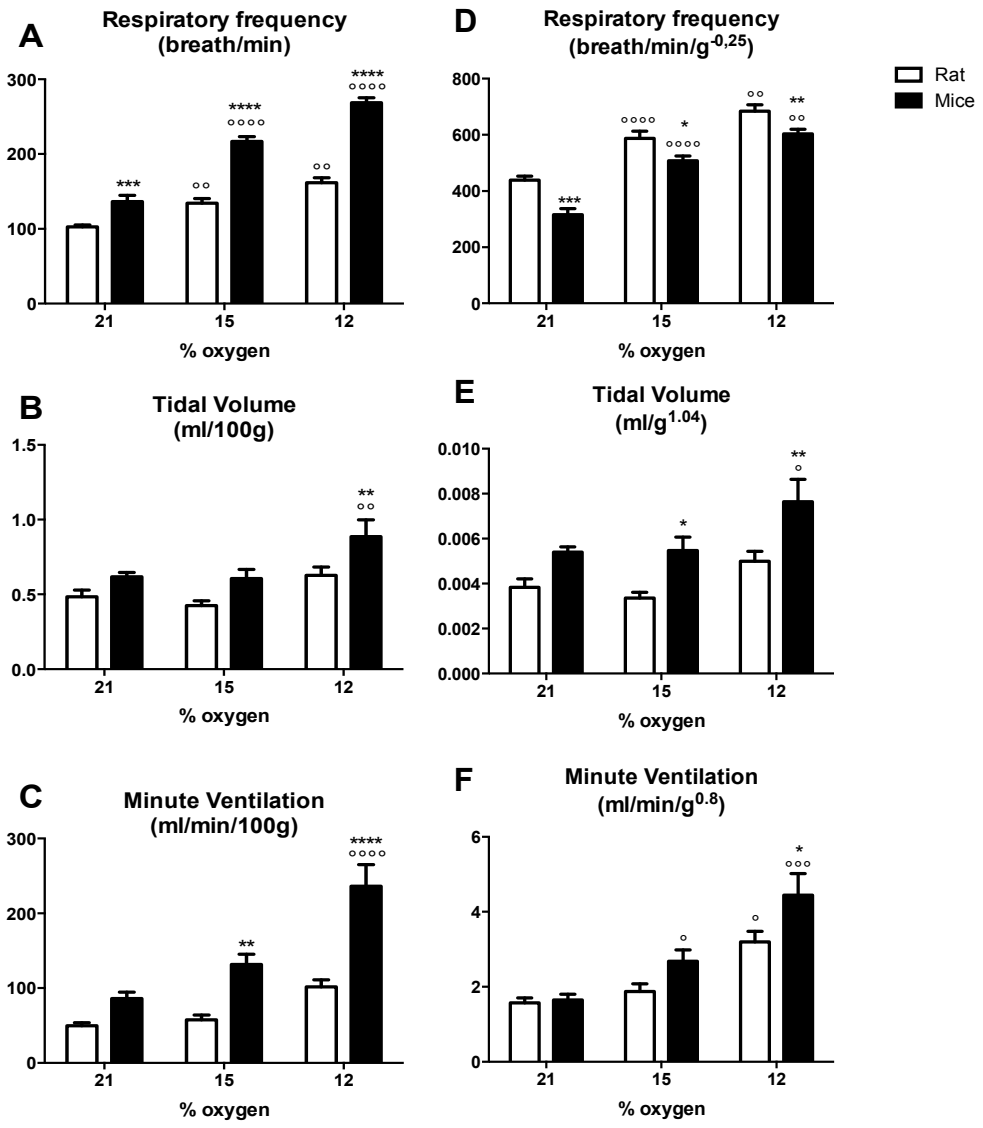


**Figure III.6: Ventilatory variables in response to normoxia and sustained hypoxia – 6h at 21, 15 and 12% $O_2$ – in sea level rats and mice.**

(A,D) minute ventilation ($V_E$ – ml/min/100g and ml/min/$g^{0,8}$), (B,E) tidal volume ($V_T$ – ml/100g and ml/$g^{1,04}$), (C,F) respiratory frequency (fR – breaths/min and breaths/min/$g^{-0,25}$), in 2-month-old rats and mice. A and B are mass-specific values, and D-F are mass-corrected values; means+s.e.m. **P*<0.05, ***P*<0.01 and ****P*<0.001 mice versus rats. °*P*<0.05, °°*P*<0.01, °°°*P*<0.001 and °°°°*P*<0.0001 15% versus baseline (21% $O_2$) or 12% versus 15% $O_2$.

### 4.3.2. Metabolic rate, respiratory quotient, and body temperature

There was no significant effect of species in mass-corrected $O_2$ consumption ( $\dot{V}_{O_2}$ ) and $CO_2$ production ( $\dot{V}_{CO_2}$ ) but there was a significant effect of hypoxia ($P$<0.0001) and a significant interaction between hypoxia and species only in $\dot{V}_{CO_2}$ ($P$<0.5 – Figure III.7 C,D). However, compared with rats, mice had higher mass-specific $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ in response to normoxia and graded level of hypoxia that decreased in response to sustained hypoxia with a significant interaction between species and hypoxia ($P$<0.0001 for species and hypoxia, $P$<0.01 for species x hypoxia – Figure III.7 A,B).

Compared with rats, mice had lower respiratory quotient in all exposure conditions. There was a significant effect of species and hypoxia without interaction ($P$<0.05 for species and hypoxia – Figure III.7E).

Compared with rats, mice decreased significantly their body temperature in response to hypoxia ($P$<0.0001 for species and $P$<0.001 for hypoxia – Figure III.7F).

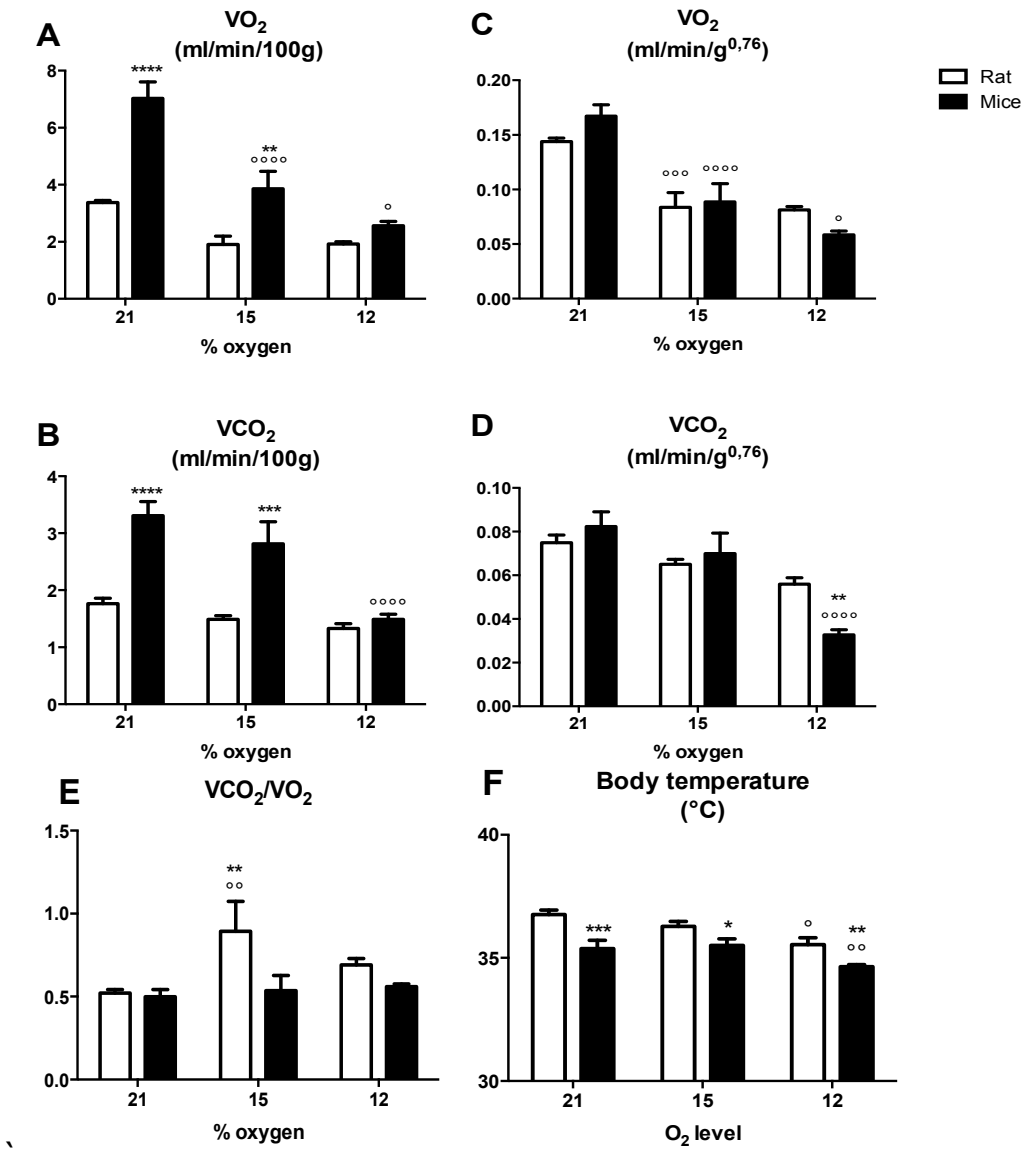


**Figure III.7: Metabolic variables in response to normoxia and sustained hypoxia – 6h at 21, 15 and 12% $O_2$ – in sea level rats and mice.**

(A,C) $O_2$ consumption ( $\dot{V}_{O_2}$ – ml/min/100g and ml/min/$g^{0,76}$), (B,D) $CO_2$ production rate ( $\dot{V}_{CO_2}$ – ml/min/100g and ml/min/$g^{0,76}$) in 2-month-old rats and mice. A and B are mass-specific values, C and D are mass-corrected values. (E) Respiratory exchange ratio ( $\dot{V}_{CO_2}/\dot{V}_{O_2}$ ) and (F) Body temperature (°C). Means+s.e.m.

**P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 mice versus rats. °*P*<0.05, °°*P*<0.01, °°°*P*<0.001 and °°°°*P*<0.0001 15% versus baseline (21% $O_2$) or 12% versus 15% $O_2$.

### 4.3.3. Respiratory exchange ratio and correlation between respiratory and metabolic variables

Mice had significantly higher ventilatory equivalent for $O_2$ and $CO_2$ than rats in all exposure conditions ($V_E/\dot{V}_{O_2}$ and $V_E/\dot{V}_{CO_2}$ $P<0.05$ and 0.001 for species respectively and $P<0.0001$ for hypoxia). There was a significant interaction between species and hypoxia for $V_E/\dot{V}_{CO_2}$ only ($P<0.0001$ – Figure III.8)

To further emphasize the relationship between ventilation and metabolism in response to sustained hypoxia, we plotted the minute ventilation $V_E$ against the $O_2$ consumption ($\dot{V}_{O_2}$ – Figure III.9) or the $CO_2$ production ($\dot{V}_{CO_2}$ – Figure III.10). Below 15% $O_2$, rats are no longer able to decrease their metabolic rate and only slightly increase their ventilation whereas mice decrease their metabolic rate between 15 and 12% $O_2$ exposure and further increase their ventilation.

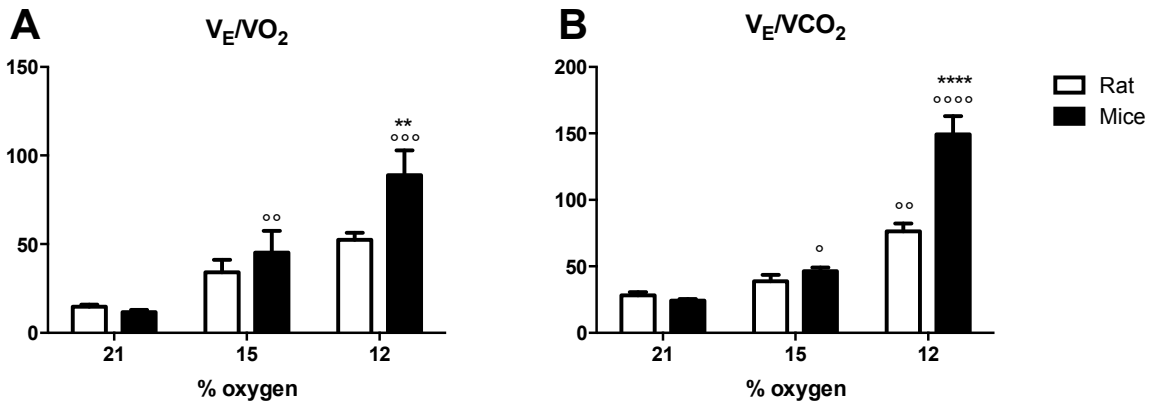


**Figure III.8: Respiratory exchange ratio under baseline conditions (21% oxygen for 6h) and in response to sustained hypoxia (15 and 12% $O_2$ for 6h) in sea level rats and mice.**

(A) $O_2$ convection ratio ($V_E/\dot{V}_{O_2}$, ml air/ml $O_2$), (B) $CO_2$ convection ratio ($V_E/\dot{V}_{CO_2}$, ml air/ml $CO_2$) in 2-month-old rats and mice. Means+s.e.m.

***P*<0.01 and ****P*<0.0001 mice versus rats. °*P*<0.05, °°*P*<0.01, °°°*P*<0.001 and °°°°*P*<0.0001 15% versus baseline (21% $O_2$) or 12% versus 15% $O_2$.

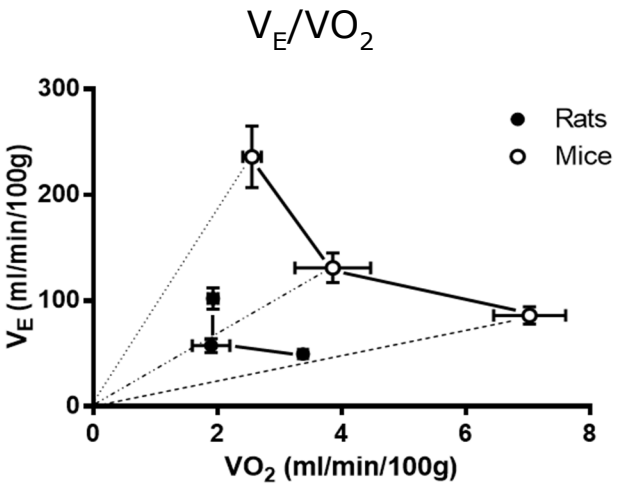


**Figure III.9 Correlation between $V_E$ against $\dot{V}_{O_2}$ showing the effect of hypoxia in rats and mice.**

The dotted lines represents the values of $V_E/\dot{V}_{O_2}$ reported for mice at each $O_2$ levels 21% $O_2$ (------ ), 15% $O_2$ (------) and 12% $O_2$ (.....) respectively from bottom to top.

$V_E/VCO_2$

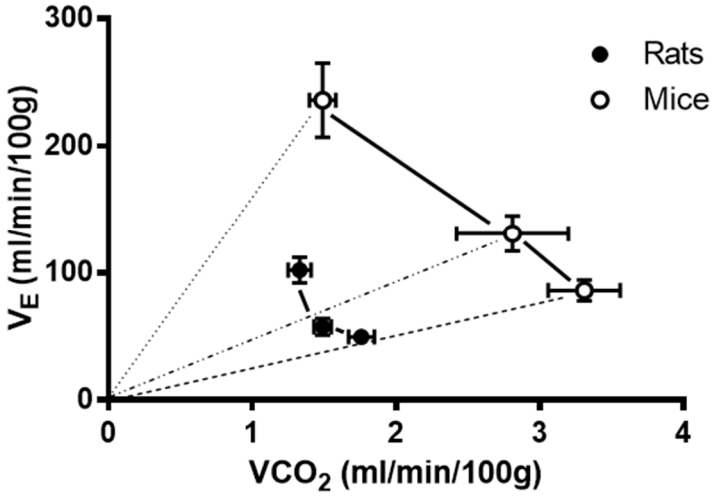


**Figure III.10: Correlation between $V_E$ against $\dot{V}_{CO_2}$ showing the effect of hypoxia in mice and rats.**
The dotted lines represents the values of $V_E/\dot{V}_{CO_2}$ reported for mice at each $O_2$ levels 21% $O_2$ (------), 15% $O_2$ (------) and 12% $O_2$ (.....) respectively from bottom to top.

## 4.4. HIF-1α expression in the brainstem of sea level rats and mice

The dosage of the expression of HIF-1α in 15 and 12% $O_2$ was normalized to the baseline value for each species. The HIF-1α expression was measured exclusively in the nuclear protein fraction of the brainstem cell. Mice but not rats increased their expression level of HIF-1α after 6h of hypoxic exposure at 15% and 12% $O_2$ ($P$<0.0001 for species and hypoxia and $P$<0.01 for species x hypoxia – Figure III.11).

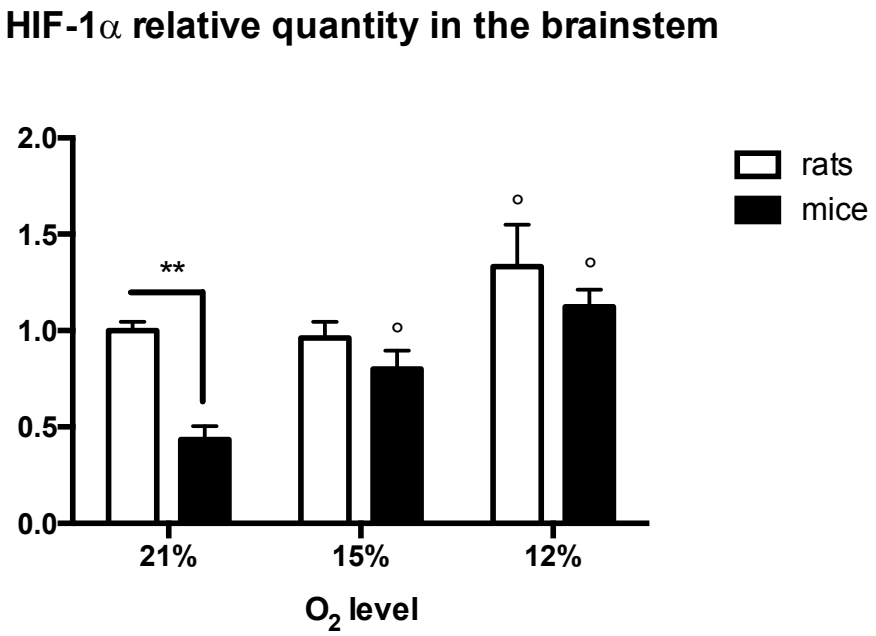


**Figure III.11: Expression level of HIF-1α in the brainstem in response to sustained hypoxia (15 and 12% $O_2$ for 6h) corrected for the baseline values (21% $O_2$) in sea level rats and mice.**
Relative quantity versus baseline. Means+s.e.m.
***P*<0.01 mice versus rats. °*P*<0.05 15% versus baseline (21% $O_2$) or 12% versus 15% $O_2$.

# 5. Discussion

We compared the physiological and molecular responses in laboratory rats and mice raised at SL in Quebec, Canada to ask whether part of the physiological responses we previously reported at HA (La Paz, Bolivia 3600m – see chapter 2 p69) are present without generation of acclimatization to environmental hypoxia. The present comparative study demonstrated that SL rats and mice display no difference regarding cardiac and hematological variables. Regarding the lungs, mice had higher lung mass and lower $L_m$ (indicating smaller alveolar units in the lungs in mice). In response to acute hypoxia, rats and mice had similar arterial $O_2$ saturation and heart rate; only at 9% $O_2$ rats had

a significantly higher heart rate compared with mice. In response to sustained hypoxia, mice had higher minute ventilation, lower metabolic rate resulting in higher respiratory exchange ratio in response to 6h at 15 and 12% $O_2$. In addition, mice increase brainstem expression of HIF-1$\alpha$ after 6h at 15 and 12% $O_2$ whereas it increases in rats only after 6h at 12% $O_2$.

## 5.1. Sea level rats and mice have similar lung architecture, right-to-left ventricle ratio and hematocrit level

SL rats and mice had right-to-left ventricle ratio and Hct level in the range of what is expected in their species at SL[1,294].

The only difference between rats and mice regarding lung architecture and morphology was a higher lung mass and a lower $L_m$ in mice compared with rats. This is in line with the images of lung slices showing that mice have smaller alveolus and higher tissue density, and corresponds to the different body size between the two species. Indeed, when results of the relative or total alveolar surface areas are presented as mass-corrected, mice and rats are similar. Thus, based on this result, mice do not present specific advantage regarding the lung morphology that would predispose them to better withstand HA hypoxia.

## 5.2. HIF expression is increased in response to sustained hypoxia in the brainstem of mice but not rats

### 5.2.1. Methodological considerations

The protocol used to assess the brainstem expression of HIF-1α might raise some concerns. We used the whole brainstem to assess the expression of HIF-1α in response to hypoxia; however, the brainstem regroups several nuclei that influence the respiratory control in different ways. For example, the pontine respiratory regions, the pre-Bötzinger complex, the rostral and caudal ventral respiratory groups, the nucleus tractus solitarius (NTS), and the raphé[316,317] are all located in the brainstem. Previous studies of the HIF brainstem expression in rats revealed that the neuronal expression of HIF-1α was located mainly in two regions: (i) in an extensive dorsomedial region that includes the NTS, the dorsal motor vagal nucleus and the hypoglossal nucleus and (ii) a more restricted region located in the ventrolateral part of the brainstem[309]. Moreover, the brainstem is very heterogeneous in terms of neuronal population and their role over the control of breathing, thus as our experiment was performed using the entire brainstem, we cannot further discriminate which nuclei might be more implicated in the HIF expression we observed in response to hypoxia in rats and mice. For future perspective, a way of differentiating the expression of the protein in each nucleus would be to use immunochemical straining of HIF-1α directed towards the nuclei of cells in brainstem slices.

We choose to use the whole brainstem as a first step to question the role of the expression of HIF-1α in the divergent responses to hypoxia we observed between rats and mice. Also, further investigations are currently on-going to quantify the protein expression of array HIF-

target genes known to be implicated in the genetic adaptation to hypoxia, such as the inducible nitric oxide synthase or the adenosine monophosphate-activated protein kinase catalytic subunit alpha 1[318,319] and nine other proteins. Because these analyses are performed on the cytosolic and nuclear fractions of cellular extracts by Gas Chromatography, and Mass-spectrometry, we had to use the whole brainstem extracts.

### 5.2.2. Mice and rats have increased expression of HIF-1$\alpha$ in response to hypoxia

When SL natives are exposed to hypoxia, they hyperventilate and as described in the introduction, the ventilatory response to hypoxia varies in time and mostly depends from the carotid bodies (CB see chapter 1 p26). Studies in goat showed that a bilateral denervation of the CB attenuates or abolishes the apparition of the ventilatory acclimatization to hypoxia (VAH)[112] and that the sensory activity of the organ increases progressively the afferent discharge in prolonged hypoxia (4h)[320]. During VAH, the hypoxic ventilatory response (HVR) is increased, this result from enhanced CB $O_2$-sensivity[321] and increased responsiveness of the central nervous system (CNS) to the CB afferent inputs[126,150]. Furthermore, several studies reported that an $O_2$ chemosensitive network exists in the brainstem and that this network is responsible for modulating the ventilation under hypoxic conditions[317,322].

HIF is a master regulator of the cellular and systemic homeostasis under hypoxic condition. In response to hypoxia, HIF will induce the transcription of genes involved in several biological processes[323,324], as such HIF might have a role in the activation of the VAH, activating the

transcription of gene implicated in the neural circuits controlling breathing. For example, heterozygous *knockout* (KO) mice for HIF-1α preserved the apparition of the HVR in response to acute hypoxia but lack the VAH response to chronic hypoxia. Furthermore, the absence of adaptation to chronic hypoxia seems to be linked to a depressed $O_2$-sensitivity in the CB observed in response to acute hypoxia ex-*vivo*[315]. The second hypothesis concerning the apparition of the VAH involves an increase in the CNS response to the CB afferent inputs. Some studies have been conducted using KO mice with a CNS-specific deletion of the HIF-1α gene[307] (it is important to precise that HIF-1α gene was not deleted in the CB of these mice[308]) reporting results similar to the heterozygous KO mice for HIF-1α, a normal HVR in response to acute hypoxia but no increase in the HVR in response to chronic hypoxia[325], indicating that the CNS also plays a role in the apparition of the VAH. In our previous studies, we proposed that HIF or HIF-regulated proteins might have a role in the hematological, ventilatory and metabolic responses we reported in HA rats and mice (Chapter 2 p99 and reference [2]). In the present study, compared with rats, mice had lower expression of HIF-1α in the brainstem under normoxic condition but similar level of expression in response to sustained hypoxia.

In rats, a study investigating the protein expression level of HIF-1α (using immunocytochemical techniques) in the brainstem of rats exposed to physiological hypoxia (10% for 1 to 6h) reported that hypoxia increased the level of HIF-1α protein in restricted cardiorespiratory areas of the brainstem[309]. In our study, we have not been able to detect any changes of HIF-1α expression in response to 6h at 15% $O_2$ but HIF-1α expression significantly increase at 12% $O_2$ in rats, thus, it is possible that the protein HIF-1α in the rats brainstem

needs a certain threshold of $O_2$ level to be activated and translocated in the nucleus of cells.

In endemic HA animals, HIF-1$\alpha$ has been investigated in the plateau pikas who demonstrated an increased protein level in the nucleus of lung, liver, spleen and kidneys cells compared with SL mice. The authors also found that HIF-1$\alpha$ expression was altitude dependent, it was increased with the increase in the altitude range of habitation of the animal[326]. These results are in concordance with what we observed in the brainstem of mice after 6h of sustained hypoxia at 15 and 12% $O_2$. This can be interpreted as a certain form of preadaptation to hypoxia in mice that is not present in rats. Thus, the variety of genes regulated by HIF-1$\alpha$ could be the early determinant inducing a cascade of events leading to the functional plasticity responsible for acclimatization to hypoxia[124,308,327].

## 5.3. Ventilatory response to sustained hypoxia in rats and mice

Mice displayed a stronger hyperventilation in response to sustained hypoxia than rats. The increase in ventilation in response to hypoxia in rats and mice has been attributed mainly to the peripheral chemoreceptors sensibility to low $O_2$ and particularly the CB[328] but also, as described in 5.2.2. p130, to the plasticity of the CNS that integrates the afferent inputs from the CB and regulates the breathing accordingly.

In response to sustained hypoxia, compared with rats, mice had lower mass corrected respiratory frequency, higher tidal volume and higher minute ventilation. It has been proposed that the plasticity occurring in the CNS in response to sustained hypoxia implicates the

glutamatergic signaling path in the NTS[126,150] and a recent study pointed out that HIF coordinate the transcriptional activation of multiple genes encoding glutamate transporters and glutamate receptors in response to hypoxia in cancer cells[329]. It is possible that in the CNS also HIF expression in response to hypoxia influence the glutamatergic signaling path. The different respiratory responses to hypoxia between rats and mice might also be linked to differences in the oxygen sensing ability of the peripheral chemoreceptors. In the CB, the $O_2$-sensing system is located in the glomus cells[94], thus, it is possible that the differences observed between rats and mice in the ventilatory response to hypoxia implicate differences in the sensitivity of the CB glomus cells to $O_2$ between the two species.

## 5.4. Metabolic response to sustained hypoxia in rats and mice

Mice had reduced metabolic rate in response to hypoxia, which is seen as protective and contributes to the preservation of the arterial $O_2$ pressure. We did not record the arterial $O_2$ pressure between rats and mice however, we expect that it would have remained higher in mice compared with rats after 6h in hypoxia at 12% $O_2$. Moreover, mice had significantly decreased their body temperature in response to acute graded levels of hypoxia compared with before the experiment and after 6h at 12% $O_2$ mice had significantly lower body temperature than rats (it is of note that the body temperature in response to sustain hypoxia was recorded in anesthetized animals which is known to have an impact in core temperature; nevertheless, anesthetized mice had lower body temperature than anesthetized rats after 6h sustained hypoxia at 12% $O_2$). It is well known that low metabolic rate in response to hypoxia is

the result of an active process leading to a reduction of the thermoregulatory set point initiated in the pre-optic hypothalamic area. Indeed, literature reported that hypoxia activates cyclic adenosine monophosphate dependent pathways in the hypothalamic pre-optic area, causing an elevation of the thermal sensitivity of pre-optic warm-sensitive neurons, in return leading to an inhibition of thermogenesis and an activation of heat loss[261,263,330]. However, there is also a link between basal metabolic rate and the ability of hypoxia to reduce $O_2$ consumption, the general rule being that the higher the relative $O_2$ consumption, the stronger the down regulation of metabolic rate in response to hypoxia is[305], which is also evident when comparing adult mice with rats.

Rats had increased respiratory quotient after 6h at 15% $O_2$. Calculating the $VCO_2/VO_2$ gives information on the fuel source that is being metabolized to produce ATP. The oxidation of glucose produces the highest ratio of ATP synthesized for each molecule of $O_2$ consumed compared with other metabolites. The glucose metabolism has a respiratory quotient of 1 and the lipids 0.76. In rats, after 6h at 15% $O_2$, the respiratory quotient was 0.89±0.18 while it was 0.53±0.09 in mice suggesting that rats used relatively more oxidation of glucose molecules to produce energy, which is an effective way to optimize the ATP synthesis to meet the energy demand under hypoxic condition[304]. Furthermore, this strategy has been previously described in male rats living at HA for several generations who had a respiratory quotient close to 1 (Chapter 2 p97 and reference [2]), while mice maintained a lower respiratory quotient (Chapter 2 p97). Accordingly, the metabolic strategy to respond to hypoxia might also be different in rats versus mice, and would provide another advantage for mice because mice would have a better use of the $O_2$ molecule by diminishing their $O_2$

consumption without diminishing the respiratory quotient (which in the wild would provide better survival chances for the fight or flight reflex).

We conclude from this study that adult rats and mice living at SL that have never been exposed to hypoxia have divergent ventilatory and metabolic responses to sustained hypoxia that are not linked to differences in the lung architecture, but might be linked to a different pattern of expression in HIF-1$\alpha$ in the central respiratory control nuclei between the two species. Furthermore, our results suggest that adult mice, who had never been exposed to altitude, display conclusive signs of innate traits that would predispose them for a better survival under hypoxia.

# IV. CHAPTER 4

## DIVERGENT PHYSIOLOGICAL RESPONSES TO POSTNATAL HYPOXIA IN RATS AND MICE RAISED AT SEA LEVEL

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* Manuscript in preparation for publication

# 1. Abstract

We previously showed that rats and mice raised over generations at high altitude have different physiological responses to hypoxia, likely explaining the different altitude distribution of these 2 species. In the present study, we ask whether similar differences occur at sea level following exposure to postnatal hypoxia. We exposed Sprague-Dawley rats and FVB mice to postnatal hypoxia (13.5% $O_2$ between postnatal days 4-14). We then compared their right ventricular hypertrophy (index of pulmonary hypertension) and alveolar surface area in the lungs and measured minute ventilation, metabolic rate, and pulse oximetry oxygen saturation ($P_{O2,sat}$) under normoxia and acute hypoxia. When necessary, all variables were corrected for body mass using allometric corrections (mass-corrected variables). Compared with control rats, control mice had lower right ventricular weight, higher lung alveolar surface area, similar ventilation but higher metabolic rate. In rats and mice postnatal exposure to hypoxia induced right ventricular hypertrophy and decreased the relative alveolar surface area, while lung volume increased in mice but not in rats. Right ventricular hypertrophy was lower in mice exposed to postnatal hypoxia versus rats. After postnatal hypoxia the hypoxic ventilatory response was increased in mice, but reduced in rats. We conclude that newborn mice and rats have divergent responses to postnatal hypoxia; hence phenotypic plasticity of the respiratory system during postnatal development appears as a key element determining their different phenotype at high altitude.

# 2. Introduction

Rats and mice that have been raised for several generations at high altitude (HA – La Paz, Bolivia, 3600m) have divergent physiological responses to the low ambient $O_2$ partial pressure ($PO_2$). Rats showed high hematocrit (Hct – 60.5±1.1%) and right ventricular hypertrophy (right-to-left ventricle weight ratio: 59.7±5.6%) whereas mice had a Hct level (47.0±1.7%) and right-to-left ventricle weight ratio (32.0±2.0%) only slightly above the values reported at SL. Mice had also a higher mass-corrected tidal volume than rats, and larger lungs (relative to their body mass) with an enhanced alveolar surface area (Chapter 2 p69).

In rats, most of the phenotype reported at HA is linked to hypoxic exposure during the early postnatal period[2]. Indeed, if rats living at HA are exposed to sea level (SL) $PO_2$ during the first 2 postnatal weeks, Hct and hemoglobin (Hb) levels in adults are reduced, and right ventricular hypertrophy and alterations of lung morphology are attenuated[2]. Similarly, if SL rats are raised under postnatal hypoxia, they develop a higher right ventricular hypertrophy and elevated Hct when subsequently exposed to chronic hypoxia as adults[1]. These data suggest that rats present an excessive sensibility to hypoxia during the critical window of postnatal lung development, which in rodents occurs between postnatal days 4 and 14[331-333]. Indeed, exposure to hypoxia during this period impairs alveolar development of the lungs by reducing the alveolar surface area in rats[334,335], but this occurs also in mice[336,337]. Similarly postnatal hypoxia delays the maturation of peripheral chemoreceptors[338,339] leading to an attenuated ventilatory response to acute[340] and chronic hypoxia[1,2] that is known to persists in adults. This so called "developmental plasticity"[341] likely contributes to the phenotype observed in HA residents[342,343] and appears to play also a

role in species that are considered as being genetically adapted to hypoxia, such as the deer-mice in which developmental hypoxia increases aerobic performance in adults[344].

In the present study we postulated that: (i.) laboratory rats and mice would have a different developmental plasticity in response to hypoxia during early postnatal life, and that: (ii.) compared with rats, in mice these responses will be in line with the phenotype reported at HA (Chapter 2 p69). To test these hypotheses, we used newborn FVB mice and Sprague-Dawley rats raised between postnatal days 4-14 under normoxia or at a $PO_2$ level that would be similar to the ambient conditions in La Paz (inspired $PO_2$ – $PiO_2$ – ≈100 mmHg $O_2$ or 13.5 % $O_2$ in inspired air at SL). At the end of the postnatal hypoxic exposure we measured respiratory and metabolic variables under normoxia and in response to graded levels of hypoxia. Then we used standard morphometric approach to determine the effects of postnatal hypoxia on the alveolar surface area. Our results suggest that differences in physiological plasticity during postnatal development contribute to establish the differences reported in adult rats and mice that have been living at HA for several generations.

# 3. Material and methods

## 3.1. General experimental design

Primiparous females from each species (Sprague-Dawley rats and FVB mice – Charles-River – St Constant, Quebec, Canada) were housed with males for mating for at least 7 consecutive days. Animals were housed under standard conditions, had access to food and water ad-libitum, and were exposed to a 12h:12h light/dark cycle. All protocols have been reviewed and approved by the local committee of animal care and use of Laval University and are in concordance with the guidelines of the Canadian Council of Animal Care.

Once pregnancy was confirmed by weight gain, the females were left alone, and the gestation continued under normal SL barometric pressure in normoxia. At birth, rat litters were culled to 12 pups with an equal number of male and female if possible. In mice all newborns were kept, as the litter size was 9 to 11 pups. Four days after birth, animals (dam and pups) were placed under normobaric hypoxia in a 50-liter plexiglas chamber, connected to an Oxycycler (A84XOV, BioSpherix, Redfield, NY, USA). On the 1$^{st}$ day of exposure, $O_2$ level in the chamber was progressively decreased to 13.5% (1% $O_2$ every 20 minutes), and kept constant at this level for 10 consecutive days (until postnatal day 14 – P14). Considering a mean atmospheric pressure in Quebec of 760 mmHg[20], this corresponds to an inspired $PpO_2$ around 100 mmHg, which is the $PpO_2$ found in La Paz. To avoid humidity and $CO_2$ accumulation, Drierite (anhydrous calcium sulfate; Hammond Drierite, Xenia, OH, USA) and Amsorb Plus (calcium hydroxide; Armstrong Medical, Coleraine, North Ireland) were placed inside the chamber. Control animals were kept in their normal room and left undisturbed (except for normal cage cleaning – once a week).

At P14, the animals were taken out of the chamber and blood was immediately drawn to assess the Hct level and the animals were used to measure ventilation, arterial $O_2$ saturation, and heart rate in normoxia, and during exposure to gradual hypoxia (18, 15, 12, 9% $O_2$ for 10 minutes each).

## 3.2. Recording of ventilatory parameters, arterial oxygen saturation, and heart rate in unrestrained, unanesthetized rats and mice

Respiratory recordings were performed using a whole body plethysmograph chamber for adult mice (Emka Technologies, Paris, France) flushed constantly with fresh room air (180-200 ml/min for rats, ≈100 ml/min for mice). The chamber was calibrated with a known volume of air (0.5 ml). Rectal temperature was measured at the beginning of the experiment and the animals were weighed, and equipped with a neck collar allowing recordings of pulse oximetry capillary $O_2$ saturation ($P_{O2,sat}$) and heart rate (Mouse OX® STARR Life Sciences Corp, USA). The animal was then placed in the chamber for a period of tranquilization (10 - 15 minutes), and baseline recordings were initiated for 20 min, followed by acute exposure to graded levels of hypoxia by switching the inflowing tube to a nitrogen gas line calibrated to obtained the desired $O_2$% (18, 15, 12, and 9% $O_2$, each for 10 minutes). Rectal temperature was measured immediately at the end of the last hypoxic exposure.

During recordings, subsampling of inlet and outlet gas were dried and directed toward an oxygen analyzer and a carbon dioxide analyzer (S3A and CD3A analyzers; AEI Technologies – previously calibrated with a certified gas tank), the airflow through the chamber was recorded

continuously by a mass flowmeter (TSI series 4140 mass flowmeter; TSI, Shoreview, MN). All signals (from the plethysmograph, gas analyzers and flowmeter) were directed toward a computer for storage and analysis using the Spike 2 software (Cambridge Electronic Design, Cambridge, UK).

For analysis, we selected a period of at least 30s with a stable breathing pattern during the last 3 minutes of each condition. $O_2$ consumption and $CO_2$ production rates were calculated as described previously in newborn mice[345].

## 3.3. Dissection of hearts and lungs

At postnatal day 15, the animals were deeply anesthetized, and used for lung and heart sampling. All experimental procedures for measurements of right ventricular hypertrophy have already been described in chapter 2 p75. After cardiac perfusion with PBS, in 2 males and 2 females of each litter, a catheter was fixed in the trachea, the lungs were inflated with 4% PFA for 30 minutes at a constant pressure of 24 $cmH_2O$, then dissected. The total volume of the inflated lungs was measured by liquid displacement, and they were kept in 4% PFA for 24 hours at room temperature. The next day, the lungs were separated into left and right lung and automatically embedded in paraffin (Tissue-Tek VIP, Miles scientific). 24 hours later, the samples were included in paraffin and stored until they were processed to determine lung histology (see also chapter 2 p76 and 3 p108).

## 3.4. Lung morphology

After deparaffinization, coloration and capture of the images of each lung section, we randomly selected 3 non-overlapping images from each slide using 3 slides per animal and 10 animals per group (4 males and 6 females for the rats and 5 males and 5 females for the mice). The Mean Linear Intercept ($L_m$), relative and total alveolar surface areas were determined as previously described in chapter 2 p77 and 3 p108.

## 3.5. Allometric scaling in rats and mice

As described previously, to compare physiological and morphological variables between control mice and rats we used allometric scaling (see chapter 2 p77 and 3 p113). In the text, data corrected for the allometric scaling variables are referred to as mass-corrected whereas data compared for body mass are referred to as mass-specific. We assumed that 14 days olds rats and mice have already completed a large portion of their postnatal maturation, and because allometric scaling parameters at this age are not available, we used the allometric coefficients calculated for adult mammals (the coefficients used for each variable are detailed in chapter 2 and 3).

## 3.6. Statistical analysis

We used the GraphPad Prism 6.0c (for 2-way ANOVA) and JMP 11.0 (for 3-way ANOVA and 2-way-ANOVA with repeated measures) for statistical analysis. All values are reported as means±s.e.m., and the significant *P*-value was set at 0.05.

For all values recorded or measured under baseline condition we first performed 3-way ANOVAs (in JMP) with species, sex, and postnatal hypoxia as grouping variables. Since no significant effect of sex, or significant interactions between sex and species, or sex and postnatal hypoxia appeared for these values, data from males and females were pooled, and we performed 2 way ANOVAs with species and postnatal hypoxia as grouping variables. When significant effects appeared, a *post-hoc* analysis with a Fisher's LSD was performed. ANOVA *P* values are reported in the text, while *post-hoc P* values are presented in the figures.

For parameters measured at different levels of $PpiO_2$ we used a MANOVA model (in JMP) with species, sex, and postnatal hypoxia as grouping variables, and $PpiO_2$ as the repeated term. Since no significant effect of sex, or significant interactions between sex and species, sex and postnatal hypoxia, or sex and $PpiO_2$ appeared for these values, data from males and females were pooled. When significant effects appeared, a *post-hoc* analysis with a Fisher's LSD was performed for each $PpiO_2$ level, when values are normalized as % changes from baseline, the Fisher's LSD test was performed on normalized data. *P* values from the MANOVA analysis are reported in the text, while *post-hoc P* values are presented in the figures.

# 4. Results

Body mass, Hct, and absolute values for lung morphology, ventilation, and metabolic rate are presented in Table IV.1. In rats there was an effect of sex ($P$=0.02) and hypoxia on body mass ($P$=0.01 – rats exposed to hypoxia had lower body mass than controls), whereas no effect was found in mice ($P$=0.11 for hypoxia). The Hct values increased

both in rats and mice after exposure to postnatal hypoxia. In rats, lung volume, lung mass, and the total alveolar surface area decreased after exposure to postnatal hypoxia, but were not altered in mice.

| | Rats | | Mice | |
|---|---|---|---|---|
| | Normoxia | Hypoxia | Normoxia | Hypoxia |
| *Body mass (g) – males* | 34.4±0.7 (n=34) | 31.9±1.0 (n=31) | 7.70±0.1 (n=14) | 7.97±0.2 (n=19) |
| *Body mass (g) - females* | 32.1±0,8 (n=29) | 29.9±1.1 (n=35) | 7.73±0.2 (n=25) | 8.00±0.2 (n=13) |
| *Hematocrit (%)* | 35.0±0.6 (n=20) | 36.4±0.6 (n=20) | 38.1±1.1 (n=7) | 39.2±0.7 (n=15) |
| **Lung morphology** | | | | |
| *Lung volume (ml)* | 2.68±0.14 (n=12) | 2.19±0.12 ** (n=16) | 0.57±0.04 (n=16) | 0.77±0.04 (n=15) |
| *Lung mass (g)* | 0.52±0.02 (n=12) | 0.45±0.02 *** (n=16) | 0.10±0.002 (n=16) | 0.11±0.002 (n=15) |
| *Total alveolar surface area ($m^2$)* | 0.37±0.05 (n=12) | 0.26±0.02 ** (n=16) | 0.18±0.02 (n=16) | 0.21±0.02 (n=15) |
| **Ventilatory variables** | | | | |
| *Resp. frequency (bpm)* | 153±5 (n=19) | 155±6 (n=17) | 224±5 (n=19) | 254±8 (n=22) |
| *Tidal volume (ml)* | 0.27±0.01 (n=19) | 0.30±0.02 (n=17) | 0.08±0.01 (n=19) | 0.09±0.04 (n=22) |
| *Minute ventilation (ml/min)* | 44.9±4.2 (n=19) | 51.5±3.7 (n=17) | 17.7±1.6 (n=19) | 21.5±1.6 (n=22) |
| **metabolism** | | | | |
| *$O_2$ consumption (ml/min)* | 1.39±0.08 (n=22) | 1.31±0.08 (n=24) | 0.89±0.08 (n=9) | 0.82±0.11 (n=8) |
| *$CO_2$ production (ml/min)* | 0.88±0.05 (n=22) | 0.84±0.04 (n=24) | 0.64±0.07 (n=9) | 0.58±0.08 (n=8) |

**Table IV.1: Values of body mass (g), Hct (%), and absolute values for lung morphology (volume - ml, mass - g, and total alveolar surface area – $m^2$), ventilatory (fR – breath/min, $V_T$ – ml, and $V_E$ – ml/min), and metabolic variable ( $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ – ml/min) at 14 or 15 (lung morphology) days of age in rats and mice raised under normal conditions (normoxia) or 13.5% $O_2$ (hypoxia).**

Means±s.e.m. number of animal used indicated in each group (n=). **$P$<0.01, and ***$P$<0.001 hypoxia versus normoxia in corresponding species. Cells in grey indicate significant differences between rats and mice.

## 4.1. Right ventricular hypertrophy

A significant effect of species and hypoxia ($P$<0.0001 for both effects) was found for the right-to-left ventricular mass ratio, without significant species x hypoxia interaction ($P$=0.2). Control mice had a lower right-to-left ventricular mass ratio compared with rats (27.6±0.7 versus 33.9±1.0%, Figure IV.1), and the same difference was present in the animals exposed to postnatal hypoxia (40.6±1.6 versus 50.1±1.9 %, Figure IV.1). There was no significant effect of sex, or significant sex x hypoxia, or sex x species interaction.

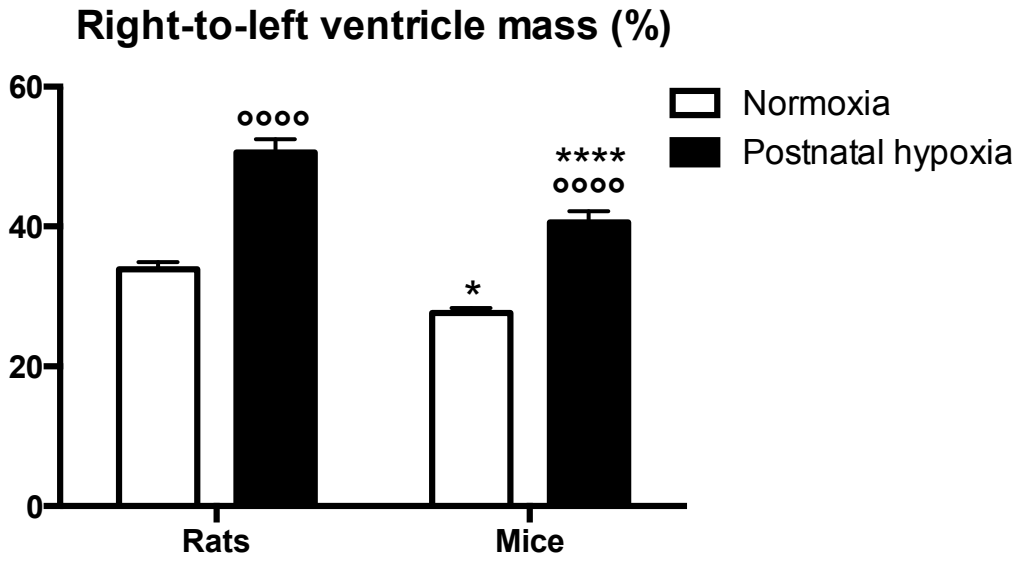


**Figure IV.1: Right ventricular hypertrophy in 14-day-old rats and mice raised under normoxia or postnatal hypoxia.**
Right-to-left ventricle mass (%) in 14-day-old rats and mice. Means+s.e.m. *$P$<0.05 and ****$P$<0.0001 mice versus rats. °$P$<0.05 and °°°°$P$<0.0001 postnatal hypoxia versus normoxia.

## 4.2. Lung morphology

Representative examples of lung slices showing alveolar architecture in rats and mice raised under control condition or postnatal hypoxia are presented in Figure IV.2. The effects of postnatal hypoxia on alveolar structure in rats and mice are clearly apparent, as well as the striking difference between both species. There was no significant effect of sex, or significant sex x hypoxia, or sex x species interaction, so males and females are pooled. The mean linear intercept ($L_m$ – Figure IV.3A) was lower in mice than in rats ($P<0.0001$ for species), and was increased by hypoxia in both species ($P<0.0001$ for hypoxia). The magnitude of the effect was stronger in rats than in mice ($L_m$ increased from 24.6±0.4 to 35.4±0.7 µm in rats – and from 12.5±0.3 to 15.8±0.3 µm in mice – $P<0.0001$ for species x hypoxia).

Mass-corrected relative alveolar surface area calculated from the values of $L_m$ ($S=4V/L_m$, were $V$ is the volume of each slice) was 40% lower in control rats (0.269±0.006 $m^2/cm^3/g^{-0.13}$) compared with control mice (0.446±0.013 $m^2/cm^3/g^{-0.13}$ Figure IV.3B), and decreased following hypoxic exposure by about 30% in rats (to 0.191±0.006 $m^2/cm^3/g^{-0.13}$) and 21% in mice (to 0.354±0.008 $m^2/cm^3/g^{-0.13}$, Figure IV.3B), with a significant effect of species and hypoxia for the mass-corrected relative alveolar surface area ($P<0.0001$) without significant species x hypoxia interaction.

For the mass-corrected lung volume, there was a significant effect of species ($P=0.003$) but no significant effect of hypoxia ($P=0.07$), or significant species x hypoxia interaction ($P=0.07$). The *post-hoc* analysis indicated that following postnatal hypoxia mice had higher mass-corrected lung volume (0.094±0.005 ml/g) than rats (0.068±0.004 ml/g, Figure IV.3C). The mass-corrected total alveolar surface area

(relative alveolar surface x lung volume) was higher in mice than in rats ($P$<0.0001 for species) and there was no significant effect of hypoxia ($P$=0.5), or significant species x hypoxia interaction ($P$=0.06, Figure IV.3D).

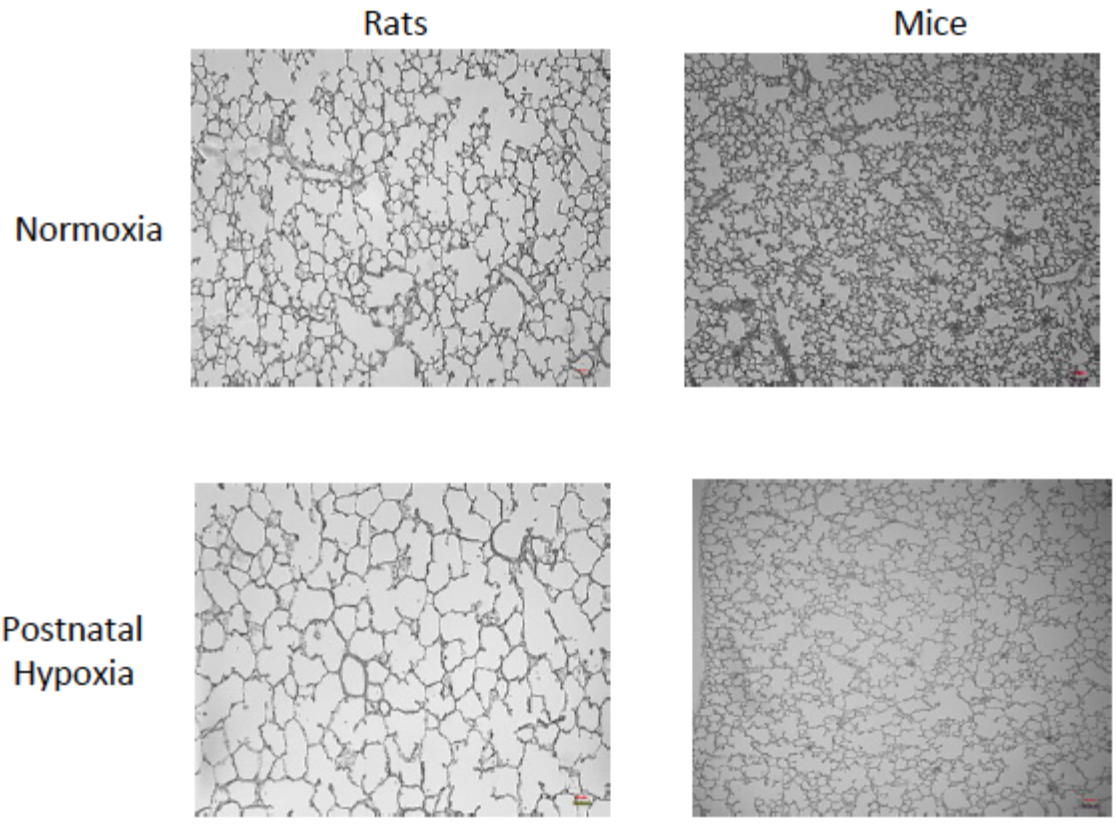


**Figure IV.2: Typical images of the lungs in 14-day-old rats and mice raised under normoxia or postnatal hypoxia.**
Scale bars, 20 µm.

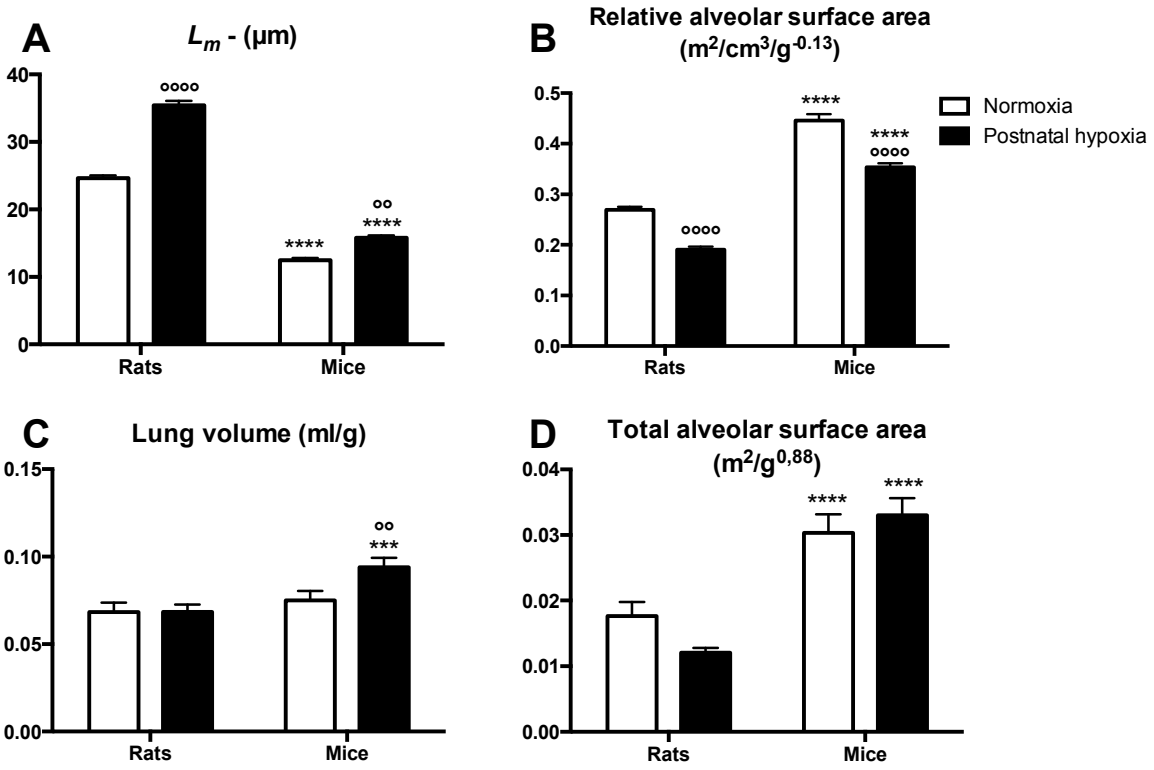


**Figure IV.3: Parameters of lung architecture in 14-day-old rats and mice raised under normoxia or postnatal hypoxia.**
(A) mean linear intercept ($L_m$ – µm), (B) mass-corrected relative alveolar surface area ($m^2/cm^3/g^{-0.13}$), (C) mass-specific lung volume (ml/g), (D) mass-corrected total alveolar surface area ($m^2/g^{0.88}$) in 14-day-old rats and mice. Means+s.e.m.
***$P$<0.001 and ****$P$<0.0001 mice versus rats. °°$P$<0.01 and °°°°$P$<0.0001 postnatal hypoxia versus normoxia.

## 4.3. Respiratory and metabolic values recorded under baseline conditions

Mass-corrected respiratory frequency (fR) was similar between species and there was no effect of postnatal hypoxia (Figure IV.4A). For mass-corrected tidal volume ($V_T$) and minute ventilation ($V_E$) there were significant effects of postnatal hypoxia ($P$=0.002 an $P$=0.009 respectively). However, the *post-hoc* analysis showed that postnatal

hypoxia slightly increased $V_T$ and $V_E$ in rats, but not in mice (Figure IV.4 B,C). For the mass-corrected $O_2$-consumption rate ( $\dot{V}_{O_2}$ ) and $CO_2$-production rate ( $\dot{V}_{CO_2}$ ) there were significant effects of species ($P$<0.0001 for both values) with mice showing higher values than rats (Figure IV.4 D,E) without significant effect of hypoxia, or interaction between species and hypoxia.

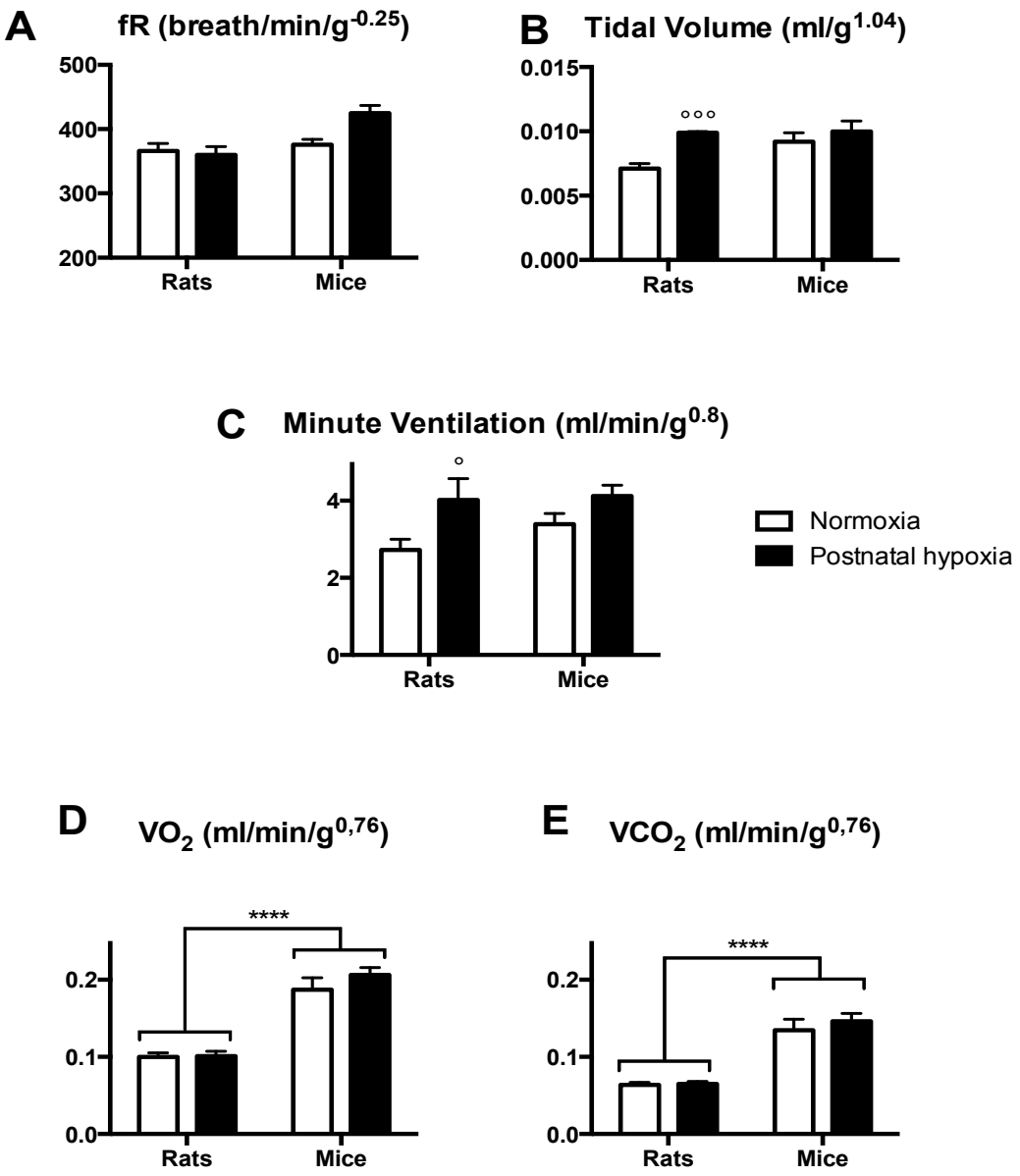


**Figure IV.4: Ventilatory and metabolic variables recorded under baseline condition (21% oxygen) in 14-day-old rats and mice raised under normoxia or postnatal hypoxia.**

(A) Mass-corrected respiratory frequency (fR – breaths/min/$g^{-0.25}$), (B) mass-corrected tidal volume ($V_T$ – ml/$g^{1.04}$), (C) mass-corrected minute ventilation ($V_E$ – ml/min/$g^{0.8}$), (D) mass-corrected $O_2$ consumption rate ( $\dot{V}_{O_2}$ – ml/min/$g^{0.76}$), and (E) mass-corrected $CO_2$ production rate ( $\dot{V}_{CO_2}$ – ml/min/$g^{0.76}$). Means+s.e.m.

***P<0.001 and ****$P$<0.0001 mice versus rats. °$P$<0.05 and °°°$P$<0.001 postnatal hypoxia versus normoxia.

## 4.4. Pulse oximetry oxygen saturation and heart rate under baseline conditions and during hypoxic exposure

$P_{O2,sat}$ and mass corrected HR values recorded in mice and rats are presented in Figure IV.5. There was no significant effect of sex, or significant sex x hypoxia, or sex x species interaction, accordingly data from males and females have been pooled. $P_{O2,sat}$ decreased steadily as the $O_2$% in the chamber decreased, with an overall effect of postnatal hypoxia ($P<0.0001$), and a significant interaction between $O_2$%, species, and hypoxia ($P=0.002$ – Figure IV.5 A,B). Rats exposed to postnatal hypoxia had lower $P_{O2,sat}$ than control rats at all levels of oxygen below 21% (Figure IV.5A). In mice this effect was less important, and appeared only at 15 and 12% $O_2$ (Figure IV.5B). Mice exposed to postnatal hypoxia were able to maintain higher $P_{O2,sat}$ than rats under moderate levels of acute hypoxia (only at 18 and 15% $O_2$).

Under baseline conditions mice raised in normoxia had higher mass-corrected heart rate than rats (1108 bpm/$g^{-0.25}$ in control mice vs. 1000 bpm/$g^{-0.25}$ in control rats), but mice reached lower values of heart rate than rats at 12% and 9% $O_2$ (971 and 757 bpm/$g^{-0.25}$ in control mice versus 1045 and 911 bpm/$g^{-0.25}$ in control rats – Figure IV.5 C,D). Rats raised under postnatal hypoxia had higher mass-corrected heart rate than control rats under all $O_2$% (Figure IV.5) whereas in mice exposed to postnatal hypoxia mass-corrected heart rate was higher than in controls at 12% and 9% $O_2$ only (971 and 757 bpm/$g^{-0.25}$ in control mice *vs*. 1076 and 921 bpm/$g^{-0.25}$ in mice exposed to postnatal hypoxia – Figure IV.5 C,D).

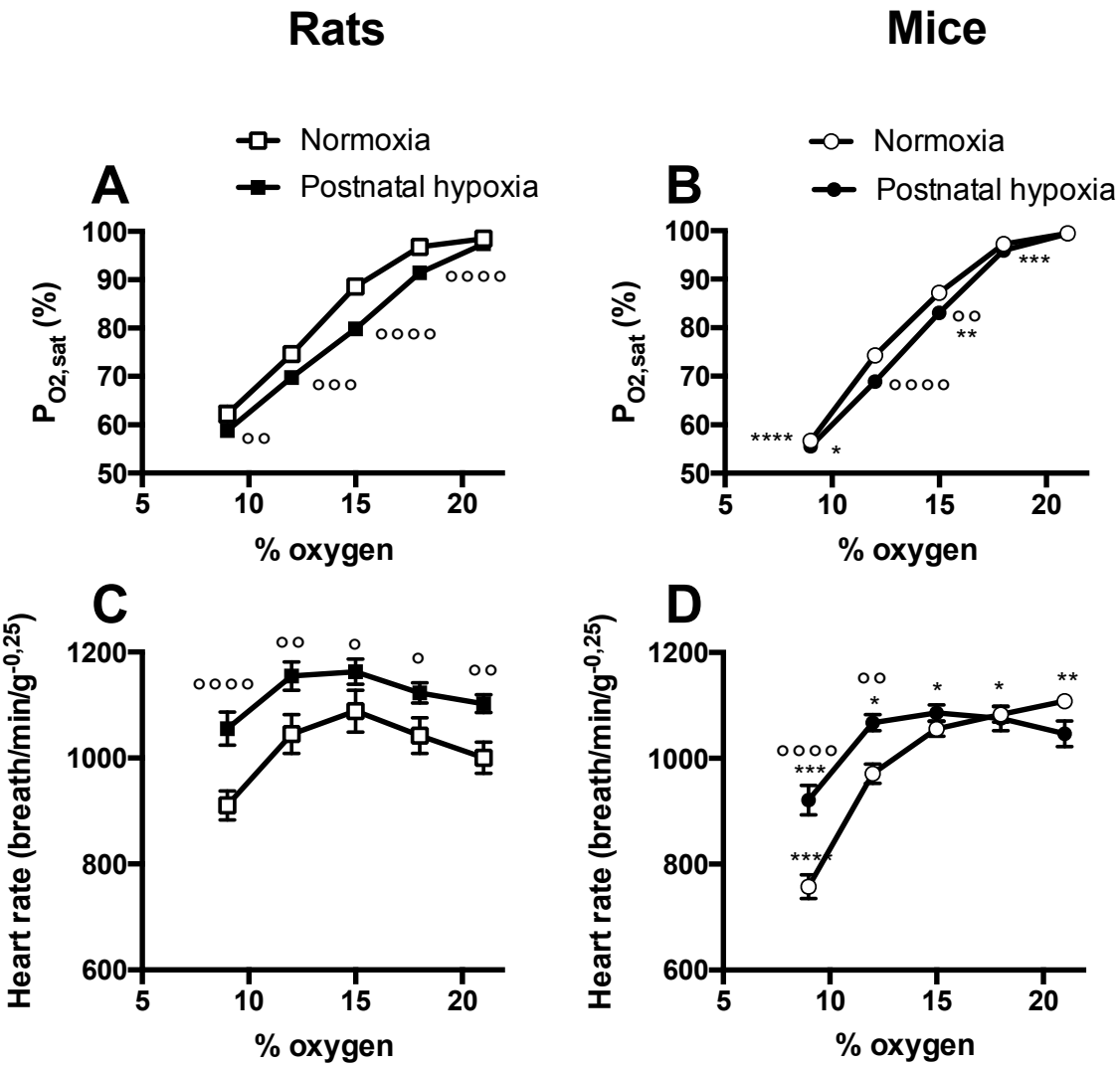


**Figure IV.5: Response to changes of inspired $O_2$ of arterial oxygen saturation and heart rate in 14-day-old rats and mice raised under normoxia or postnatal hypoxia.**

(A,B) pulse oximetry oxygen saturation ($P_{O2,sat}$ – %) and (C,D) mass-corrected heart rate (beats/min/$g^{-0.25}$). Means±s.e.m.

**P*<0.05, ***P*<0.01, ****P*<0.001, and *****P*<0.0001 mice versus rats. °*P*<0.05, °°*P*<0.01, °°°*P*<0.001, and °°°°*P*<0.0001 postnatal hypoxia versus normoxia.

## 4.5. Hypoxic ventilatory response

Overall, control mice had a different response to hypoxia than control rats (Figure IV.6): rats displayed a progressive increase of fR and $V_E$ with decreasing $O_2$ level, and they maintained their $V_T$ close to baseline values. Mice had only a limited increase of fR but they reduced $V_T$ and $V_E$ during hypoxic exposure. In rats raised under postnatal hypoxia the $V_T$ declined below baseline levels during exposure to 12% and 9% $O_2$. By contrast, in mice exposed to postnatal hypoxia, the hypoxic ventilatory response was increased compared with control mice at all level of hypoxia below 21% (Figure IV.6).

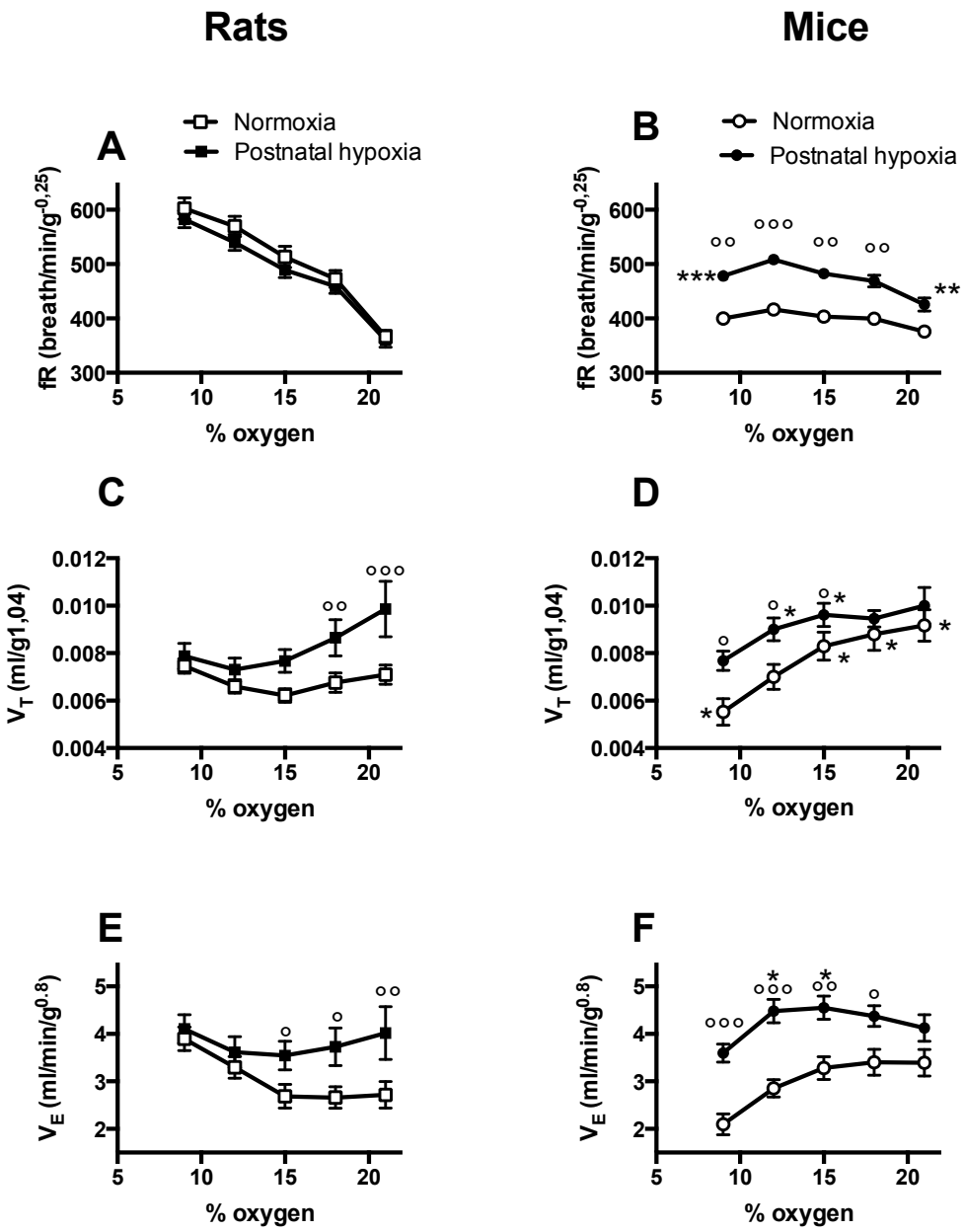


**Figure IV.6: Response to changes of inspired $O_2$ of respiratory variables (mass-corrected values) – in 14-day-old rats and mice raised under normoxia or postnatal hypoxia.**

(A-B) respiratory frequency (fR – breath/min/$g^{-0.25}$), (C-D) tidal volume ($V_T$ – ml/$g1^{.04}$), (E-F) minute ventilation ($V_E$ – ml/min/$g^{0.8}$). All values are mass-corrected. Means±s.e.m.

**P*<0.05, ***P*<0.01, and ****P*<0.001 mice versus rats. °*P*<0.05, °°*P*<0.01, °°°*P*<0.001, and °°°°*P*<0.0001 postnatal hypoxia versus normoxia.

Under hypoxia rats and mice had a decrease of metabolic rate (Figure IV.7). Interestingly, the difference between rats and mice under normoxia (higher mass-corrected $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ in mice versus rats) was progressively reduced as the $O_2$ level in the recording chamber decreased (Figure IV.7 A,B). However, when expressed as % of changes versus normoxic values, there was no difference between species, and no effect of postnatal hypoxia for $\dot{V}_{O_2}$ or $\dot{V}_{CO_2}$ (Figure IV.7 C-F). Body temperature decreased during hypoxic exposure, and control mice had a more important decrease than control rats (33.3±0.23 and 31.8±0.21°C respectively at the end of the experiment, Figure IV.8). However mice exposed to postnatal hypoxia had a less important decrease of rectal temperature during acute hypoxic exposure (32.9±0.20°C at the end of the experiment, Figure IV.8), the same effect appeared for rats, but was less pronounced.

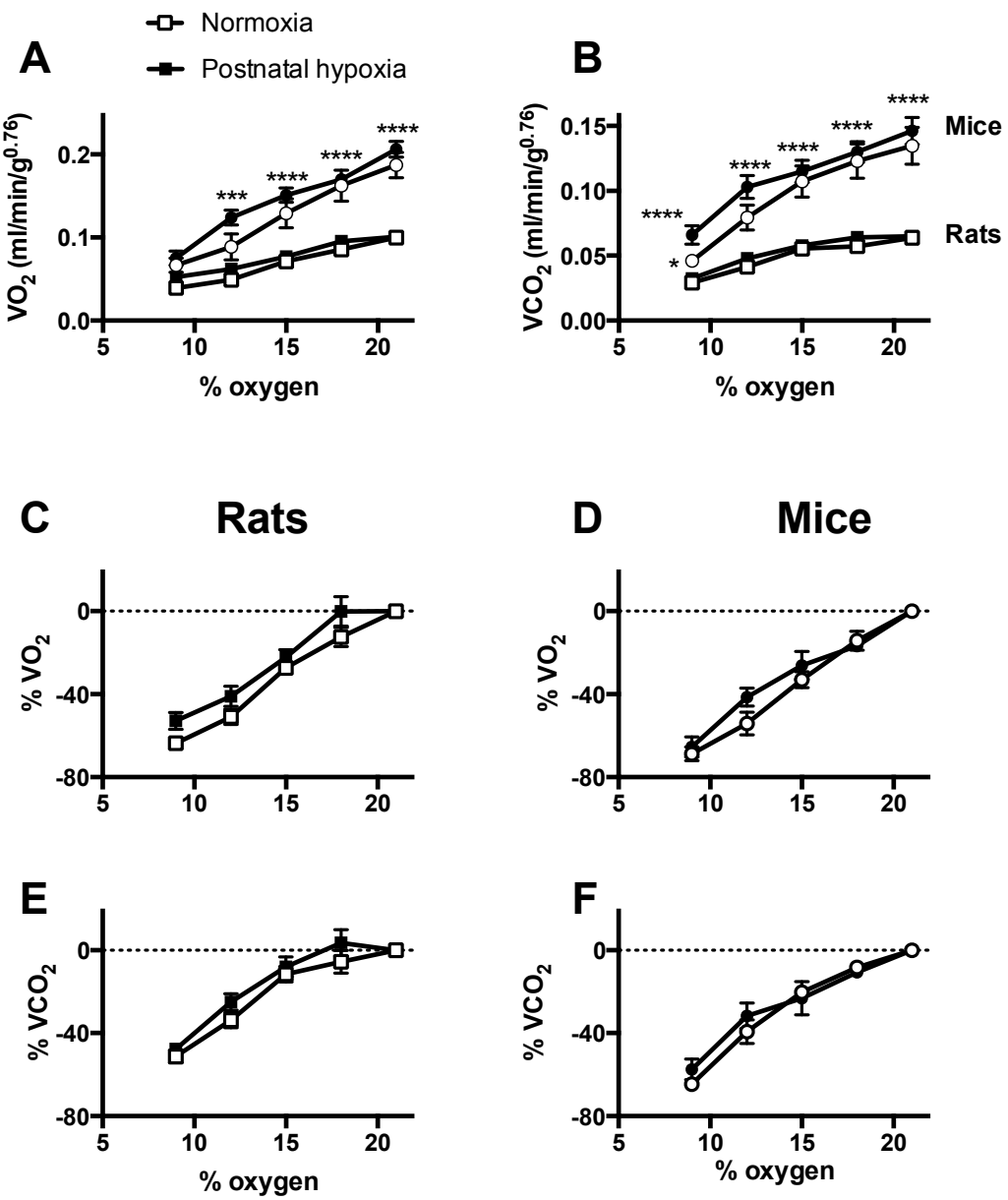


**Figure IV.7: Response to changes of inspired $O_2$ of metabolic variables (mass-corrected and % versus baseline) – in 14-day-old rats and mice raised under normoxia or postnatal hypoxia.**

(A-B) $O_2$ consumption rate and $CO_2$ production rate ( $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ ml/min/$g^{0.76}$) as mass-corrected values, (C-F) $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ as % fo changes versus baseline. Means±s.e.m.

*$P$<0.05, ***$P$<0.001, and ****$P$<0.0001 mice versus rats.

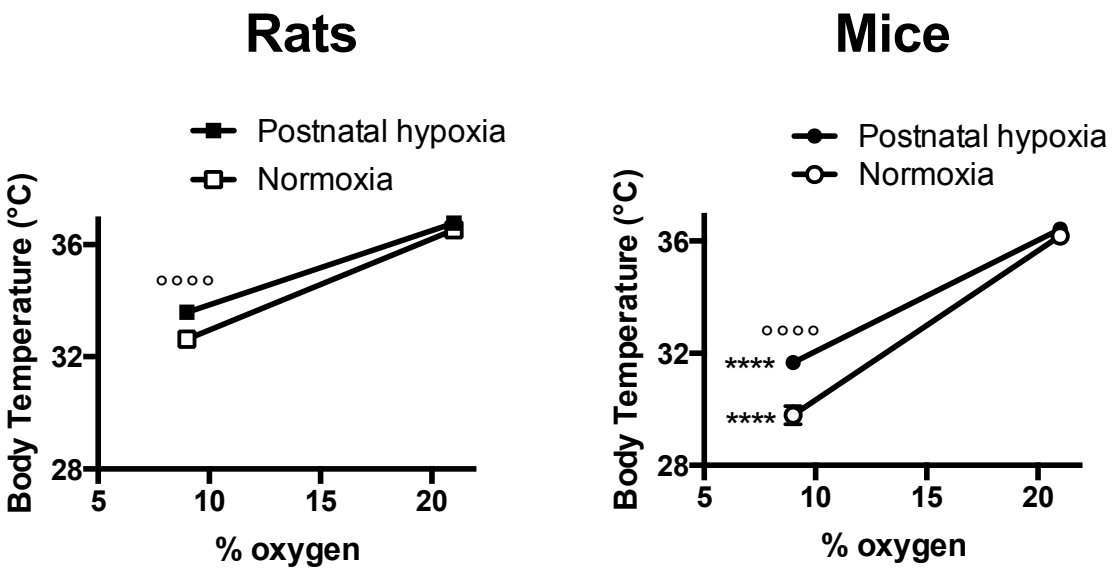


**Figure IV.8: Response to changes of inspired $O_2$ of body temperature in 14-day-old rats and mice raised under normoxia or postnatal hypoxia.**
Means±s.e.m.
***$P$<0.001 mice versus rats. °°°°$P$<0.0001 postnatal hypoxia versus normoxia.

The respiratory exchange ratio ($\dot{V}_{CO_2}/\dot{V}_{O_2}$) increased progressively during hypoxic exposure ($P$<0.0001 for hypoxia) in rats and mice (Figure IV.9), to reach a maximum value at 12% $O_2$. While there was no significant effect of group ($P$=0.25), or group x hypoxia interaction ($P$=0.6), it is noteworthy that in mice exposed to postnatal hypoxia, $\dot{V}_{CO_2}/\dot{V}_{O_2}$ was lower than in control mice during the hypoxic exposure (the *post-hoc* analysis showed a $P$ value of 0.05 and 0.005 for postnatal hypoxia in mice at 15 and 12% $O_2$ when the ANOVA is performed only in mice).

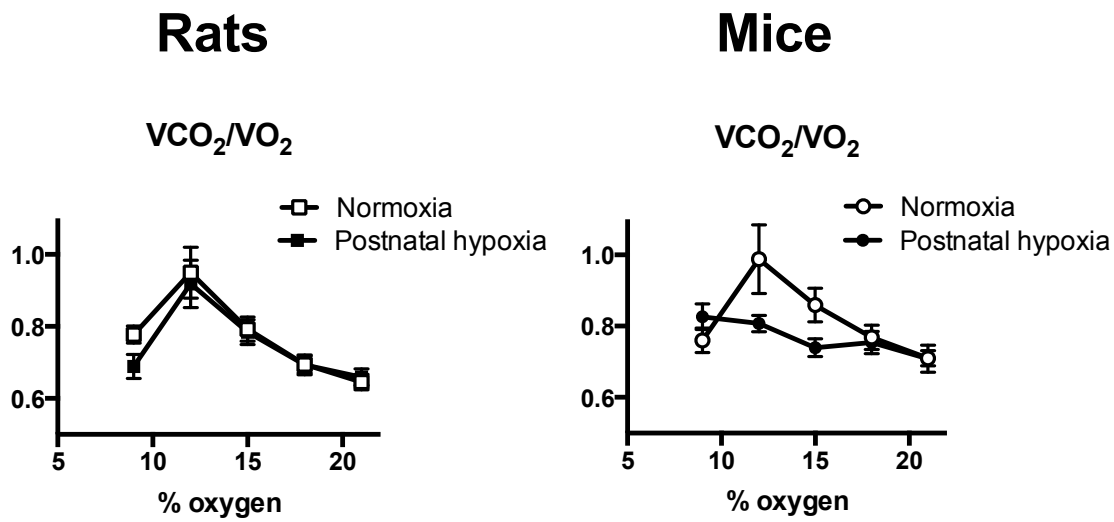


**Figure IV.9: Response to changes of inspired $O_2$ for the respiratory exchange ratio ( $\dot{V}_{CO_2} / \dot{V}_{O_2}$ ) in 14-day-old rats and mice raised under normoxia or postnatal hypoxia.**
Means±s.e.m.
****P*<0,001 and *****P*<0,0001 mice versus rats. °°*P*<0.01, and °°°°*P*<0,0001 postnatal hypoxia versus normoxia.

# 5. Discussion

We showed previously that adult laboratory rats and mice that have been raised over generations at HA (3600m La Paz, Bolivia – for more than 20 years) have different physiological responses to the ambient hypoxia (Chapter 2 p69). Furthermore, in rats living at HA postnatal hypoxia had deleterious long-term consequences, leading to exaggerated Hct values and right ventricular hypertrophy[2], which likely explain that under natural conditions wild mice can be found at HA, but not rats.

In the present study, we tested the hypothesis that at SL, newborn rats and mice would have different physiological responses to chronic postnatal hypoxia. Our results show that after 10 days of

exposure to a moderate level of hypoxia (corresponding to an altitude of 3600m) mice had a lower right ventricular hypertrophy, higher relative and total alveolar surface areas than rats, higher lung volume, and, when exposed to low $O_2$ levels, higher $V_E$, and higher arterial $O_2$ saturation than rats. Furthermore, compared with control rats (raised in normoxia), control mice have a lower relative mass of the right ventricle, likely indicating a lower arterial pulmonary pressure, higher relative and total alveolar surface areas in the lungs, higher metabolic rate and higher $P_{O2,sat}$ in hypoxia. Accordingly, we conclude that newborn mice display innate traits that could probably explain that mice are able to establish stable colonies at HA under natural conditions[277], while this is not the case for rats (see Chapter 2). Therefore the differences reported between rats and mice in HA colonies (See chapter 2 p69) likely result from a mix of predisposition, and phenotypic plasticity in response to hypoxic exposure during postnatal development[341].

## 5.1. High altitude versus sea level rats and mice

We have previously discussed that the subspecies characterization of the mice we used at HA have not been completely elucidated (chapter 2 p72). However because the colony has been established by using mice provided by a standard laboratory mice breeder they are most likely *Mus musculus domesticus*[299]. We performed a genetic background analysis (service provided by Charles-River) for these mice based on allele variations of 384 single-nucleotide-polymorphisms of a sample of DNA, that indicated that the mice scored as 73.11 % FVB, and that they were likely a mix of FVB and 2 other strains, or an outbred strain. Accordingly, we used FVB mice in the present study as the closest strain. A genetic survey in wild mice captured in La Paz showed that

they were either related to *Mus musculus domesticus* or *Mus musculus castaneus*[277] - another Mus subspecies. Although we might not discard the possibility that lung morphology or physiological responses to hypoxia might be different in another inbreed line of mice, we are still confident that the FVB strain of *Mus musculus domesticus* is a reliable model to understand the physiology of the HA mice.

Similarly, the colony of HA rats used in the previous study at HA has been established using Sprague-Dawley rat breeders originally purchased from IFFA-CREDO, and they have been used in recent (reference [2], and chapter 2 p69) and previous studies at HA[313,346]. We used the same strain in the present study, and therefore we believe that it is reasonable to use these animals to better understand the phenotype of the HA rats. Moreover, regardless of the strain or the species, ecological data on distributions of wild rats uniformly reports that they are not found at altitude whether reporting data from New-Zealand[347], Corsica[348], or Bolivia[32]. Accordingly, this is a widespread observation, and using laboratory Sprague-Dawley rats to understand this appears as a reliable tool.

### 5.1.1. "Predispositive traits": sea level control newborn mice have higher alveolar surfaces but similar levels of $P_{O2,sat}$ in acute hypoxia compared with control rats

In terms of lung structure the most significant difference between rats and mice appears to be the higher mass-corrected relative and total alveolar surface areas in mice. This likely contributes to the differences observed for the hypoxic ventilatory response by ensuring a higher diffusion of $O_2$ through the lungs without requiring excessive increase of

$V_E$ (cf. Figure IV.6) despite the decrease in $O_2$ consumption (cf. Figure IV.7). In parallel with their high alveolar surface areas, mice also had higher metabolic rate than rats, which appears consistent with the notion that $O_2$ consumption and growth of the lungs during postnatal development are tightly linked[349]. However, previous studies performed in adult rats show that they have a highly efficient gas exchange function, as evidenced by their ability to increase arterial $PO_2$ by an efficient hyperventilation (decreased arterial $PCO_2$) during exercise in hypoxia[350], but we are not aware of systematic comparisons between rats and mice, particularly during postnatal development.

Most of these traits have been previously reported in adult mice compared with adult rats after several generations at HA (see chapter 2 p69), these physiological, morphological, and metabolic traits argue in favor of a greater predisposition of newborn (and therefore adults) mice for survival at HA, without requiring long-term selection and genetic adaptations.

### 5.1.2. "Developmental plasticity": newborn mice have larger lungs, and higher HVR than rats after exposure to postnatal hypoxia

Postnatal exposure to hypoxia decreased the relative alveolar surface area in the lungs in the two species, as previously reported in rats[334,335] and mice[336,337]. Accordingly, there is no sign of specific resistance of mice for the deleterious consequences of postnatal hypoxia on alveolar development. It should be mentioned that when rats[188,351] or guinea pigs[174] are exposed to hypoxia after the 3$^{rd}$ or 4$^{th}$ postnatal week, the effects observed are opposite, with an enhanced lung growth, and higher alveolar surface areas. Nonetheless, as observed in our

colony of HA rats, these effects are apparently not sufficient to reverse the deleterious effects of hypoxia on lung development during the 1st and 2nd postnatal weeks, as previously reported[334]. After exposure to postnatal hypoxia, mice had higher lung volume than rats and the total alveolar surface area showed a tendency to be reduced in rats but not in mice. Previous studies in deer mice showed that upon hypoxic exposure adult animals are able to increase their heart mass and lung volume[175], thus suggesting that this strategy is common between the two species.

In rats, as expected, postnatal hypoxia decreased the magnitude of the ventilatory response to hypoxia, a well-known effect[338,339] which is associated with a reduction of the sensitivity of carotid body type 1 cells to hypoxia[352]. In mice the opposite effect was apparent as the hypoxic ventilatory response increased after exposure to postnatal hypoxia. These results are surprising, and it is likely that these differences are linked to different responses to postnatal hypoxia of the peripheral chemoreceptors and central pathways involved in respiratory control. Interestingly, in the previous chapter p126, we assessed the expression of HIF-1$\alpha$ in the brainstem of adult SL Sprague-Dawley rats and FVB mice exposed to sustained hypoxia, and found a higher response in mice than in rats. Accordingly, a higher response of HIF and HIF's target genes in mice compared with rats might underlie these differences of postnatal respiratory responses to hypoxia.

## 5.2. Different metabolic response to hypoxia in rats and mice?

In rats and mice raised in normoxia, the respiratory exchange ratio ($\dot{V}_{CO_2}/\dot{V}_{O_2}$) increased in response to acute hypoxic exposure, likely

reflecting a progressive increase of glycolysis, a well known process that helps sustain cellular metabolism in hypoxia by ensuring the most efficient production of ATP molecules for each molecule of $O_2$ used[353]. Interestingly, mice exposed to postnatal hypoxia maintained lower respiratory exchange ratio than control mice during exposure to acute hypoxia (Figure IV.9). However, it needs to be pointed out that the hypoxic exposure protocol is only acute and the duration of all hypoxia combined last for less than 2 hours, thus it is possible that the animals have not yet reached steady state and that blood $CO_2$ level might still be falling. Thus the $\dot{V}_{CO_2} / \dot{V}_{O_2}$ ratio calculated is not necessary an accurate reflection of cellular fuel use but rather reflects the accentuation of the $CO_2$ loss (which occur until steady state of blood $CO_2$ are reach).

In animals from our HA colony, under baseline conditions rats had a higher respiratory exchange ratio than mice (chapter 2 p84), which could help them avoiding a reduction in arterial $O_2$ saturation. On the contrary, mice had lower respiratory exchange ratio, a high metabolic rate ($O_2$ consumption and $CO_2$ production), and much larger lungs and higher alveolar surface area than rats. Interestingly, deer mice are also able to maintain a low respiratory exchange ratio and elevated levels of fatty acid oxidation when simultaneously exposed to cold stress and hypoxia[305]. It has been suggested that this pattern promotes "thermogenic endurance" in a cold and hypoxic environment, but that specific mechanisms ensuring adequate $O_2$ delivery to the tissues should be present to compensate for the higher $O_2$ demand required by this strategy. In HA deer mice, there is evidence for an enhanced hemoglobin affinity that plays this role[282,283], and we proposed that in FVB mice raised at HA the high exchange surface area of the lungs can be sufficient to maintain adequate $O_2$ delivery in hypoxia (see chapter 2 p69). The present data further expand this view, showing that newborn

mice exposed to postnatal hypoxia respond to acute hypoxia while maintaining a lower respiratory exchange ratio than rats, providing another advantage for mice to withstand hypoxia and HA during postnatal development.

## 5.3. Control of heart rate after chronic hypoxia in newborn rats and mice

Several studies have shown that chronic exposure to hypoxia induces a desensitization of the sympathetic control of heart rate through the β-adrenergic pathway[354] and increases the parasympathetic control through the cholinergic/muscarinic pathway[355] to prevent excessive stimulation of the heart. Species adapted to HA such as the plateau pika (*Ochotona curzoniae* – a lagomorph from the Tibetan plateau) demonstrate a desensitization of the cholinergic and adrenergic system in the heart[285], whereas in guinea-pigs born and raised at 4300m above SL (Peru) there is a desensitization of the sympathetic system and a sensitization of the parasympathetic system[356]. In our laboratory colonies of HA rats and mice, rats had higher heart rate than mice (Chapter 2 p95). However, in the present study, mass-corrected heart rate during acute hypoxia increases in mice but not in rats after postnatal hypoxic exposure – another response that might favor $O_2$ delivery to the tissues in mice. It is possible that rats and mice display differential plasticity of the cholinergic and adrenergic systems in the heart. However, at least in guinea pigs, it has been suggested that this plasticity of the cardiac sympathetic/parasympathetic control is a response to the metabolic demand of the heart rather than to hypoxemia, and has not been considered as an exclusive adaptation to

hypoxia[356]. The same cautious statement could be applied to our observed differences of heart rate between control rats and mice.

We conclude that newborn rats and mice have different physiological responses to postnatal hypoxia. Since most of the differences observed between these two species are similar to the differences reported in rats and mice that have been living at HA for more than 30 generations, the most important difference between the two species likely relies on different phenotypic plasticity in response to hypoxic exposure. In mice this phenotypic plasticity would confer an adaptive advantage in the sense that it seems to favor survival and colonization of the HA environment[277]. The fact that this plasticity occurs early during postnatal development is in line with a large number of studies showing that postnatal hypoxia is a key determinant of the phenotypic response to altitude and hypoxia in different mammalian species[2,274,340,342-344] and confirm our earlier hypothesis that newborn rats have detrimental responses to postnatal hypoxia[1,2], likely explaining that under natural conditions rats are not able to establish stable colonies at HA.

# V. CHAPTER 5

## HIGH ALTITUDE 14-DAY-OLD LABORATORY RATS HAVE ACQUIRED CHARACTERISTICS THAT PROTECT LUNG GROWTH AT 100 mmHg

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*Preliminary exploitation of the results

# 1. Abstract

We previously showed that adult rats and mice raised over generations at high altitude (HA) have different physiological responses to hypoxia, likely linked to an increased gas-exchange surface area present in mice but not in rats. However, at sea level (SL), adult rats and mice display similar lung architecture, and postnatal exposure to hypoxia in rats at SL resulted in an alteration of the architecture of the alveolar surface area compared with control rats. Thus, in this study we asked how laboratory rats were able to survive and breed for more than 30 generations at HA, and tested the hypothesis that newborn rats living at HA might display a form a phenotypical adaptation to HA in the lungs architecture, that might be the result of a traits selection over generations that would thus not be present in SL animals. To test this hypothesis, we compared arterial $O_2$ saturation, and lung morphology in response to hypoxia in 4 and 14 days old rats living at HA in La Paz at 3600m or at SL in Quebec. In Quebec, animals were exposed in ambient air or in hypoxia (13% $O_2$, corresponding at SL to the $PO_2$ of the altitude of La Paz, Bolivia – 100 mmHg) during postnatal days 4 to 14 (P4-P14). In La Paz, animals were exposed in ambient air or in 32% $O_2$ (this $O_2$ percentage in La Paz relieve the ambient hypoxic stimulus and mimics the ambient condition of sea level $PO_2$ – 160 mmHg). The result showed that HA control P14 rats presented architectural lung structure similar to the control SL P14 rats, suggesting that over the generations, HA rats were able to acquire traits preventing the destruction of the lung architecture. The preservation of the lung architecture in the HA rat is certainly in part responsible for improving their arterial $O_2$ saturation.

# 2. Introduction

High altitude (HA) environment is a challenging place to live for many organisms including humans and animals. Different species have been able to colonize and adjust at different altitudes: the mice (*Mus musculus*) are commonly found up to an altitude of 4000m[277] whereas the rats (no matter the strain or species) are notably absent in high montane environment[32,348]. However, we have been able to raise Sprague-Dawley rats (*Rattus norvegicus*, originally imported from Charles River France) at the Bolivian Institute of High Altitude Biology (IBBA – La Paz, 3600m above sea level – SL), for more than 20 years and over 30 generations. Compared with mice that have been raised in the same conditions, we observed that rats had numerous signs of inadequate responses to altitude environment such as important right ventricular hypertrophy (a sign of pulmonary hypertension) and altered alveolar structure with enlarged airspaces in the lungs, and lower arterial $O_2$ saturation when exposed to moderate hypoxia (chapter 2 p69). Previous studies demonstrated that most of the phenotype observed in the adult HA rats is reversible if newborn are raised under SL $PO_2$ (inspired $PO_2$ – $PiO_2$ – ≈160 mmHg $O_2$ or 32 % $O_2$ in inspired air at 3600m) during the first two weeks of life[2]. In the previous chapter, we observed that postnatal hypoxia decreased arterial $O_2$ saturation and impaired alveolar formation in SL 14-day-old rats (chapter 4 p137). These data suggested that rats present impaired developmental plasticity to hypoxia during the critical window of postnatal lung development (which in rodent occurs between postnatal days 4 to 14)[169,170] and are in line with previous studies showing that postnatal hypoxia is a key determinant of the phenotypic response to altitude and hypoxia in different mammalian species[154,274,340,342,344]. Key element for survival and adaptation at HA is to enhance $O_2$ uptake and distribution,

which can be achieved by improving lung morphology and diffusion capacity. Several studies documented that postnatal hypoxia in mammal results in immediate changes such as enhancing alveolar septal growth and acinar remodeling[125,165,176,334-336]. When we compared our previous results regarding lung architecture between adult rats living at SL and HA (Appendix 2) we observed that rats raised at HA for 30 generations had similar lung volume, mean linear intercept ($L_m$) and total alveolar surface area than SL rats whereas, raising SL rat in hypoxia between postnatal days 4-14 had deleterious consequences in the lung architecture increasing the $L_m$ and the relative alveolar surface area and a strong tendency to reduce the total alveolar surface area. Based on these observations, we tested the hypothesis that newborn rats living at HA preserved lungs architecture to improve $O_2$ uptake and delivery. To test this hypothesis, we used newborn Sprague-Dawley rats raised at HA (La Paz, Bolivia) or SL (Quebec, Canada). In Canada, animals were raised under normoxia or under conditions that would be similar to the ambient conditions in La Paz ($PiO_2$ ≈100 mmHg $O_2$ or 13.5 % $O_2$ in inspired air at SL). In Bolivia, animals were raised under ambient air or under a $PiO_2$ that would be similar to the SL ambient condition ($PiO_2$ ≈160 mmHg or 32% $O_2$ in inspired air in La Paz, 3600m above SL). All exposure were performed during postnatal days 4-14. Prior to, and, at the end of, the postnatal exposure, we recorded the arterial $O_2$ saturation in 4- and 14-day-old SL and HA laboratory rats. Then, we compared right ventricular hypertrophy (a sign of pulmonary hypertension) and lung architecture in P15 rats living at SL or HA.

# 3. Materials and methods

## 3.1. General experimental design

### 3.1.1. Animals

Rats come from 20 primiparous females Sprague-Dawley rats (12 were ordered in Charles-River – St Constant, Quebec, Canada and 8 were born in our HA colony, La Paz, Bolivia) that were housed with males for mating for at least 7 consecutive days. Once pregnancy was confirmed by weight gain, the females were left alone. In Quebec, at birth, rat litters were culled to 12 pups with an equal number of male and female. In La Paz, all the pups were kept as the litter size are between 10-13 pups. In total, experiments at SL were performed on 67 males and 60 females and experiments at HA were performed on 27 males and 28 females. SL P14 rats data are used as comparison in this chapter and are the same as the previous data presented in chapter 4 p137.

### 3.1.2. Oxygen exposure

At SL, four days after birth, rats were exposed under normoxia or under normobaric hypoxia at 13.5% $O_2$ in a 50 liters Plexiglas chamber connected to an Oxycycler (A84XOV, BioSpherix, Redfield, NY, USA – as described in chapter 4 p106). At HA, four days after birth, rats were exposed under room air or under hypobaric normoxia at 32% $O_2$ in a 50 liters Plexiglas chamber. $O_2$ level inside the chamber was maintained by a continuous flow of calibrated gas permanently flushed through the chamber. The chamber was equipped with a $CO_2$ sensor that was checked twice daily and never exceeded 0.3%. The air inside the

chamber was continuously mixed with a small fan. The chamber was not opened during the 10 days of exposure. At postnatal day 4, the $O_2$ inside the chamber increased progressively to 32% and at postnatal day 14 (P14), the chamber is opened and animals are taken out and immediately used for measurements.

In both countries, animals were housed under standard conditions, had access to food and water ad-libitum, and exposed to a 12h:12h light/dark cycle. All protocols have been reviewed and approved by the local committee of animal care and use of Laval University in Canada or by the scientific committee of IBBA in Bolivia and are in concordance with the guidelines of the Canadian Council of Animal Care.

## 3.2. Recording of arterial oxygen saturation in unrestrained, unanesthetized rats

The animal was placed in a whole body plethysmograph chamber for adult mice (Emka Technologies, Paris, France) flushed constantly with fresh room air (150-200ml/min). Before starting the experiment, animals were weighed, and equipped with a pulse oximeter to record arterial $O_2$ saturation. Animals were then placed in the chamber for a period of tranquilization (10 - 30 minutes), and baseline recordings were initiated for 20 min.

In Canada, we used the neck collars of the Mouse OX® STARR (Life Sciences Corp, USA) to record the pulse oximetry capillary oxygen saturation ($P_{O2,sat}$). The acute exposure to 15 and 12 % $O_2$ was done by

switching the inflowing tube to a nitrogen gas line calibrated to obtain the desired $O_2$% (15 and 12 % $O_2$ at SL correspond to an $PiO_2$ of 110 and 90 mmHg respectively – each exposure lasted for 10 minutes – as previously described in chapter 3 p109 and 4 p142). The signals from the pulse oximeter were directed toward a computer for storage and analysis using the Spike 2 software (Cambridge Electronic Design, Cambrige, UK).

In Bolivia, the arterial $O_2$ saturation was recorded using a tail sensor put around the neck of the pup at P4 and the front limb at P14 (MouseSTAT – Kent Scientific, Torrington, CT, USA) and data were observed by experimenter in the monitor and collected every 3 minutes, as described previously in chapter 2 p73. The acute exposure to 18% $O_2$ for 10 minutes and 32% $O_2$ for 15 minutes (corresponding to an $PiO_2$ of 90 and 160 mmHg respectively) was done by mixing an $O_2$ and a nitrogen gas tank calibrated to obtain the desired $O_2$% (see also chapter 2 p73).

## 3.3. Dissection of hearts and lungs

Prior to lung and heart sampling, all animals, animals were deeply anesthetized by an intraperitoneal injection (0.1 ml/100g of body weight) of ketamine (87.5 mg/ml) and xylazine (12.5 mg/ml). Perfusion was performed through the left ventricle with ice-cold PBS (pH 7.2) at a constant pressure of 24 $cmH_2O$. The heart was quickly dissected and used to measure the ratio of RV/(LV+S), an index of right ventricular hypertrophy and pulmonary hypertension as previously described in chapters 2 to 4. After cardiac perfusion with PBS, a catheter was fixed in the trachea of the animals used for lung histology assessments, the lungs were inflated with 4% PFA for 30 minutes at a constant pressure

of 24 cm$H_2O$, then dissected. The total volume of the inflated lungs was measured by liquid displacement, and they were kept in 4% PFA for 24 hours at room temperature before being embedded manually (in Bolivia) or automatically (in Canada) in paraffin using the same techniques described in the previous chapters. The samples included in paraffin in Bolivia were then shipped to Quebec City where they were processed to determine lung histology.

## 3.4. Lung histology and morphology

All the experimental procedure for the measurements of lung histology and morphology have been extensively described in the previous chapters. Paraffin embedded lungs were cut at 5 µm thick. Sections were then deparaffinized, colored with Harris hematoxylin solution and mounted as previously described. The images were captured using an optical microscope at a magnification of x100. We randomly selected 3 non-overlapping images from each slide using 3 slides per animal and 8 animals per group in Bolivia and 7 controls and 12 postnatal hypoxia in Canada (9 males and 10 females at SL and 8 males and 8 females at HA). The Mean Linear Intercept ($L_m$) was determined as previously described (chapter 2 to 4) and from $L_m$ values, we calculated the relative alveolar surface area and an estimation of the total alveolar surface area as the product of the relative alveolar surface area and lung volume (measured by water displacement after fixation see chapters 2 to 4 for detailed descriptions).

## 3.5. Allometric scaling in rats and mice

As described previously, to compare physiological and morphological variables between species of different size we used allometric coefficients. This study was only performed in rats, however, the body mass between SL and HA P14 rats is significantly different (see Figure V.1); thus we used allometric coefficients to compare SL P14 rats and HA P14 rats. However, data not corrected for allometric scaling are presented as comparison in the result section.

The allometric coefficients used for the lung mass and lung volume, were calculated by Stahl et al.[292]. For relative and total alveolar surface, we respectively used -0.13 and 0.88 as reported for mammals by Maina[293]. In the text, data corrected for the allometric scaling variables are referred to as mass-corrected whereas data compared for body mass are referred to as mass-specific.

## 3.6. Statistical analysis

We used the GraphPad Prism 6.0c (for 2-way ANOVA and *post hoc* analysis, nonlinear regression and t-tests) for statistical analysis. All values are reported as means±s.e.m., and the significant *P*-value vas set at 0.05.

It is of note that the results and figures presented here are drafts and preliminary exploitation of the data. I assessed t-tests between male and females in HA rats and no significant effect appeared. Thus, because P14 SL rats did not present sex specific effect either (see chapter 4 p144) except if precision are given regarding sex-effect, male and female are pooled in the following sections.

# 4. Results

## 4.1. Body mass

In SL rats, there was a slight effect of sex regarding body mass ($P$=0.049); accordingly, only male rats are presented in Figure V.1. In 4-days old rats, there was a significant effect of altitude ($P$<0.0001 – Figure V.1A). In 14-day-old rats, there was a significant effect of altitude without significant effect of postnatal exposure or significant interaction in body mass ($P$<0.0001 – Figure V.1B). SL 14-day-old rats had significantly higher body mass than HA 14-day-old rats and postnatal hypoxia did not influence body mass. Accordingly, it seems justified to use allometric coefficients to compare SL and HA 14-day-old rats.

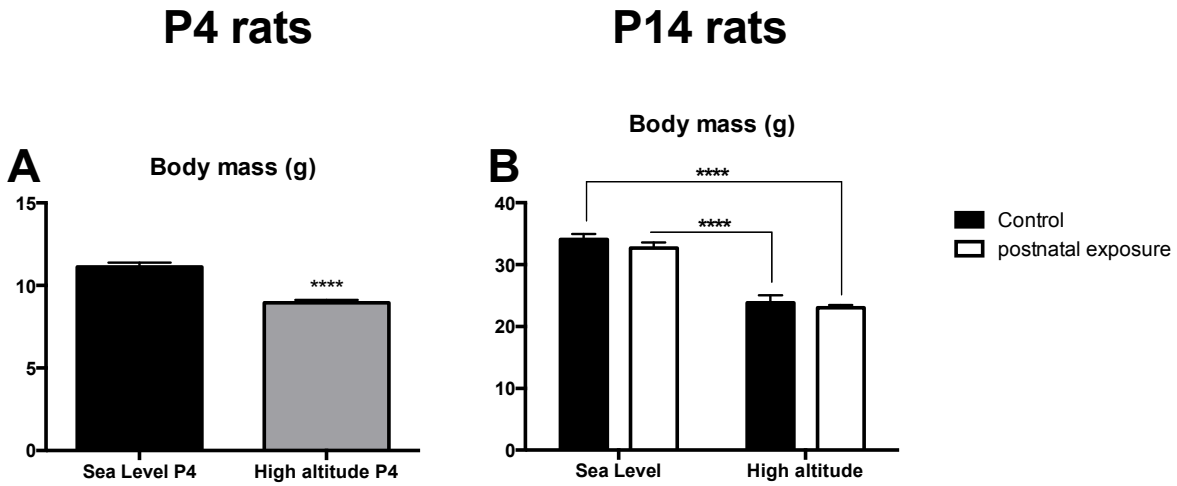



**Figure V.1: Body mass in 4- and 14-day-old SL and HA male rats.**
(A) Body mass (g) in 4-day-old rats raised at SL or HA (B) body mass in 14-day-old SL and HA rats raised under normoxia or with postnatal exposure to hypoxia (at SL) or normoxia (at HA). Means+s.e.m.
****$P$<0.0001 SL versus HA rats at the same $PiO_2$ (SL control versus HA postnatal exposure and SL postnatal exposure versus HA control) in 14-day-old rats and SL versus HA in 4-day-old rats.

## 4.2. Right ventricular hypertrophy

A significant effect of altitude ($P$<0.0001) with a significant postnatal exposure x altitude interaction effect ($P$=0.0006) was found for the right-to-left ventricular mass ratio without significant effect of the postnatal exposure ($P$=0.6). HA control P14 rats had higher right-to-left ventricular mass ratio compared with SL P14 rats that have been exposed to postnatal hypoxia (53.2±1.5 versus 48.8±1.6 %, Figure V.2) and postnatal normoxia in HA rats decreased the right-to-left ventricular mass ratio but in values that stayed higher than the SL control rats (39.6±0.8 versus 33.9±1.0 %, Figure V.2).

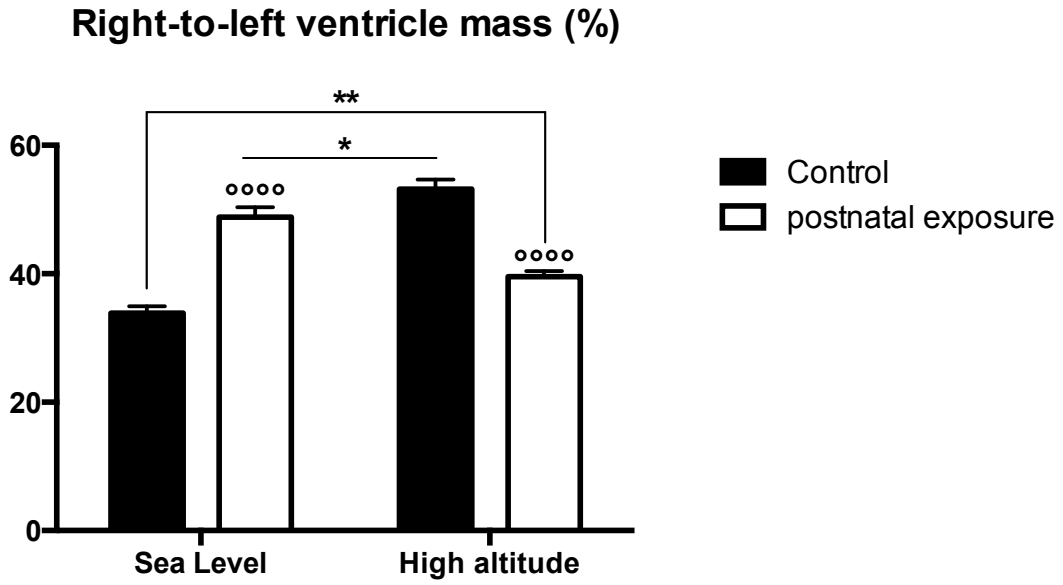



**Figure V.2: Right ventricular hypertrophy in 14-day-old SL and HA rats raised under normoxia or with postnatal exposure to hypoxia (at SL) or normoxia (at HA).**
Right-to-left ventricle mass (%) in 14-day-old rats raised at SL or HA. Means+s.e.m.
*$P$<0.05 and **$P$<0.01 SL versus HA rats at the same $PiO_2$ (SL control versus HA postnatal exposure and SL postnatal exposure versus HA control). °°°°$P$<0.0001 postnatal exposure versus control.

## 4.3. Lung morphology

There was a significant effect of altitude ($P$=0.004) without significant effect of postnatal exposure or significant interaction between altitude and postnatal exposure in the mass-corrected lung volume between SL and HA rats (Figure V.3A). HA rats had significantly higher mass-corrected lung volume compared with SL rats especially, the *post-hoc* analysis indicated that following postnatal normoxia, HA rats had higher mass-corrected lung volume than control HA rats but also SL rats. SL and HA 14-day-old rats had similar values of lung mass (Figure V.3B).

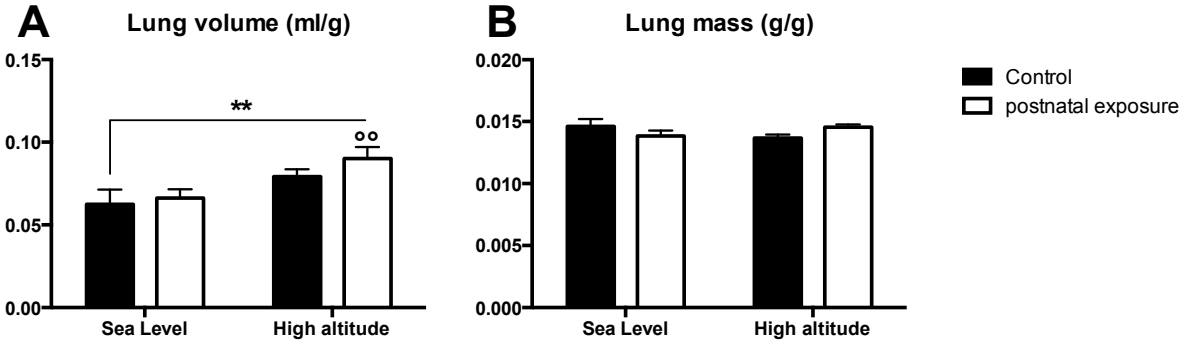



**Figure V.3: Lung volume and lung mass in 14-day-old SL and HA rats raised under normoxia or with postnatal exposure to hypoxia (at SL) or normoxia (at HA).**
(A) mass-specific lung volume (ml/g), and (B) mass-specific lung mass (g/g). Means+s.e.m.
**$P$<0.01 SL versus HA rats at the same $PiO_2$ (SL control versus HA postnatal exposure and SL postnatal exposure versus HA control). °°$P$<0.01 control versus postnatal exposure.

Representative examples of lung slices showing alveolar architecture in SL and HA, control and after postnatal exposure, rats are presented in Figure V.4. The effect of postnatal exposure on alveolar structure is clearly apparent. The mean linear intercept ($L_m$ – Figure V.4E) was lower in HA rats than in SL rats ($P$<0.0001 for altitude), and

was altered by postnatal exposure in both colonies ($P$<0.0001 for postnatal exposure). The magnitude of the effect was stronger at SL than at HA ($L_m$ increased by 44% from 24.6±0.4 to 35.4±0.7 μm in SL rats and decreased by 16% from 23.84±0.6 to 19.9±0.5 μm in HA rats – $P$<0.0001 for altitude x postnatal exposure). Remarkably however, $L_m$ values were similar between controls SL and HA rats.

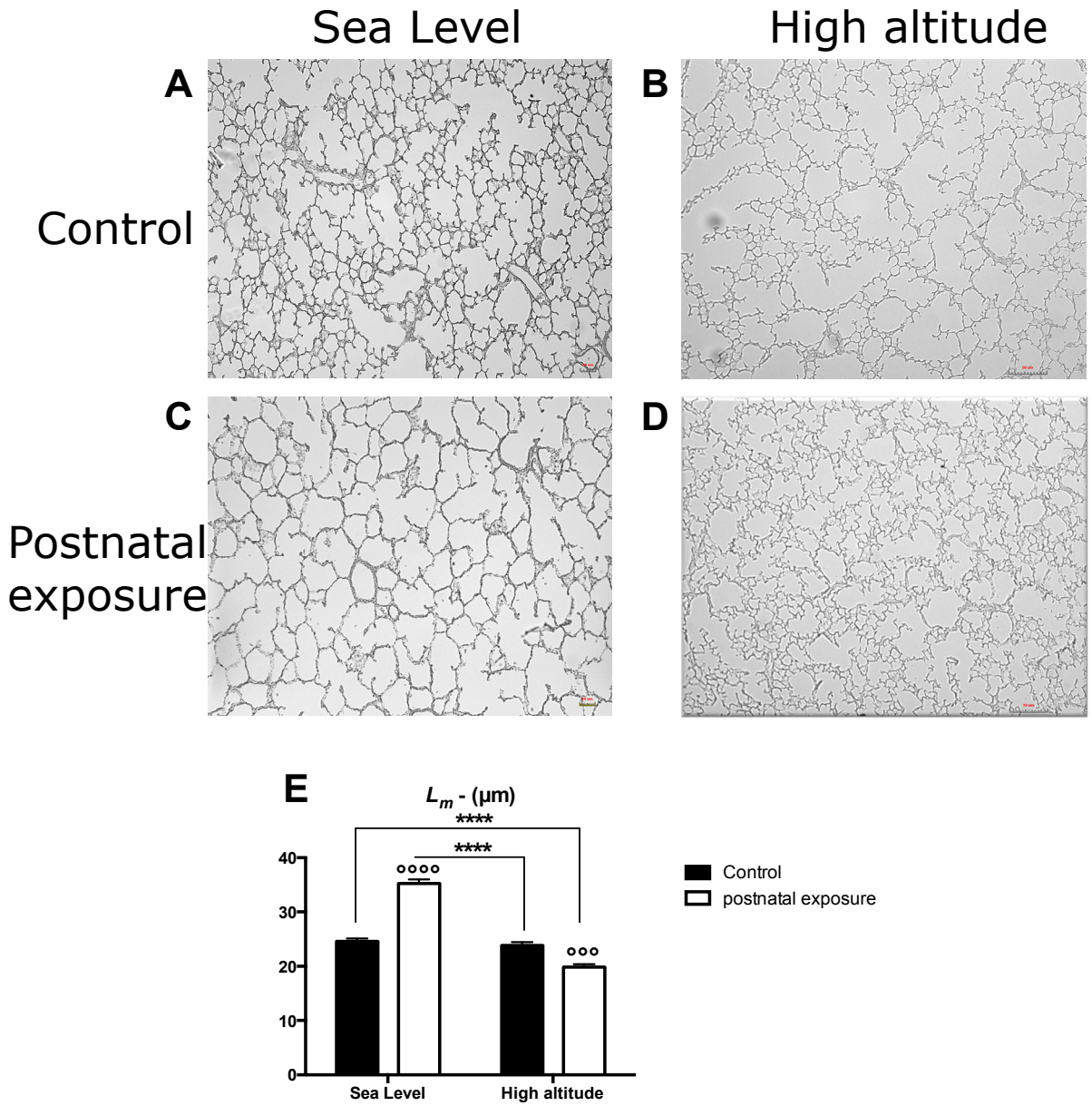



**Figure V.4: Typical lung architecture (A-D) and $L_m$ (E) in 14-day-old SL and HA rats raised under normoxia or with postnatal exposure to hypoxia (at SL) or normoxia (at HA).**

Typical images obtained for rats living at SL (A) control and (C) after postnatal exposure to 100mmHg $PiO_2$ and HA rats (B) control and (D) after postnatal exposure to 160 mmHg $PiO_2$. (E) mean linear intercept ($L_m$ – µm). Means+s.e.m.

Scale bars in A and C, 20 µm and in B and D 50 µm.

****$P$<0.01 SL versus HA rats at the same $PiO_2$ (SL control versus HA postnatal exposure and SL postnatal exposure versus HA control). °°°$P$<0.001 and °°°°$P$<0.0001 control versus postnatal exposure.

Mass-corrected relative alveolar surface area calculated from the values of $L_m$ ($S=4V/L_m$, were $V$ is the volume of each slice) was lower in control SL rats ($0.269\pm0.006$ $m^2/cm^3/g^{-0.13}$) compared with HA rats that have been exposed to postnatal normoxia ($0.320\pm0.008$ $m^2/cm^3/g^{-0.13}$ Figure V.5B). Following postnatal exposure, the relative alveolar surface area decreased by about 33% in SL rats (to $0.191\pm0.002$ $m^2/cm^3/g^{-0.13}$) and increased by about 16% in HA rats (to $0.226\pm0.005$ $m^2/cm^3/g^{-0.13}$, Figure V.5B). There was a significant effect of altitude between SL and HA rats ($P<0.0001$) but no significant effect of postnatal exposure ($P=0.08$). The mass-corrected total alveolar surface area (relative alveolar surface x lung volume) was higher in HA rats than in SL rats ($P<0.0001$ for altitude) and there was significant altitude x postnatal exposure interaction ($P=0.0003$, Figure V.5D) but no significant effect of postnatal exposure ($P=0.4$).

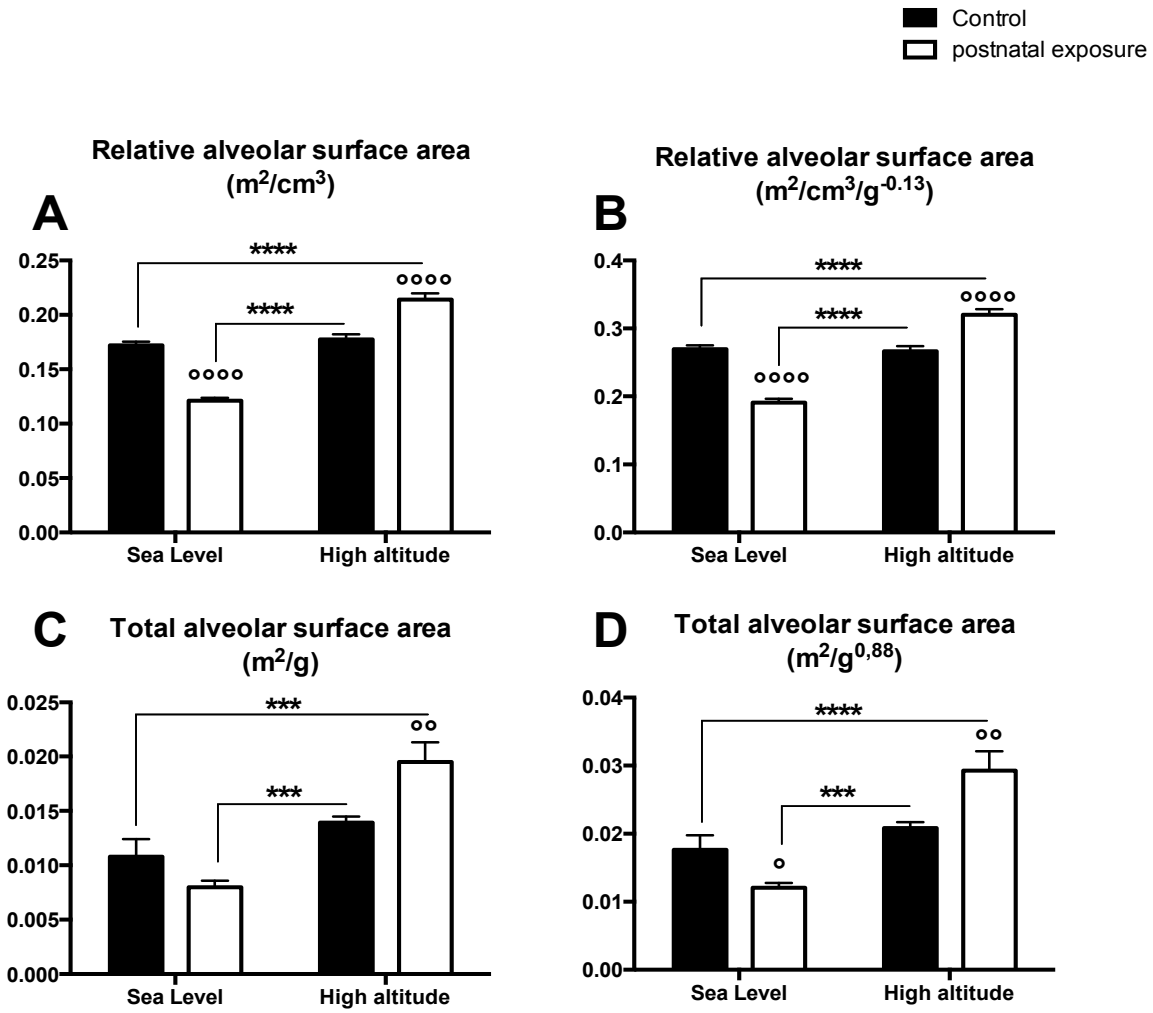



**Figure V.5: Variables of lung architecture in 14-day-old SL and HA rats raised under normoxia or with postnatal exposure to hypoxia (at SL) or normoxia (at HA).**

(A) relative alveolar surface area ($m^2/cm^3$), (B) mass-corrected relative alveolar surface area ($m^2/cm^3/g^{-0.13}$), (C) mass-specific total alveolar surface area ($m^2/g$), (D) mass-corrected total alveolar surface area ($m^2/g^{0.88}$) in 14-day-old rats and mice. Means+s.e.m.

***$P$<0.001, and ****$P$<0.0001 SL versus HA rats at the same $PiO_2$ (SL control versus HA postnatal exposure and SL postnatal exposure versus HA control). °$P$<0.05, °°$P$<0.01, and °°°°$P$<0.0001 control versus postnatal exposure.

## 4.4. Arterial $O_2$ saturation under baseline condition and during hypoxic and hypobaric normoxic exposure

$P_{O2,sat}$ values recorded in SL and HA rats at 4 and 14 days are presented in Figure V.6. There was no significant effect of sex, accordingly data from males and females have been pooled. $P_{O2,sat}$ remained >90% in 4-day-old rats living at HA even when the $PpiO_2$ reached 90 mmHg (which correspond to an inspired fraction of 12% $O_2$ at SL) whereas in SL 4-day-old rats, the $P_{O2,sat}$ decreased at 82% in response to 90 mmHg $PpiO_2$ (Figure V.6A). Overall, HA P4 rats had higher $P_{O2,sat}$ than SL P4 rats ($P$<0.0001 for altitude – Figure V.6A). At 14 days of age, this tendency is reversed; SL rats have higher $P_{O2,sat}$ than HA rats in response to 160 mmHg and 90 mmHg ($P$<0.0001 for altitude – Figure V.6 B and C). It is of note that in 14-day-old rats, we plotted recordings of $P_{O2,sat}$ in ambient room air in La Paz ($PpiO_2$ around 100 mmHg) with $P_{O2,sat}$ recorded in response to 15% $O_2$ in Quebec, ($PiO_2$ around 110 mmHg at SL); thus, as the $PpiO_2$ is not the same between 100 and 110 mmHg, no 2-way ANOVA has been done to compare these data and only t-tests between control and postnatal exposure of each altitude were made.

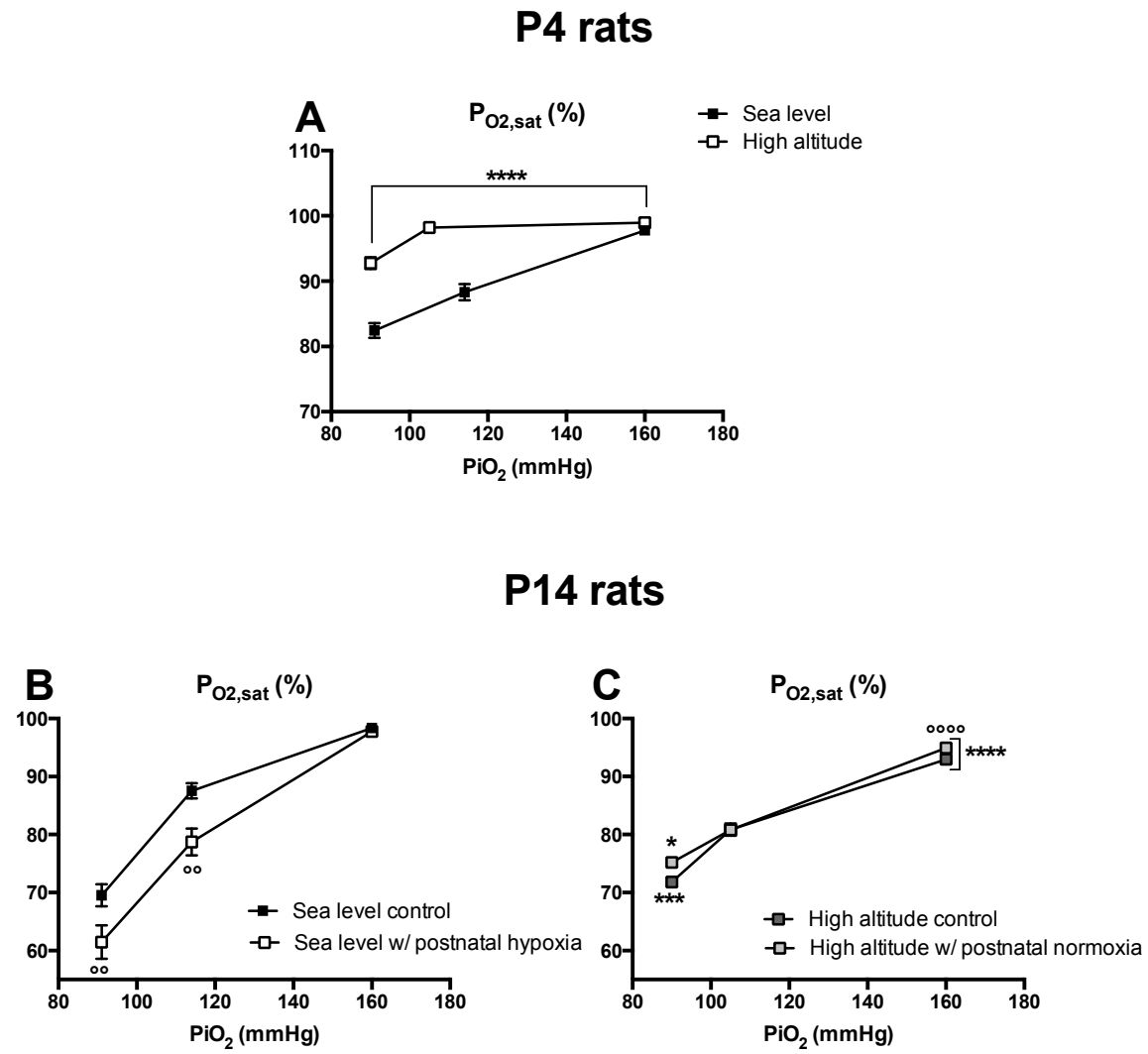



**Figure V.6: Arterial $O_2$ saturation in response to changes $PiO_2$ in 4- and 14-day-old SL and HA rats.**

(A) arterial $O_2$ saturation ($P_{O2,sat}$ – %) in P4 rats raised at SL or HA, (B and C) arterial $O_2$ saturation ($P_{O2,sat}$ – %) in 14-day-old SL and HA rats raised under normoxia or with postnatal exposure to hypoxia (at SL) or normoxia (at HA). $PiO_2$ are 160, ≈100 and 90 mmHg. Means+s.e.m.

A: ****$P$<0.0001 SL data versus HA data.

B and C: *$P$<0.05, ***$P$<0.001, and ****$P$<0.0001 SL versus HA rats at the same $PiO_2$ (SL control versus HA w/postnatal exposure and SL w/postnatal exposure versus HA control) in 14-day-old rats. °°$P$<0.01, and °°°°$P$<0.0001 control versus postnatal exposure.

# 5. Discussion

## 5.1. Developmental plasticity, acquired traits or epigenetics changes in the lung architecture in rat at HA?

14-day-old HA control rats had higher mass-corrected relative and total alveolar surface areas compared with SL 14-day-old rats exposed to postnatal hypoxia. Rats living at HA further increased the total alveolar surface area in response to postnatal normoxia. This might reflect specific resistance of the HA rats against the deleterious consequences of postnatal hypoxia on alveolar development. And HA rats seem to be able to maintain this characteristic throughout life, as adult HA rats have similar lung morphology as SL adult rats (Appendix 2). Previous studies in dogs raised at SL or HA (3800m – for 5 months) during maturation showed that after 1-2 years following return to SL, adult HA raised dogs still had improved lung functions compare with SL raised animals[357] suggesting that HA exposure during maturation induce structural adaptation, which may be permanent. It is interesting to note that dogs are a species particularly well adapted to HA, and it is a common experience to encounter large groups of dogs wandering in the streets of La Paz or on the Altiplano above 4000m of altitude. Therefore, the persistent beneficial effects of postnatal hypoxia on lung architecture observed in dogs might be a critical trait for their adaptation to HA and the same phenomenon might be occurring in the lungs of the rats living in the colony implanted in La Paz (3600m) in Bolivia in 1992.

The gas exchange surface area is composed of the pulmonary alveoli and the pulmonary capillary. During postnatal lung development, in all mammals, pulmonary alveoli are formed in part by subdivision of the alveolar sac into smaller units; this process is called "septation".

Alterations occurring during the alveolarization process will compromise further alveolar formation and have long-term consequences through adulthood. This aspect has been investigated in rats[125,165,334,349] but also in humans[358]. The mechanisms implicated in the maintenance of the lung structure in the rats living at HA for several generations could implicate an accelerated angiogenesis with an increase apoptosis of the interstitial cells which would increase the formation of the septa in the lungs. One other explanation could implicate the A vitamin that have been implicated in the maturation of the lungs in rats where supplementing rats with A vitamin between postnatal days 4-13 induce an increase in alveolarization with additional septas [334,349]. Because postnatal hypoxia in SL rats impaired lung postnatal development (see chapter 3 p149), it is likely that developmental plasticity in rats does not have favourable consequences in response to environmental hypoxia. Then, because 30 generations might not be enough to have had the apparition of fixed characters in rats, we suggest that the maintenance of the lung architecture in HA rats is the result of epigenetics modifications however further investigations are required to confirm this hypothesis.

It is of note that in humans also, environmental hypoxia during development influences the lung morphology and functional phenotype. It has been shown since the 70's that postnatal hypoxia contributes to the enlargement of human chest cavity and lungs volume leading to enhanced aerobic capacity in altitude native but also in lowlanders born and raised at HA[154,155].

## 5.2. Developmental plasticity in the arterial $O_2$ gas exchange surface area: P4 HA rats maintain elevated levels of $P_{O2,sat}$ in acute hypoxia compared with P4 SL rats but this characteristics is lost in P14 HA rats.

4-day-old HA rats maintained higher arterial $O_2$ saturation compared with SL P4 rats. This result was not expected but is of significance. $P_{O2,sat}$ does not directly reflect the tissue $O_2$ saturation however, it gives a good indicator of how well the $O_2$ is moving across the alveolar-capillary membrane and into the blood to be carried to the tissue. Thus this result suggests that 4-day-old rats born and raised at HA for several generations have better gas-exchange functions in the lungs compared with 4-day-old rats born and raised at SL, which might be interpreted as a physiological adaptation to HA in 4-day-old rats[65]. However, this "adaptation" seems to be vulnerable with aging as 14-day-old HA rats have lower arterial $O_2$ saturation in response to 160 mmHg $PiO_2$ when compared with SL P14 rats exposed to postnatal hypoxia. Postnatal normoxia in HA rats significantly increase the arterial $O_2$ saturation at 160 mmHg however, it stays significantly lower compared with 14-day-old SL control rats. Furthermore, under ambient air at HA ($PiO_2$ of 100 mmHg at the altitude of La Paz), $P_{O2,sat}$ in 4-day-old HA rats was at 98% whereas in 14-day-old HA rats it drops at 81% (similar to the value recorded in HA adult rats of 80% – see chapter 2 p79). This drop in the arterial $O_2$ saturation in P14 rats living at HA can lead to a drop in the $O_2$ cascade and a decrease in the body oxygenation[56-58].

A methodological caution needs to be addressed here. The arterial $O_2$ saturation recordings in 4-day-old rats raised at HA were measured using sensors placed on the neck whereas in 14-day-old (and adult) rats raised at HA the sensor was placed on the limb. However, when exposed

to 160 mmHg inspired $O_2$ pressure (corresponding to a $FiO_2$ of 32% to relieve the hypoxic stress present in the ambient air in La Paz); we observed a significant increase in the $P_{O2,sat}$ (93% in control rats, and to 95% in rats raised under high $O_2$), accordingly, the $P_{O2,sat}$ recordings appear to be reliable.

We conclude that P4 and P14 rats that have been living at HA for several generations display signs of improved gas-exchange functions, alveolar surface area and lung growth. These characteristics probably reflect some sort of phenotypic plasticity and/or adaptation to cope with environmental HA hypoxia in rats, which are triggered early in life. However, these characteristics are not sufficient to reverse the deleterious effects of hypoxia in a long-term manner and it seems that there is a loss of adaptation in rats with aging. Furthermore, this hypothesis is in concordance with previous studies observing the life-long consequences of postnatal chronic hypoxia in HA and SL adult rats[1,2]. This might in part explain why rats have not been able to colonize HA environment in wildlife.

# VI. CHAPTER 6

## GENERAL DISCUSSION

All the work reported in this thesis shows that compared with laboratory rats, laboratory mice have physiological responses to the ambient hypoxia that correlates with better acclimatization and survival capacities at high altitude (HA). As explained in the introduction, the ambient hypoxic stimulus present at HA is challenging and organisms living at HA will need to adjust their physiological responses to improve $O_2$ uptake and distribution to survive and colonize such an environment. Literature shows that at HA improving $O_2$ uptake and distribution involve increasing ventilation, Hb-$O_2$ affinity and larger lungs with high exchange surface area; these traits were clearly present in mice living at HA for several generations, but were notably absent in rats. Furthermore, some of these traits were present in mice at sea level (SL), indicating a predisposition for mice to survive at HA, and likely explaining their success for colonization of some ecological niches at HA.

# 1. Compared with adult rats, adult mice display better physiological responses to hypoxia

In chapter 2, we compared basic physiological responses in adult rats and mice that have been living at HA (under an ambient $O_2$ partial pressure – $PO_2$ – of 100 mmHg) in laboratory conditions for more than 30 generations (at 3600m in the city of La Paz, Bolivia). The results showed that mice and rats have divergent physiological responses to HA environment. Compared with rats, mice had lower hematocrit and hemoglobin levels, lower right ventricular hypertrophy, enhanced lung characteristics, higher tidal volume and $O_2$ consumption with a similar arterial $O_2$ saturation in ambient conditions. In response to the exposure to graded levels of acute hypoxia, compared with rats, mice had

reduced metabolic rate and rectal temperature and maintained higher arterial $O_2$ saturation.

In chapter 3, we compared the physiological and molecular responses to acute graded level of hypoxia (18, 15, 12 and 9% $O_2$, 10 minutes each) and to sustained hypoxia (6h exposure at 15 and 12% $O_2$) in adult rats and mice living at SL that had never been exposed to hypoxia. The results showed that SL adult rats and mice display similar cardiac, hematological and lung characteristics, but had divergent ventilatory and metabolic responses to sustained hypoxia. Compared with rats, mice had higher minute ventilation and lower metabolic rate in response to 6h at 12% $O_2$. Theses differences in the ventilatory and metabolic responses to hypoxia were correlated with an increase in the expression of the Hypoxia Inducible Factor 1 alpha (HIF-1$\alpha$ – main mediator of the responses to hypoxic environment in the cells) in the brainstem of mice but not rats.

*It is of note that specifics aspects regarding the results depicted above are thoroughly discussed at the end of each chapter. This general discussion will focus on providing hypothesis to further expand specific explanations regarding the divergent physiological responses between adult rats and mice under hypoxic conditions.*

## 1.1. Hypothesis 1: mice exposed to chronic hypoxia for several generations maintain $O_2$ consumption using hyperplasic mitochondria

In the cells, $O_2$ is used permanently in the mitochondria to produce energy. Measuring the metabolic rate can assess the quantity of energy consumed at the cellular level. Compared with rats, mice

exposed to short-term (several minutes to a few hours) hypoxia at SL and HA had reduced metabolic rate. However, at HA under ambient conditions, mice maintained higher metabolic rates than rats.

In some species living under constant hypoxia, such as the naked mole rats, the metabolic rate is reduced, this strategy allows them to maintain higher arterial $O_2$ pressure[261,263]. Compared with mice, HA rats have a reduced metabolic rate but similar arterial $O_2$ saturation; this might be the result of a strategy similar to the one used by the naked mole rat to cope with ambient hypoxia. Nevertheless, this strategy was pointed out as being deleterious for growth, reproduction and physical activity[264] and studies using the deer mice (a rodent with a range of distribution from SL to HA) reported that compared with low altitude deer mice, HA deer mice have increased $O_2$ consumption because of higher energy demands[359].

Mice decreased their metabolic rate in response to acute hypoxia at HA and under sustained hypoxia for 6h at SL, thus it is possible that in the common mice (*Mus musculus*), exposure to short-term hypoxia (few minutes to 6h) triggers an immediate response reducing the metabolic rate to preserve the arterial $O_2$ pressure, whereas in conditions of long-term hypoxic exposure such as living at HA for several generations, other long-term adjustments are triggered to maintain an adequate $O_2$ delivery to the tissue. In fact, HA mice have similar baseline metabolic responses than SL mice, this can be considered as an adaptive response as it gives them the ability to further increase this consumption if needed (in situation implicating a fight or flight response for instance).

HA implies to cope with low ambient $PiO_2$ but also with low ambient temperature. In homoeothermic mammals (mammals that

regulate their internal temperature at high and constant level) small animals require more energy to maintain their inner temperature than large animals (because the surface/volume ratio is increased). Thus in an environment where the ambient $PiO_2$ and temperature are low, increasing the metabolic rate would show better adaptation skills than reducing it.

Faced with an environmental stressor, animal populations have to adjust. These adjustments can be achieved either by genetics changes in response to selection (evolution) or by plastic phenotypic adjustments to conditions experienced within the lifetime of individuals (either reversible – phenotypic plasticity – or irreversible)[280]. The deer mice have been extensively used to understand phenotypic plasticity and evolution at HA. Studies regarding the globin chains in the deer mice reported diverse hemoglobin polymorphisms that correlate with native altitudes and clearly state in favor of an evolutionary response to altitude[236,360]. This influence over blood-$O_2$ affinity also influences the maximal aerobic performance during exercise and thermogenesis [361]. In the common mice however, a previous genetic study showed that there is no adaptive modification of the hemoglobin function in wild *Mus musculus* caught in La Paz compared with other specimens at SL (Lima, Peru)[277]; therefore, as explained in chapter 2, it is tempting to speculate that the physiological responses observed in mice that have been bred in La Paz might explain the ability of this species to successfully withstand the HA hypoxic environment.

The energy production takes place at the cellular level in the mitochondria; and it has been established that permanent or long-term exposure to severe environmental hypoxia decreases the mitochondrial content of muscle fibers (mainly using human muscular tissue)[252-254,270].

However, in a recent study conducted by Qi et al., they compared the numerical density on area, volume density, specific surface and surface density of the mitochondria in the heart tissue of pikas versus Sprague-Dawley rats. They found that the plateau pikas had an increased density of surface of the mitochondria compared with Sprague-Dawley rats[284]. We can hypothesize that mice living at HA for several generations might also display hyperplastic mitochondria compared with HA rats, resulting in an increased density of the mitochondrial respiratory chain that would enhance the $O_2$ uptake at the cellular level resulting in a higher $O_2$ consumption and $CO_2$ production in mice compared with rats.

## 1.2. Hypothesis 2: Mice exposed to chronic hypoxia for several generations have enhanced lung morphology and architecture to cope with the decreased $O_2$ level

At HA, the decrease in $O_2$ availability can be overcome by increasing the surface area for $O_2$ transport (that is by increasing the alveolar surface area in the lungs for example). In the deer mice, HA natives have larger lungs than low altitudes natives[177]. Using the *Phyllotis darwini* (a rodent belonging to the superfamily of the muroidae same as *Rattus* and *Mus)* Pearson and Pearson compared a while ago the ultrastructure of the lung in wild caught rodents living at high and low altitude. They found that the HA living animals had a greater volume percentage of tissue component linked to a hyperplasia of the cells composing the tissue. They also postulated that the larger lungs of the HA animals had more respiratory units that were either smaller or modified in some other manner increasing the ratio between the surface area of the alveolar epithelium and the volume of the alveolar air

space[179]. These observations are in concordance with our results between SL and HA laboratory mice. Compared with SL laboratory mice, HA laboratory mice have lower body mass and lower $L_m$ (indicating smaller alveolus units) but higher lung volume, lung mass and a strong tendency toward having higher relative alveolar surface area in $m^2/cm^3$ ($P$=0.054; it is possible that the small size sample, only 4 animals account for the absence of significance in the result) and higher mass-corrected total alveolar surface area (Figure VI.1).

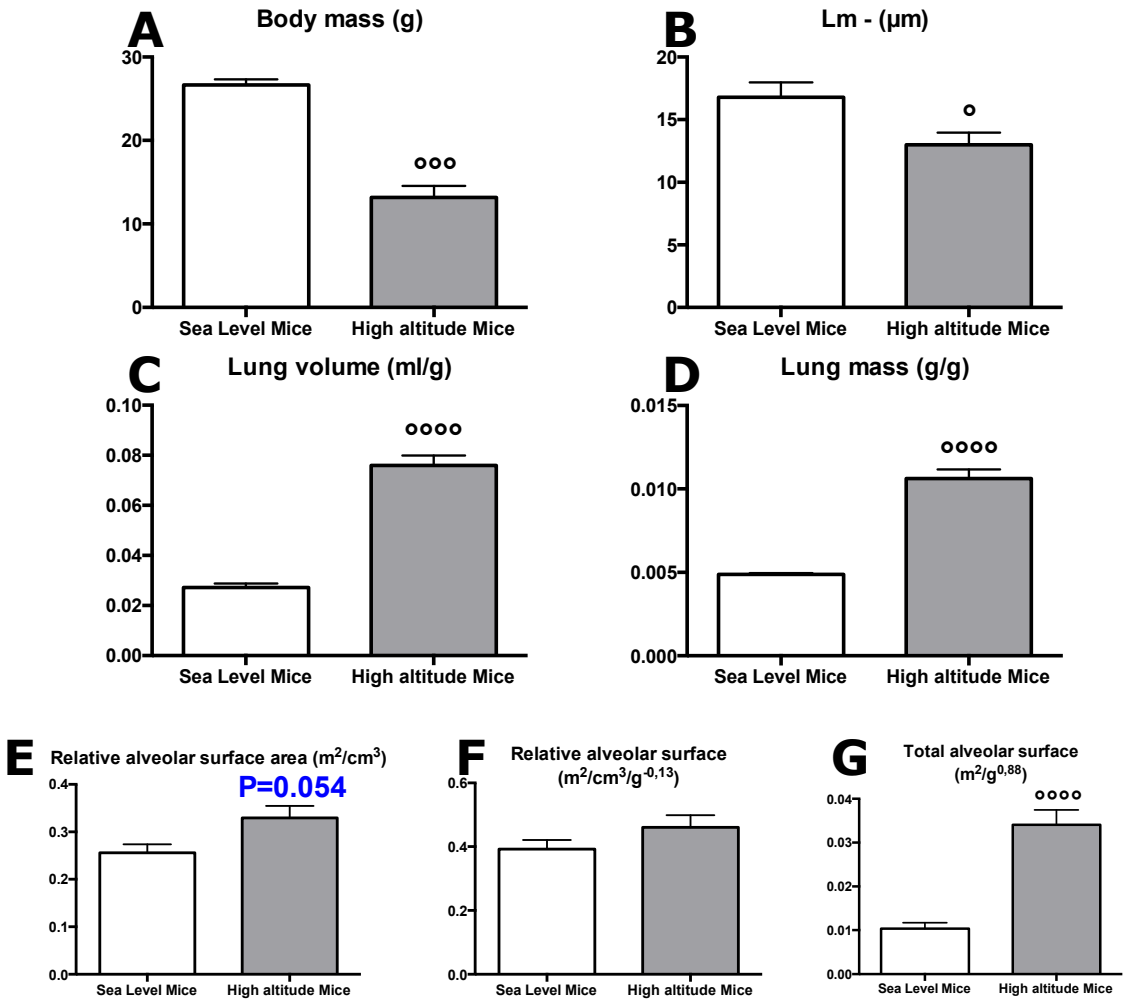



**Figure VI.1: Variables of body mass, lung morphology and architecture in adult mice raised at SL and HA.**

(A) body mass (g), (B) mean linear intercept ($L_m$ – µm), (C) mass-specific lung volume (ml/g), (D) mass-specific lung mass (g/g), (E) relative alveolar surface area ($m^2/cm^3$), (F) mass-corrected relative alveolar surface area ($m^2/cm^3/g^{-0.13}$), (G) mass-corrected total alveolar surface area ($m^2/g^{0.88}$). Means+s.e.m. °$P$<0.05 and °°°$P$<0.001 and °°°°P<0.0001 SL versus HA mice.

### 1.3. Hypothesis 3: the increased expression of HIF-1α in mice could be the early determinant inducing a cascade of events leading to the functional plasticity responsible for a better acclimatization to hypoxia in HA mice compare with rats

HIF, the hypoxia inducible factor was discovered in the early 90's and since its discovery it has emerged as a central component of many $O_2$-dependant physiologic and pathophysiologic processes. HIF is now recognized as a master regulator of $O_2$ homeostasis.

The regulation of HIF-1α is $O_2$ dependent. In normoxia, HIF is continuously ubiquitinilated in the cytosol of the cell resulting in its degradation by the proteasome whereas upon hypoxic exposure, HIF-1α is translocated to the nucleus where it will form a dimer with HIF-1β and regulate the transcription of various target genes via hypoxic response elements bindings sites (Figure VI.2).

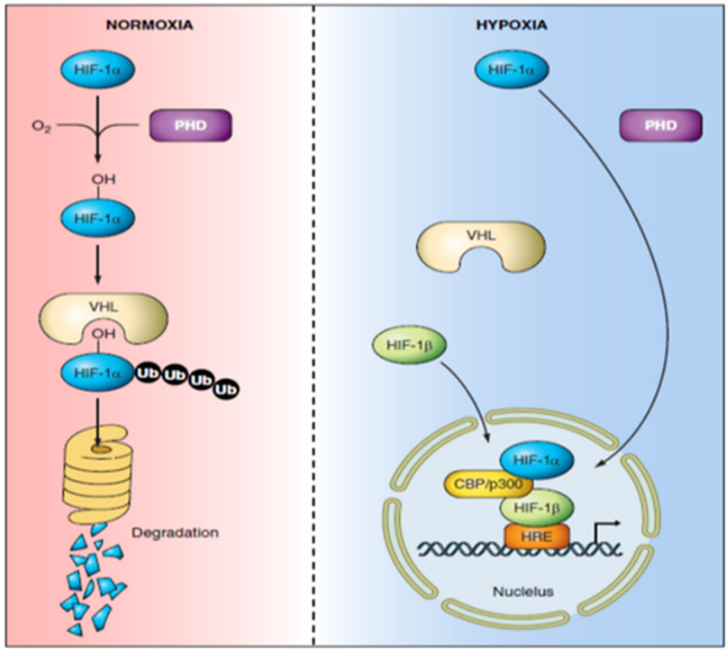



**Figure VI.2: Schematic illustration of HIF-1α regulation.**
(A) normoxic conditions: the prolyl hydroxylase domain (PHD) proteins uses the molecular $O_2$ as a substrate to hydroxylate HIF-1α. Once hydroxylated, HIF-1α binds the von Hippel-Lindau (VHL) protein and becomes polyubiquitylated (Ub) and targeted for proteosomal degradation. (B) hypoxic conditions: the PHD activity is reduced and HIF-1α escapes hydroxylation, accumulating and translocating to the nucleus where it binds with HIF-1β and CBP/p300 to the hypoxia response element (HRE).
*Adapted from: Shimoda, 55th Bowditch Lecture: Effects of chronic hypoxia on the pulmonary circulation: role of HIF-1. 2012.*

HIF-1α is known to act over the transcription of ≈100 genes in response to hypoxia leading to the up-regulation of genes that are involved in erythropoiesis, iron metabolism, apoptosis, glucose metabolism, angiogenesis/vascular tone, cell proliferation/survival etc.[324,362-365] HIF-1α might also be implicated with the reactive oxygen species (ROS), studies have proposed that ROS have a role in the stabilization of HIF-1α particularly in low $PO_2$ environment: in normoxia, the continuous degradation of HIF-1α is achieved by the hydroxylation by proline hydroxylase of a critical prolyl group of the protein[366]; the activity of the proline hydroxylase has been pointed out as being very

sensitive to inhibition by ROS[367]. Furthermore, recent studies reported that HIF-2$\alpha$ prevents the apparition of ROS whereas HIF-1$\alpha$ promotes it[368,369].

As discussed in chapter 3, HIF might have a role in the activation of the ventilatory acclimatization to hypoxia (VAH), activating the transcription of genes implicated in the neural circuits controlling breathing but also in the $O_2$-sensivity of the CB[308,315,325,370]. Also, study showed that transgenic mice heterozygous for HIF-1$\alpha$ deletion exhibit delayed development of polycythemia, and pulmonary hypertension during chronic hypoxia compared with control mice[324]. Investigation in the expression of HIF-1$\alpha$ in the plateau pikas demonstrated an increased protein level in the nucleus of lung, liver, spleen and kidneys cells compared with SL mice with an expression of the protein that was altitude dependent (HIF-1$\alpha$ expression was increased with the increase in the altitude range of habitation in the pikas)[326].

In our studies, the expression level of HIF-1$\alpha$ increased in the brainstem of adult mice in response to sustained exposure to hypoxia, but remained lower in rats. Furthermore, in mice, the increased level of HIF-1$\alpha$ correlated with the $O_2$ percentage exposure (because mice had increased expression of HIF-1$\alpha$ in response to 15% $O_2$ compared with baseline that further increased when they were exposed for 6h at 12% $O_2$ see Figure III.11), and with the degree of ventilation, thus it is tempting to establish a formal link between the expression of HIF in the brainstem and the ventilation level. Unfortunately, while we tried to assess HIF expression in the lungs of these animals, the assay procedure was apparently not sensitive enough. A study using pulmonary arterial endothelial and aortic endothelial sheep cell culture (exposed at 1% $O_2$ and 5% $CO_2$ for 6h at 37°C) and ferrets intubated

and ventilated with 16% $O_2$ for 30 minutes followed by 0,1,4,7,10 or 16% $O_2$ for up to 8h (all gas mixtures containing 5% $CO_2$) showed an increased expression of HIF-1$\alpha$ in the lung coupled with $O_2$ concentration in vivo. However, the physiological role of HIF-1$\alpha$ in the lungs remained unclear[371]. And, studies using knock out mice (KO) HIF-1$\alpha$+/- demonstrated a reduced pulmonary vascular remodeling leading to a reduced right ventricular hypertrophy and pulmonary pressure in response to 1-6 weeks at 10% $O_2$ in the KO mice compared with the wild type [372,373].

## 2. Sensibility to hypoxia in the lung growth during postnatal life in rats and mice at SL and HA

In chapter 4, we compared the influence of the hypoxic stimulus on the physiological responses during postnatal development in 14-day-old (P14) rats and mice. Rats and mice were exposed in ambient air or in hypoxia (13% $O_2$, corresponding at sea level to the $PO_2$ of a 3600m altitude same as La Paz, Bolivia) during postnatal days 4 to 14 (which correspond in rodent to the phase of the alveolar formation in the lungs)[169,170,331]. Results showed that after postnatal hypoxic exposure, mice had lower right ventricular hypertrophy, higher lung volume and higher ventilation and higher saturation in responses to graded level of hypoxia (18, 15, 12 and 9% $O_2$, 10 minutes each) than rats. Moreover, prior to the hypoxic exposure, control mice had lower relative weight of the right ventricle, higher metabolic rate and lower arterial saturation in response to hypoxia.

In chapter 5, we compared arterial $O_2$ saturation, and lung morphology in response to hypoxia in 4- and 14-day-old rats living at HA in La Paz at 3600m or at SL in Quebec. In Quebec, animals were exposed in ambient air or in hypoxia (13% $O_2$, corresponding at SL to the $PO_2$ of the altitude of La Paz, Bolivia – 100 mmHg) during postnatal days 4 to 14. In La Paz, animals were exposed in ambient air or in 32% $O_2$ (this $O_2$ percentage in La Paz relieve the ambient hypoxic stimulus and mimics the ambient condition of SL $PO_2$ – 160 mmHg). The result showed that HA control P14 rats presented architectural lung structure similar to the control SL P14 rats, indicating that over the generations, rats were able to acquire traits to preserve their lung architecture under low ambient $PO_2$. The preservation of the lung architecture in the HA rat is certainly in part responsible for improving their survival at HA.

## 2.1. Hypothesis 1: accelerated lung growth during postnatal development at HA is a necessity to ensure future adequate gas-exchange functions in adulthood

In organisms living in an environment where $O_2$ is low an adequate and well functioning gas-exchange surface area is required. The gas exchange surface area is composed of the pulmonary alveoli and the pulmonary capillary. During postnatal lung development, in all mammals, pulmonary alveoli are formed in part by subdivision of the alveolar sac into smaller units; this process is called "septation". The timeline of septation during development varies considerably among species. In rodents, the septation takes place between the postnatal days 4-14 whereas in humans it starts around the 7$^{th}$ month of pregnancy and continues until the end of the 1$^{st}$ or 2$^{nd}$ year of life[374].

Alterations occurring during the alveolarization process will compromise further alveolar formation and have long-term consequences through adulthood. This aspect has been investigated in rats[125,165,334,349] but also in humans[358]. Moreover, this aspect is important, as it is a serious condition in premature birth, babies born before the 28$^{th}$ week often suffer from septationnal alteration that will impede the efficiency of the gas exchange functions. Theses infants often develop bronchopulmonary dysplasia associated with less enlarged alveoli[375,376].

Studies had previously shown that postnatal hypoxia is deleterious for the lungs integrity in rats resulting in a reduced alveolar surface area with a lower count of alveoli[334,349]. However, older studies conducted by Cunningham and Mortola in 1974 and 1986 respectively found that postnatal hypoxic exposure increased lung growth, alveolar size and pulmonary surface area[125,165].

In 14-day-old rats living at SL, the $L_m$ (mean linear intercept) is increased after postnatal hypoxia, the lung volume unchanged and the total alveolar surface area show a tendency to be reduced in rats exposed to postnatal hypoxia compared with control animals when comparison is made using the *post-hoc* analysis of the 2-way ANOVA with SL 14-day-old mice, and is significantly reduced when comparison is made using the *post-hoc* analysis of the 2-way ANOVA with HA 14-day-old rats. It is of note that the $L_m$ does not measure the alveolar size but it gives direct indications regarding size of the structural lung units (the acinus that regroups a respiratory bronchiole giving birth to 3 to 6 alveoli canals. Each alveoli canal is subdivided 2 to 3 times forming the terminal alveolus canal that into the atrium up to 2 to 3 air sacs) and allows comparisons between different groups[377]. Thus, at SL, rats exposed to chronic hypoxia in postnatal life have increased alveolar size

which reduces the alveolar surface area compared with control animals; which is in concordance with findings reported by Massaro et al. and Blanco and al. in 1990 and 1991 but in contradiction with part of the results reported previously by Cunningham et al. and Mortola et al. indicating that postnatal hypoxia increase lung growth and pulmonary surface area (but in the same time also increased the size of the alveoli). Just a remark here, in the study of Cunningham and Mortola, the lungs were inflated at a constant pressure of 20 $cmH_2O$ for 90 minutes and 48h respectively whereas we used 24 $cmH_2O$ for only 30 minutes. It has been established that $L_m$ is significantly affected by lung volume, which can be altered by inflation conditions or altered elastic recoil[378]. Also, it is of note that the study conducted by Cunningham et al. assessed the lung architecture in 3 weeks old rats, however, studies have pointed out that in rats, after septation, the number of alveoli continues to increase until about 40 days of life[349,379,380]. In the study conducted by Mortola et al., the animals were exposed at 10-11% $O_2$, which is lower than the level that we used (13,5% $O_2$), thus it is possible that in rats, using $O_2$ percentage below 12% triggers HIF-1$\alpha$ expression and hypoxia-related responses that are not present if the $O_2$ percentage is higher[82]. In the same line of idea, a study just published compared the consequences of the degree of hypoxia (10 versus 13% $O_2$) to the fetal growth during pregnancy in mice. The authors reported that 10% $O_2$ exposure affected the placenta of pregnant mice resulting in a decrease in the fetal resource allocation whereas 13% $O_2$ exposure triggers beneficial changes in the placenta morphology, nutrient transport and metabolic signaling to preserve fetal growth[381]. Thus, the level of hypoxia is apparently a very strong determinant of physiological responses, and it is difficult to compare results obtained in different studies at different $O_2$ levels with, often, different techniques. Also, the hypoxic exposure in all the previous studies started either at birth or

prior to birth, this might influence other developmental aspects of the lung formation and maturation and explain the differences with our study where the animals were exposed only during the alveolarization phase. Nonetheless, all these hypotheses are in line with the proposition that postnatal hypoxia might influence the timeline of the lung development.

In 14-day-old rats at HA the $L_m$ was similar than in 14-day-old rats at SL. Furthermore, compared with 14-day-old SL rats exposed to postnatal hypoxia, the total alveolar surface area was higher in 14-day-old rats at HA. These results suggest that at HA 14-day-old rats have been able, over the generations, to preserve lung growth and architectural organization to compensate for the lower $O_2$ level. If we take a look into the timeline of lung growth by comparing pictures of the rat lung development over the time from birth to 65 days with pictures taken from the lungs of 14-day-old SL rats raised under ambient room air or exposed to postnatal hypoxia, the eye comparison will reveal similarities between the architectural organization of the lungs of a 14-days old rat exposed to postnatal hypoxia and a 6-days old control rats (Figure VI.3)[382]. This observation corroborates the hypothesis that postnatal hypoxia influences the timeline of lung development resulting in 14-day-old SL rats exposed to postnatal hypoxia in a delay in the formation of the alveoli. Furthermore, it is possible that in the HA rat colony, continuous hypoxia throughout life lead to epigenetic changes in the next generations that compensate the lung growth such that lung surface area reaches a higher value in adulthood. Thus leading to the notion that functional demand may be able to override genetically fixed developmental principles.

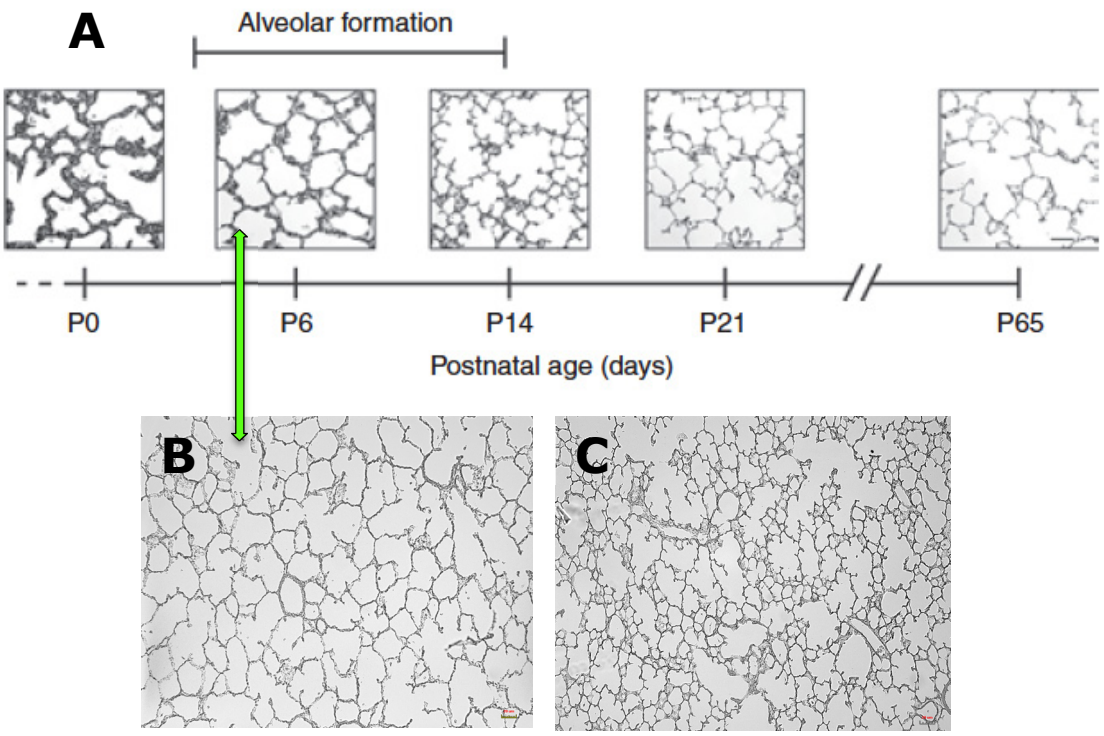



**Figure VI.3: Variables of lung morphology and architecture in adult mice raised at SL and HA.**
(A) Timeline of rat lung development. Representatives' images of postnatal days 0, 6, 14, 21 and 65. Magnification x80. Typical image of the architectural lungs obtained for rats living at SL (C) control and (B) after postnatal exposure to 100mmHg $PiO_2$. Magnification x100.
All scale bar = 50 µm.
*Adapted from Joss-Moore et al. Intrauterine growth restriction transiently delays alveolar formation and disrupts retinoic acid receptor expression in the lung of female rat pups. 2013*[382]*.*

In mice, the consequences of hypoxia in the architectural formation of the lungs have not been investigated in such an extensive way as in rats. However, it has been pointed out on several occasions that postnatal hypoxia in mice also impairs the alveolar development of the lungs[2,336,337]. In our studies, 14-days old mice that had been exposed to postnatal hypoxia have increased lung volume and enlarged lung structures compared with control mice but no differences were

found regarding the total alveolar surface area between the two groups. However, it is interesting to point out here that 14-day-old control mice living at SL display similar total alveolar surface area as HA adult mice whereas SL adult mice have a much lower surface area (Figure VI.4).

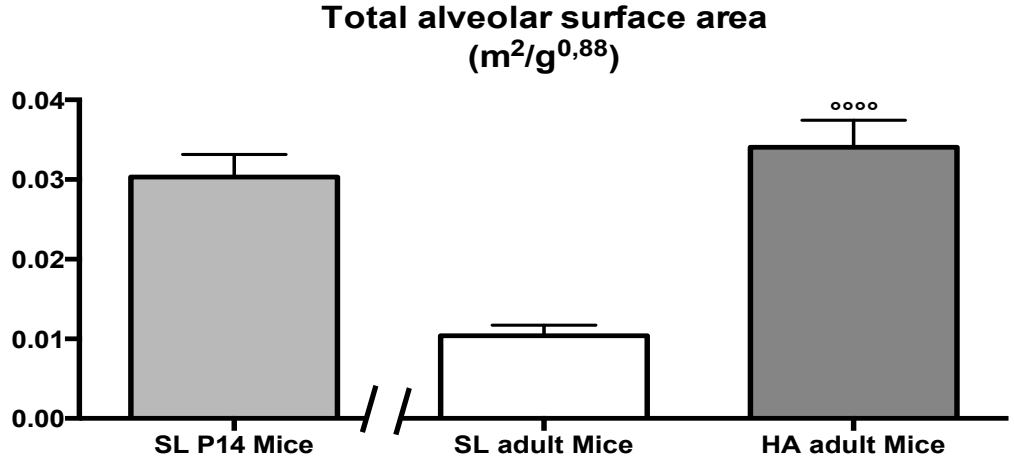



**Figure VI.4: Total alveolar surface area in SL 14-day-old mice, SL adult mice, and HA adult mice.**
All the animals are controls.
°°°°$P$<0.0001 in adult mice, SL versus HA.

Thus it is possible that one component to survive and colonize HA environment would be to accelerate the formation and growth of the lungs during the early life and maintain enhanced architectural organization to ensure adequate $O_2$ uptake through the lungs in adult. These results also point out a potential role for neoteny at HA which is the characteristic of maintaining youth traits in adulthood, this phenomenon has particularly been pointed out in reptiles that maintained a larval stage in case of food deprivation or winter conditions until better conditions are available to fully complete their maturation. Unfortunately, we were not able to assess the architectural structure of

the lungs of 14-day-old mice living at HA to confirm either of these hypotheses, but we can argue that either they will have similar lung architecture as 14-day-old control mice living at SL or maybe, they will even have enhanced lung architecture with increased alveolar surface area and lung volume compared with their SL counterparts.

# 3. The HA rats are living at the extreme limits of their physiological capacities

HA adult rats display reduced gas-exchange functions compared with HA mice, and increased heart rate baseline values compared with SL rats, (above the values expected during nighttime in this species – see chapter 2 p79 and reference [303]) associated with a low $O_2$ consumption rate in an environment where the ambient $PO_2$ is already low. Basal heart rate reported in the HA rats were recorded during daytime in La Paz; the values reported regarding the average heart rate in rats living at SL are around 250-300 beats/min; we had values around 500 beats/min in HA rats. This increased heart rate was associated with a low $O_2$ consumption rate (compare with SL rats but also with HA mice) presumably because the erythrocytes do not spent enough time close to the tissue to liberate an adequate quantity of $O_2$. It might have been interesting to measure the oxyhemoglobin dissociation curve in rats and mice living in La Paz because it might have given some insight regarding the affinity of the Hb for $O_2$ but also regarding the bohr effect (blood pH and $PCO_2$ variations) and the production of 2,3 DPG (the 2,3 diphosphoglycerate is a metabolite produced in response to anaerobic glycolysis that diminishes the Hb-$O_2$ affinity easing the release of $O_2$ close to the tissue). Furthermore, HA rats displayed a low alveolar surface area compared with HA mice; this could result in a deficit in the

$O_2$ uptake during the pulmonary ventilation in rats living at HA where the ambient $PO_2$ is low. One anecdotal fact is that female rats can be housed with male for mating for 1 to 3 month before pregnancy is confirmed; furthermore, the life expectancy for the rats living in Bolivia is around 1.5 years whereas at SL, it is around 3 years[2]. Thus, it is possible that at HA, our rat colony has reach a fragile equilibrium that allow them to survive by reducing the $O_2$ consumption to maintain arterial $O_2$ saturation, and using the oxidation of glucose molecules as a way to optimize ATP production. However, this strategy works only because they are maintained under laboratory conditions but it would not allow them to survive in the wild because of the high $O_2$ demand associated with daily activities such as searching food, building a nest or mating.

## 3.1. Hypothesis 1: Excessive hypoxia-induced production of ROS in rats is responsible for the adaptation loss with aging

Chronic-hypoxia-induced production of ROS is still controversial[383] and might need exposure to extreme levels of hypoxia to actually influence the physiology of organisms[384]. However, as mentioned in the introduction, low ambient $O_2$ influences the mitochondrial functioning, which might result in an increase in the production of ROS. Furthermore, the nature of the produced ROS species will also influence the tissue integrity. Indeed, some ROS are known to be highly toxic and to have deleterious consequences on tissues.

A study conducted by Magalhaes et al. used skeletal muscle of mice exposed 48h to severe hypoxia to show that indeed hypoxia can induce ROS-dependent damage in tissue. They also showed that a

complementation in E vitamin would attenuate the damage[384]. Later Clanton hypothesized that chronic or extreme hypoxia might induce the formation of ROS in the muscle tissue that will then fail to maintain a normal redox homeostasis resulting in cell injuries or dysfunction[385]. Animals that have to live in situation where $O_2$ becomes low or even absent, such as fish, frogs, diving seals and turtle, have enhanced antioxidant defenses[386], suggesting that coping with prolonged hypoxia involves defense strategies against increased oxidant production (channel arrest hypothesis).

At HA, 4-day-old rats have better gas-exchange functions than SL 4-day-old rats. However, this "adaptive" characteristic is no longer present in 14-day-old rats living at HA and lung architecture is similar between HA and SL 14-day-old control animals. Furthermore, the right-to-left ventricle ratio is increased in HA control animals compared with SL control animals. Unfortunately, we did not assess the architecture of the lungs in 4-day-old rats living at SL and HA but we can postulate that 4-day-old rats living at HA would present higher exchange surface area than SL 4-day-old rats, an adaptive trait to cope with HA that would no longer be present in 14-day-old and adult rats.

It is possible that rats that have been living under chronic conditions of hypoxia for several generations triggered early strategies to adjust and cope with low ambient $PO_2$ but at some point, these strategies are no longer effective. One hypothesis explaining these adaptations lost could be linked to a defect in anti-oxidant defenses leading to an accumulation of ROS resulting in a destruction of the alveoli in the lungs with aging. Furthermore, we have established that rats present an extreme sensibility to hypoxia during postnatal life (references [1,2] and chapter 4p137 of the present thesis); thus, we can

hypothesize that at SL, exposure to 13% $O_2$ during the alveolarization stage, might trigger an overexpression of ROS in rats (that would be lower or absent in mice) resulting in a delay in the alveolar formation of the lungs. It is also possible that in SL animals that have never been exposed to hypoxia, the anti-oxidant response present in early life in rats at HA because of generations under hypoxic conditions is absent explaining that at SL, hypoxic exposure in rats during postnatal life results in a delay in the alveolar formation.

However, the understanding of the levels and duration of hypoxia on the ROS production and the effectiveness of the antioxidant defense mechanisms have not really been investigated in HA endemic animals. Dosing the antioxidant enzymes (such as the superoxide dismutase) in the lungs of HA and SL animals might be a good element to provide evidence regarding the hypoxia-induced production of ROS and their role in the delay in maturation of the lung tissue in HA rats. Furthermore, supplementing rats exposed to postnatal hypoxia at SL with E vitamin (or other antioxidants) might provide insight regarding the role of the ROS in the alveolarization of the lung under conditions of chronic hypoxia.

## 3.2. Hypothesis 2: HA rats present a defect in the heart capillarization

The last fact that I would like to point out here is that we lost many adult male rats at HA during the exposure at 12% $O_2$ (corresponding at SL to an exposure at 7% $O_2$). This might be the result of a lack of capillarization in the heart of the adult rats living at HA. Indeed, HA adult rats have reached a fragile equilibrium, which allows

them to survive under laboratory conditions by reducing $O_2$ consumption. However, if this fragile equilibrium is challenged by exposure to severe hypoxia, it leads to the death of the animal. The early studies that regarded the myocardial muscle capillarization in mammals in response to hypoxia had some disagreements. In endemic HA guinea pigs, some authors found a decreased myocardial capillarization whereas some other found no differences. In rats some found an increase myocardial capillarization in both ventricles, in others, the increase was only in the right or the left ventricle and others reported no changes[65]. In endemic large mammals at HA, one study using vicunas found an important capillary density in their heart[387] and one other comparing the capillarization of the cardiac tissue of yaks showed increased capillarization of the right ventricle compared with the left ventricle as a strategy to cope with increased loading[388]. Another study from 2008 showed that pikas have increased microvessel density compared with Sprague Dawley rats[284].

Studies regarding the role of HIF in the cardiac functions showed that it is important for the formation of the heart during development. HIF increases the synthesis of angiotensin II (which is implicated in the capillary formation and the cardiac rhythm) and influences the maintenance of the heart functions throughout life. In animals lacking the expression of HIF there is a diminution in the heart muscular contractility, a diminution in the vessels density, a hypertrophy of the left ventricle and it also lead to a diminution in the synthesis of several proteins implicated in the delivery of $O_2$ to the heart cells [389,390].

Protecting the functionality of the vital organs such as the brain and heart against the deleterious consequences of hypoxia is indispensable for survival and the susceptibility of the myocardium to

hypoxic damage is real. Moreover, it seems that the heart's damage susceptibility to hypoxia is dependent on gender, females tending to be more tolerant than males[391]. This could explain why we lost half of the males when the hypoxic exposure at HA reached 12% $O_2$ (it is of note that after loosing the 3rd male, the other males and females were not exposed at 12% $O_2$) whereas females survived without critical signs of distress. It is possible that HA rats were able from one generation to the other to develop strategies to survive but these strategies are at the maximum of their adaptive possibilities and if we further push on the challenge, they cannot further adjust.

# VII. CHAPTER 7

## GENERAL CONCLUSION

Our study is the first to systematically compare physiological responses to the hypoxic environment between laboratory rats and mice. Also, the strength of this study relies on the fact that we had the unique opportunity to compare these responses in two colonies that have been living at HA, in the city of La Paz at 3600m for more than 30 generations in laboratory conditions, further exploring the consequences of living in hypoxic environment for several generations.

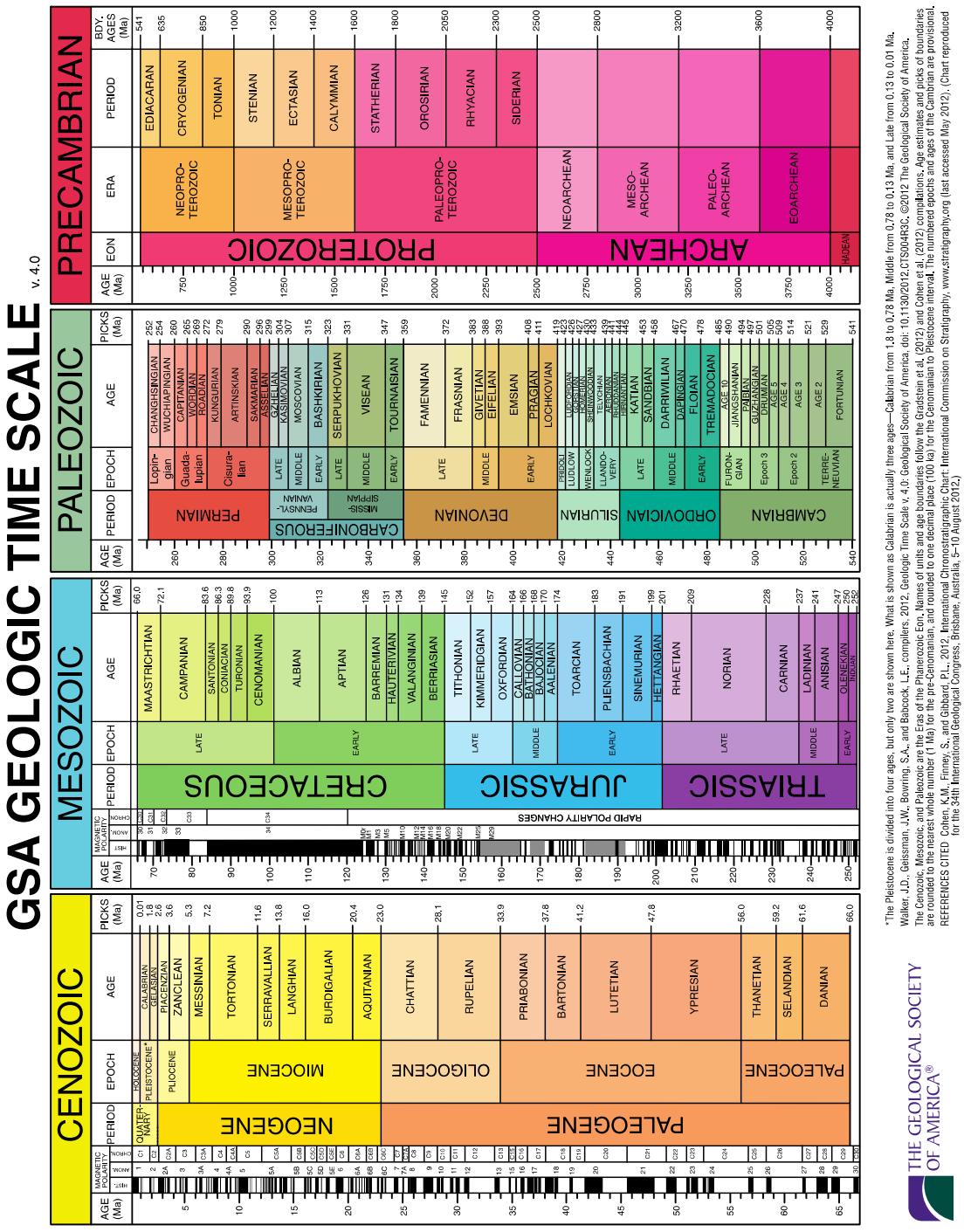
Our results show that compared with rats, mice living at HA display better physiological responses to the environment. Mice had reduced hematological variables, low pulmonary artery pressure, increased $O_2$ consumption and enhanced total alveolar surface area. When exposed to hypoxia at SL, adult mice are able to reduce metabolic rate and increase ventilation to preserve arterial $O_2$ saturation. We linked these responses with an increased expression of the hypoxia inducible factor 1 alpha in the brainstem of mice, but not rats, which is dependent upon the percentage of $O_2$ exposure.

Postnatal hypoxia at SL induced divergent phenotypical plasticity between rats and mice. After 10 days of postnatal hypoxic exposure, mice had increased lung volume, lower right ventricular hypertrophy and when exposed to low $O_2$ levels, higher minute ventilation and higher arterial $O_2$ saturation than rats. Furthermore, control mice had lower relative mass of the right ventricle and higher relative and total alveolar surface areas compared with control rats.

Rats that have been living in laboratory conditions for more than 30 generations seem to have reach a fragile equilibrium that allows them to withstand hypoxia by reducing $O_2$ consumption. They also display traits of adaptive response to HA during early life that decay with aging, probably explaining why rats are not able to establish stable colonies at HA under natural conditions.

Accordingly, we conclude that mice seem predisposed to better withstand HA environment. This predisposition might be the reflection of different genetic background between rats and mice linked to an early activation of the HIF-pathway and to differential $O_2$-sensing characteristics in the carotid bodies and in the central nervous system between rats and mice. Furthermore, recent studies indicate that environmental hypoxia can interact with epigenetics mechanisms[392-396]. Epigenetic studies the regulation of gene expression that are caused by external or environmental factors that will switch genes on and off and affect how cells read genes. Thus, studying the epigenetic mechanism of hypoxia-induced gene regulation could help understand cellular processes to cope with hypoxia. However, actually, no definitive role for the epigenetic changes has been reported regarding environmental hypoxia. Further study regarding this aspect might help understand how species adjust in HA environment but also help human medicine understanding pathology that involves cellular hypoxia.

# Appendix 1




*The Pleistocene is divided into four ages, but only two are shown here. What is shown as Calabrian is actually three ages—Calabrian from 1.8 to 0.78 Ma, Middle from 0.78 to 0.13 Ma, and Late from 0.13 to 0.01 Ma.

Walker, J.D., Geissman, J.W., Bowring, S.A., and Babcock, L.E., compilers, 2012, Geologic Time Scale v. 4.0: Geological Society of America. doi: 10.1130/2012.CTS004R3C. ©2012 The Geological Society of America.

The Cenozoic, Mesozoic, and Paleozoic are the Eras of the Phanerozoic Eon. Names of units and age boundaries follow the Gradstein et al. (2012) and Cohen et al. (2012) compilations. Age estimates and picks of boundaries are rounded to the nearest whole number (1 Ma) for the pre-Cenomanian, and rounded to one decimal place (100 ka) for the Cenomanian to Pleistocene interval. The numbered epochs and ages of the Cambrian are provisional.

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Gradstein, F.M., Ogg, J.G., Schmitz, M.D., et al., 2012, The Geologic Time Scale 2012: Boston, USA, Elsevier, DOI: 10.1016/B978-0-444-59425-9.00004-4.

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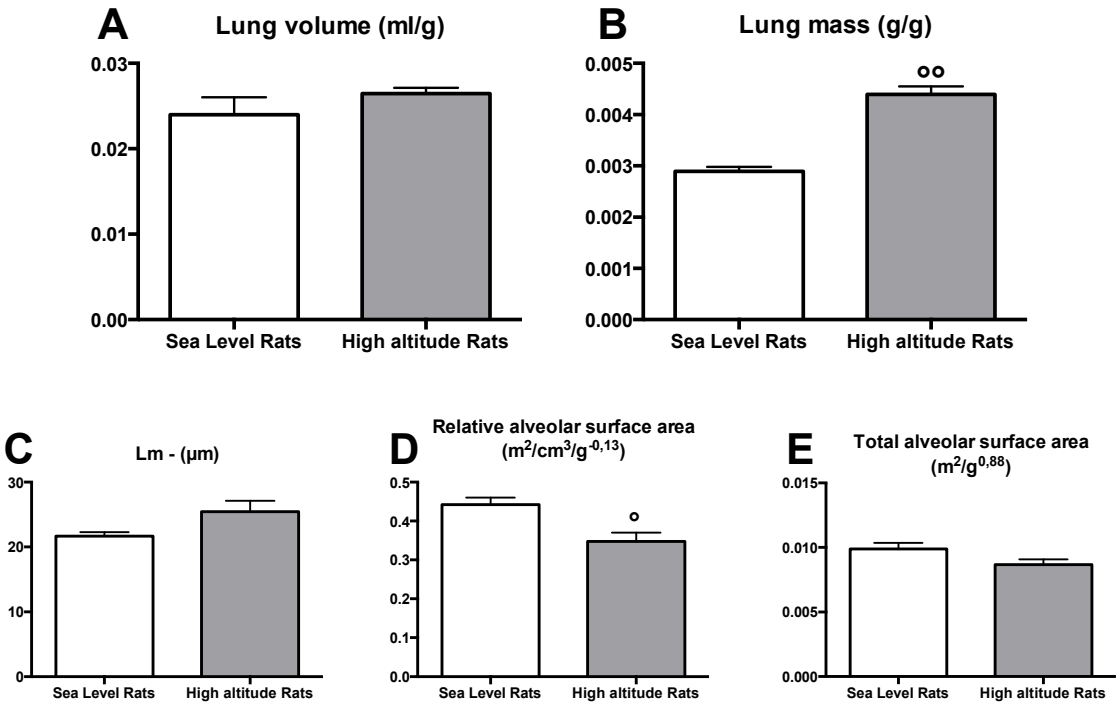
**Appendix 1: Geologic time scale**

# Appendix 2




**Appendix 2: Variables of lung morphology and architecture in adult rats raised at SL and HA.**
(A) mass-specific lung volume (ml/g), (B) mass-specific lung mass (g/g), (C) mean linear intercept ($L_m$ – µm), (D) mass-corrected relative alveolar surface area ($m^2/cm^3/g^{-0.13}$), (D) mass-corrected total alveolar surface area ($m^2/g^{0.88}$). Means+s.e.m.
°$P$<0.05 and °°$P$<0.01 SL versus HA rats.

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