UNIVERSITÀ di VERONA

UNIVERSITA' DEGLI STUDI DI VERONA

DEPARTMENT OF

Neurosciences, Biomedicine and Movement Sciences

GRADUATE SCHOOL OF

Life and Health Sciences

DOCTORAL PROGRAM IN

Neuroscience, Psychological And Psychiatric Sciences, And Movement Sciences

WITH THE FINANCIAL CONTRIBUTION OF

Italian Ministry Of Education (MUR)

Cycle / year: XXXVI / 2020

TITLE OF THE DOCTORAL THESIS

Physiological Responses to Exercise in Hypoxia: The Effect of Cold-Air Exposure and Sex Differences

S.S.D. M-EDF/02

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Exercise in extreme environments: beyond the frontiers of combined stressor exposure and sex differences in exercise physiology.

Alexa Callovini

PhD Thesis

Rovereto, 06 March 2024

UNIONE EUROPEA
Fondo Sociale Europeo
Fondo Europeo di Sviluppo Regionale

Ministero dell'Università e della Ricerca

FSC Fondo per lo Sviluppo e la Coesione

La borsa di dottorato è stata cofinanziata con risorse del
Piano Stralcio «Ricerca e Innovazione» 2015-2017 Asse "Capitale Umano", del Fondo per lo Sviluppo e la Coesione, "Dottorati A Tema Vincolato – Aree Interne/Aree Marginalizzate"

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SOMMARIO

Gli effetti dell'esposizione acuta all'ipossia sulle risposte fisiologiche durante l'esercizio sono stati ampiamente studiati negli ultimi decenni, a causa del crescente numero di individui coinvolti in attività ad alta quota (HA) sia per scopi di allenamento che ricreativi. Comprendere l'impatto della ridotta disponibilità di ossigeno sui meccanismi fisiologici coinvolti nelle risposte all'esercizio è fondamentale per migliorare la tolleranza umana alla quota, riducendo così i rischi per la salute intrinsecamente legati a questo ambiente estremo. Tuttavia, nelle situazioni reali, l'HA è spesso combinata con molteplici stressors, come ad esempio temperature ambientali estremamente rigide.

Nonostante ciò, vi è una carenza di studi specifici sull'interazione degli effetti freddo-ipossia sulle risposte dell'organismo in esercizio, soprattutto perchè tali approcci possono essere sperimentalmente molto impegnativi. Per le stesse ragioni, la maggior parte degli studi che indagano gli effetti dell'esposizione acuta all'ipossia sulle risposte fisiologiche all'esercizio ha incluso solo soggetti maschi, rendendo difficile comprendere se debbano essere sviluppati interventi personalizzati in base al sesso per garantire una più sicura esposizione alla quota per uomini e donne.

Questa tesi di dottorato affronta queste lacune investigando gli effetti combinati del freddo e dell'ipossia sulle risposte all'esercizio e esaminando l'influenza del sesso biologico su vari aspetti legati al trasporto dell'ossigeno all'interno dell'organismo ad alta quota. La ricerca fornisce preziosi approfondimenti sull'interazione complessa tra esercizio e stimolo ipossico, ma tiene anche conto delle caratteristiche dell'esposizione ad alta quota nel mondo reale e delle sue implicazioni per il corpo in esercizio.

In particolare, lo Studio 1 esplora gli effetti indipendenti e combinati del freddo e dell'ipossia sulle risposte massimali, sotto-massimali e alla soglia del lattato in soggetti maschi allenati. I risultati indicano che sia il freddo che l'ipossia influenzano in modo indipendente le risposte all'esercizio sia a livello massimale che sotto-massimale, con la combinazione dei due stressors che mostra effetti additivi sulla maggior parte delle risposte considerate.

Come prosecuzione dello Studio 1, lo Studio 2 mira a investigare possibili effetti dell'esposizione combinata a freddo-ipossia sull'affaticamento dei muscoli respiratori e sulla bronco costrizione indotta dall'esercizio, cercando di spiegare la relazione tra le risposte ventilatorie durante l'esercizio e questi meccanismi. Lo studio mostra che sia l'affaticamento dei muscoli respiratori che la bronco costrizione indotta dall'esercizio sono influenzati negativamente dall'esposizione al freddo, senza ulteriori compromissioni significative legate allo stimolo ipossico, almeno dopo un esercizio ad alta intensità e di breve durata.
Infine, lo studio 3 ha confrontato le risposte ventilatorie, la capacità di diffusione polmonare e gli adattamenti cardiovascolari in uomini e donne allenati durante l'esercizio in ipossia. Sono state riscontrate risposte simili all'esposizione alla quota simulata in entrambi i sessi, suggerendo l'assenza di influenza del sesso sulle variazioni del funzionamento dei sistemi esaminati quando è ridotta la disponibilità di ossigeno circolante.

Questa tesi di dottorato e i dati presentati in essa mirano dunque ad ampliare la nostra comprensione dell'esercizio in ipossia e a stimolare nuove ricerche su questo argomento, cercando di colmare le distanze che dividono quanto già descritto in studi di laboratorio con le caratteristiche reali dell'esposizione alla quota, sia in soggetti di sesso maschile che femminile.

ABSTRACT

The effects of acute hypoxic exposure on physiological responses during exercise have been extensively studied in recent decades, given the growing number of individuals engaging in high-altitude (HA) activities for both training and recreational purposes. Understanding the impact of reduced oxygen availability on the physiological mechanisms involved in exercise responses is crucial for enhancing human tolerance to HA, thereby mitigating the health risks associated with this extreme environment. However, real-world HA scenarios often involve multiple stressors, with one of the most common being extremely low ambient temperatures. Despite the prevalence of such conditions, there is a scarcity of specific studies investigating the interaction between cold and hypoxic effects on exercising responses. This is largely due to the experimental, temporal, and practical challenges associated with such approaches. Additionally, many studies exploring the effects of acute hypoxic exposure on exercise physiology have predominantly focused on male subjects. This limitation complicates the understanding of whether tailored interventions based on biological sex are necessary to ensure the safest high-altitude exposure for both men and women.

This doctoral thesis addresses these gaps by investigating the combined cold-hypoxic effects on whole-body exercise responses and examining the influence of biological sex on various aspects related to oxygen transportation within the organism at high altitudes. The research provides valuable insights into the intricate interaction between exercise and hypoxic stimuli while considering the real-world characteristics of HA exposure and its implications for all practitioners.

In particular, *Study 1* explores the independent and combined effects of cold and hypoxia on maximal, submaximal, and lactate threshold responses in trained male subjects. The findings indicate that both cold and hypoxia independently impact exercising responses, with the combination of the two stressors exhibiting additive effects on the majority of considered outcomes.

Continuing from Study 1, *Study 2* aims to investigate potential exacerbation effects of combined cold-hypoxic exposure on respiratory muscle fatigue (RMF) and exercise-induced bronchoconstriction (EIB). This study seeks to elucidate the

relationship between exercising ventilatory responses and the pulmonary function and respiratory muscles work at simulated freezing altitudes. Results show that both respiratory muscle fatigue and exercise-induced bronchoconstriction are negatively affected by cold exposure, with no significant additional impairments related to the hypoxic stimulus, at least after high-intensity exercise of short duration.

Finally, *study 3* compares ventilatory responses, lung diffusion capacity, and cardiovascular adaptations in trained men and women during exercise in hypoxia. Similar responses to exercise during simulated high-altitude exposure have been observed in both sexes, suggesting no influence of biological sex on the variations in the functioning of the examined systems when circulating oxygen availability is reduced.

This doctoral thesis and the data presented herein want to expand our understanding of hypoxic exercise and stimulate new research on this topic, minding the gap between experimental settings and real-world situations.

PUBLICATIONS

***Callovini A**., Fornasiero A., Savoldelli A., Decet M., Skafidas S., Pellegrini B., Bortolan L., Schena F.* 'Independent, additive and interactive effects of acute normobaric hypoxia and cold on submaximal and maximal endurance exercise' Published in European Journal of Applied Physiology, November 2023 (DOI: https://doi.org/10.1007/s00421-023-05343-9)

***Callovini A**., Fornasiero A., Savoldelli A., Dorelli G., Decet M., Bortolan L., Pellegrini B., Schena F.* 'Freezing Hypoxia and Exercise: impact of concurrent stressors on pulmonary function and respiratory muscle fatigue.' Under Review

ORAL COMMUNICATIONS

***Callovini A**., Fornasiero A., Savoldelli A., Decet M., Skafidas S., Pellegrini B., Bortolan L., Schena F.* "Independent and combined effects of hypoxia and cold on physiological and perceptual variables while exercising." *SISMES XII National Congress*. Padova, 8 – 10 Oct 2021.

***Callovini A**., Fornasiero A., Savoldelli A., Decet M., Skafidas S., Pellegrini B., Bortolan L., Schena F. "* Freezing Hypoxia and exercise: how do they affect lung function and respiratory muscle fatigue?" *European College of Sport Medicine Congress*. Sevilla, 30 Aug – 4 Sept 2022

***Callovini A**., Fornasiero A., Savoldelli A., Decet M., Skafidas S., Pellegrini B., Bortolan L., Schena F.* "Independent, additive and interactive effects of acute normobaric hypoxia and cold on submaximal and maximal endurance exercise, lung function and respiratory muscles fatigue." *9th International Congress Mountain Sport and Health*, Rovereto, 16-17 Nov 2023

POSTER

***Callovini A**., Fornasiero A., Savoldelli A., Decet M., Skafidas S., Pellegrini B., Bortolan L., Schena F.* 'Exclusive, additive, and interactive effects of cold and hypoxia on maximal endurance exercise and lactate threshold parameters.' *European College of Sport Medicine Congress*. Paris, 5-7 Jul 2023

ABBREVIATIONS

[La] blood Lactate Concentration

ANOVA Analysis of Variance

BSA Body Surface Area

C Cold Normoxia

CH Cold-Hypoxia

CO Cardiac Output

CPET Cardio Pulmonary Exercise Test;

DLCO Diffusion Capacity for Carbon monoxide

EBPT Exercise Broncho Provocation Test

GXT Graded Exercise Test

H Normothermic Hypoxia

HA High Altitude

HEAV Heavy Intensity Domain

HR Heart Rate

LF Lung Function;

LT Lactate Threshold

MEP Maximal Expiratory Pressure;

MIP Maximal Inspiratory Pressure;

MOD Moderate Intensity Domain

N Normothermic Normoxia

PaO2 Arterial O2 Pressure

PF Pulmonary Function

Rf Respiratory Frequency;

RMF Respiratory Muscle Fatigue;

RPE Rate of Perceived Exertion

SEV Severe Intensity Domain

SL Sea Level

TS Thermal Sensation

Ve Ventilation

V̇O2max Maximal Oxygen Consumption

Vt Tidal Volume;

WL WorkLoad

BACKGROUND AND AIMS

HYPOXIA

1.1 DEFINITION

Hypoxia is defined as “An abnormal condition resulting from a decrease in the oxygen supplied to or utilized by body tissue” in the Webster’s New World College Dictionary, (4th ed., 2010) but also as “an oxygen deficiency causing a very strong drive to correct the deficiency,” in the lexical database for English web site (wordnet, princeton.edu) (taken from Richalet 2021(Richalet, 2021)). This inadequate oxygen delivery to the tissues can result either from low blood supply or low partial pressure of oxygen in the arterial blood (PaO2), this latter condition being referred to as hypoxemia in the American Heritage Dictionary of Medicine (2018) (Richalet, 2021). Among the non-pathological reasons that may cause a hypoxic status in the organism and thus a disruption of homeostasis, High Altitude (HA) exposure is the most popular one. As one ascends from sea level to the top of Mount Everest (Earth’s highest mountain, elevation ≈8848 m) a nearly exponential fall in barometric pressure (i.e. from 760 mmHg at sea level to 265 mmHg at the Summit of Mt. Everest) causes the partial pressure of inspired oxygen in ambient air (PiO2) to decrease, which, in turn, leads to a cascade reduction of alveolar (PAO2) and arterial (PaO2) oxygen pressure (Fig.1) (S. A. Gallagher & Hackett, 2004). As a consequence resting arterial Oxygen Saturation (SaO2) decreases from ≈96% at sea level, to ≈92% at 3000m and ≈80% at 5000m asl.

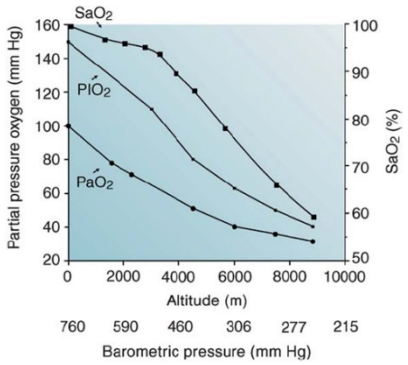

Fig. 1. Relationship of altitude and barometric pressure to PaO_2, PIO_2, and SaO_2. Increasing altitude results in decreased barometric pressure, partial pressure of arterial oxygen (PaO_2), and arterial oxygen saturation (SaO_2). Oxygen saturation is well maintained up to about 3000 m, despite a significant decrease in arterial PO_2. Above this altitude, small changes in arterial PO_2 result in large changes in arterial saturation. (*From* Hackett PH, Roach RC. High-altitude medicine. In: Auerbach PS, editor. Wilderness medicine. Philadelphia: Mosby; 2001. p. 5; with permission.)

Figure 1 Taken from Gallagher & Hackett, 2004; Relationship of altitude and barometric pressure to partial pressure of inspired oxygen in ambient air (PiO2), arterial oxygen pressure (PaO2) and Saturation (SaO2).

Health risks and exercise impairments increase with ascending altitude, which has been for this reason subdivided into three main categories: High Altitude (from 1500 to 3500 m asl),Very High Altitude (from 3500 to 5500 m asl) and Extreme Altitude (from 5500 to 8850 m asl) (S. A. Gallagher & Hackett, 2004). Interestingly, traveling to high altitude has become increasingly popular not only for the latter categories: in 2015, UK-based commercial companies offered 93 expeditions to climb Mount Kilimanjaro (5,895 m) (Shah et al., 2011), but it was also estimated that more than 120 million people visited the European Alps and reached at least 2000 m of altitude (Chatre B, Lanzinger G, Macaluso M, 2010). Furthermore, the last decades were characterized by a growing interest in activities like ski mountaineering by both recreational and competitive athletes, with an increasing number of races organized Worldwide. Interestingly, the International Ski Mountaineering Federation (ISMF), in contrast to other winter sports, does not set limits on the altitude at which competition may take place, reaching also 3000m or even, sometimes, 4000m above sea level (Bortolan et al., 2021). Considering the fact that significant health risks and performance decrements may develop already above altitudes of ≈2000m (Wehrlin & Hallén, 2006), it is easy to understand the

growing interest in scientific literature aiming at better understanding acute and chronic adaptations to HA exposure.

1.2 HYPOXIA, $VO2_{max}$ AND PERFORMANCE

When exercising at altitude, two different (and divergent) stresses are superimposed: reduced oxygen availability on one side (hypoxia), and increased oxygen requests on the opposite (exercise). Before addressing the physiological adaptations that take place in order to cope with the combined above-mentioned stressors (see Chapter 1.3), changes in $VO2_{max}$ and exercise performance at HA will be discussed.

First of all, as altitude increases, the systemic reduction in arterial O_2 content strains the cardiovascular system's ability to meet the required O_2 delivery to active musculature (Amann et al., 2006; Fulco et al., 1998), causing a linear decrease in maximal oxygen uptake ($\dot{V}O_{2max}$) corresponding to ≈6.3% per 1000 m increasing altitude in endurance trained athletes up to 3000m (Wehrlin & Hallén, 2006), this relationship becoming curvilinear at higher altitudes as a function of the non-linear relationship of PiO2 and SaO2 (which is related to $VO2_{max}$) (Macinnis et al., 2015).

O2 transport pathway can be considered as an in-series system of four transport processes: ventilation, alveolar-capillary diffusion, circulation, and muscle capillary–mitochondrial diffusion (P. D. Wagner, 2010). For whole-body exercise performed at sea level, it has been demonstrated that $VO2_{max}$ is constrained mainly by oxygen delivery and not by the mitochondria's ability to consume oxygen (i.e. central rather than peripheral factors) (D R Bassett & Howley, 2000). However, the relationship between reduced O2 availability and $VO2_{max}$ reductions at altitude has been extensively debated. Wagner and colleagues (P. Wagner, 2022; P. D. Wagner, 2010) concluded that of the four steps involved in O2 transport, only pulmonary and muscle diffusion capacity contributed equally and substantially to $VO2_{max}$ reductions at altitude (Fig.2). Conversely, Calbet et al. claimed that the main mechanism limiting $VO2_{max}$ during whole-body exercise in hypoxia is O2 delivery (thus comprising ventilatory, alveolar-capillary diffusion, and circulation reasons), while muscle O2 diffusing capacity may have a secondary role (José A.L. Calbet & Lundby, 2009).

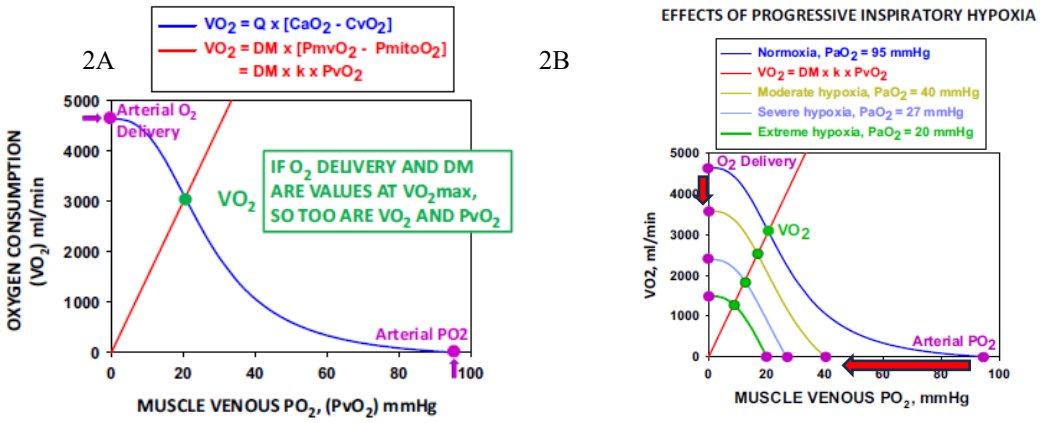

Figure 2 Convective (in blue) and diffusive (in red) properties of $VO2_{max}$ by Wagner. Fig 2A: Diagram depicting (1) in blue the Fick principle equation for VO2 as a function of venous PO2 (PvO2) for given values of blood flow (Q) and arterial [O2] (CaO2) and (2) in red the diffusion equation expressing VO2 as a linear function of mean microvascular PO2 (approximated as PvO2 multiplied by a constant k). The slope of the red line is k x the red cell-to-mitochondria O2 diffusion coefficient (DM). Conservation of mass (of O2) means that these two simultaneous equations must result in the same VO2 at the same PvO2. This in turn means that VO2 and PvO2 must be those values at the unique point of intersection of the two lines. Figure 2B represent $VO2_{max}$ at various altitudes with diffusion coefficient (DM) held constant (supposing that an acute change in FIO2 on a single day would not change the O2 DM). Altitude causes a substantial reduction in $VO2_{max}$: first, O2 delivery to the muscles is reduced in proportion to the reduction in O2 saturation (downward arrow, upper left corner) and second, the fall in arterial PO2 (leftward arrow, lower right corner) reduces the diffusion gradient between blood and mitochondria. Thus, both convective and diffusive components of O2 transport downstream of the lungs are impacted by hypoxia. Interestingly, PvO2 falls in proportion to VO2. Taken and modified from Wagner 2022.

Interestingly, $VO2_{max}$ decrease at altitude has also been linked to subjects' training status for both men and women (Faiss et al., 2014; Lawler et al., 1988; Macinnis et al., 2015; Woorons et al., 2005) (Fig.3): however, within the population of highly trained subjects, a great inter-individual variability is present; for this reason, Chapman and colleagues (1999) concluded that the degree of arterial desaturation (SaO2) during maximal exercise at sea level, and not training status *per se,* influence the ability to defend $\dot{V}O_{2max}$ in mild hypoxia. This phenomenon, known as Exercise Induce Arterial Hypoxemia (EIAH), has been primarily linked to pulmonary diffusion limitations secondary to short transit times (<0.25 s) of the erythrocyte in the pulmonary circulation (ROBERT F Chapman et al., 1999; J. A.

Dempsey et al., 1984; Derchak et al., 2000; Torre-Bueno et al., 1985). Thus, the high prevalence of EIAH (between 50 and 70% of cases for $VO2_{max}$ >68 ml/kg/min (Powers et al., 1988, Mucci et al., 2000; Connes et al., 2004a; Grataloup et al., 2005; Gaston et al., 2016, Connes et al., 2004b; Guenette et al., 2004; Constantini et al., 2017) and the consequent greater relative $VO2_{max}$ reductions at High Altitude in trained athletes could be related to higher maximal metabolic demands (4–5 L/min), with elevated maximal cardiac output (CO_{max}) that easily lead to reduced pulmonary transit time below critical values. However, this might not be the unique reason since, for example, women present a higher prevalence of EIAH than their male counterparts despite lower CO_{max} values (Durand & Raberin, 2021). Moreover, decreased SpO2 during exercise at altitude in subjects suffering from EIAH has also been linked to a decreased hypoxic ventilatory response in this population, which might contribute to hypoxemia (Derchak et al., 2000; Granger et al., 2021; Harms & Rosenkranz, 2008). Also, EIAH athletes present changes in cerebral deoxygenation and a low limb muscle blood volume during exercise at sea level, which are accentuated by the severity of O2 arterial desaturation (Raberin et al., 2020). Finally, Mollard et al. (Mollard, Woorons, Letournel, Lamberto, et al., 2007b) proposed that the difference between trained and untrained subjects in $VO2_{max}$ impairments in hypoxia lies not only in reduced maximal ventilation and a greater decrease in maximal O2 transport in trained individuals but it is associated also to a significantly lower capacity to increase tissue O2 extraction (which is already maximal in normoxia) to compensate for SaO2 reductions. To summarize, the theory expressed in Figure 2 (P. Wagner, 2022) is supposed to be the one that best characterizes the reasons for $VO2_{max}$ reductions at altitude, and different changes in convective and diffusive properties have to be expected when considering different populations.

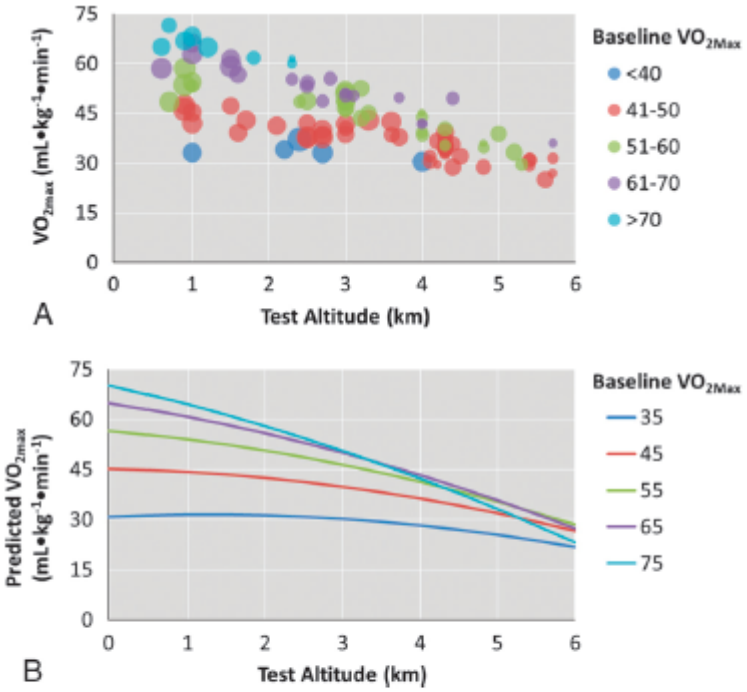

FIGURE 2—A. Observed $\dot{V}O_{2max}$ at high-altitude test (*circles*) for each independent participant group as a function of average baseline $\dot{V}O_{2max}$ and test altitude. The size of each *circle* is inversely proportional to the variance of each study. B. Predicted $\dot{V}O_{2max}$ at high-altitude test as a function of test altitude and baseline $\dot{V}O_{2max}$ (shown as separate lines). These predictions are based on the curvilinear model.

Figure 3 Taken from MacInnis et al., 2015; Observed (A) and predicted (B) $VO2_{max}$ changes as a function of baseline normoxic $VO2_{max}$ and altitude test.

This impairments in $VO2_{max}$ inevitably leads to aerobic performance decrements at high altitude: Burtscher and colleagues (M. Burtscher et al., 2006) found that for a 3200m exposure, a 30s performance showed no differences in mean Power Output (POmean) if compared to sea level values, whereas performances lasting from 5 to 50 mins showed significant decreases in POmean; these PO decrements were partially recovered after 45h of altitude exposure, even though still remaining significantly lower than sea level. Clark et al. (Clark et al., 2007) showed a 7% decrease in POmean for every 1000m of altitude gain during a 5 min Time Trial performance (TT): this decrease was comparable to the decrease in oxygen consumption, differently from the non-linear and amplified decrease in exercise performance if compared to VO2 values found by Wehrlin e Hallen (Wehrlin & Hallén, 2006). The reason why for performances under 2 min in length the

differences between sea level and altitude are negligible can be explained through greater reliance on anaerobic energy sources during such a short exercising time; on the other hand, performance differences become increasingly important as exercise duration increases due to a larger relative aerobic energetic contribution, which is inevitably affected by reduced oxygen availability (Deb et al., 2018; Wyatt, 2014). Furthermore, the state of art regarding maximal incremental test in hypoxia shows a 10 to 13% decrease in peak power output (PPO) or maximal aerobic velocity (VAM) at altitudes between 2500 and 3500m if compared to sea level (Faulhaber et al., 2021; Friedmann et al., 2005; Friedmann et al., 2004; Lorenz et al., 2006; Ofner et al., 2014; Weckbach et al., 2019), and the same happens at the intensities associated with the lactate thresholds, with a reduction in power output ranging from 12 to 19% considering different detecting methods (Faulhaber et al., 2021; Weckbach et al., 2019). Similarly, Townsend et al. (Townsend et al., 2017) found that Critical Power was reduced progressively as altitude increased from 1250 to 4250 if compared to sea level values. Interestingly, Gallagher and colleagues (C. A. Gallagher et al., 2014) reported no changes in Oxygen consumption at anaerobic threshold (VT_2) from 0 up to 4000: differently from other works, these authors tested healthy but untrained subjects, this suggesting that as for $VO2_{max}$, also changes in parameters evaluated at submaximal exercising intensities (i.e. VT_2) are related to subjects training status.

1.3 PHYSIOLOGICAL RESPONSES TO HIGH ALTITUDE

In the previous chapter it was claimed that either $VO2_{max}$, PPO or VAM are reduced in hypoxia (at least for exercising durations > 2min). However, it must be noted that when performing the same absolute Work Load (WL) at altitude as that at sea level, the O2 cost of constant-load submaximal work is not affected by hypoxia (Clark et al., 2007; Macinnis et al., 2015; R. S.Mazzeo, 2006; Wehrlin & Hallén, 2006). As a result of the decline in $VO2_{max}$, similar external WL represent greater relative exercising intensities (i.e. $\%VO2_{max}$) at higher elevations, which require greater physiological and metabolic adjustments to maintain homeostasis. This condition is reached through the regulation of both ventilatory and circulatory mechanisms, which are partially differently affected by acute vs chronic hypoxic exposure. For the purpose of this Thesis, the main focus will be on physiological responses to

acute hypoxic exposure, with only a brief summary of chronic adaptations provided in chapter 1.3.3. Moreover, different responses linked to the nature of the hypoxic stimulus (i.e. normobaric hypoxia with reduction of FiO2 vs hypobaric hypoxia with a reduction in barometric pressure) will be discussed below in a specific paragraph (1.4).

1.3.1 PHYSIOLOGICAL RESPONSES TO ACUTE HIGH ALTITUDE EXPOSURE

At altitude, both at rest and during exercise, the primary goal of the body is to ensure that an adequate amount of oxygen is delivered to the tissues despite the lack of oxygen availability. During acute exposure, the complex regulation of ventilatory and cardiovascular adjustments allows oxygen supplies to meet demands.

At first, hyperventilation occurs due to the so called Hypoxic Ventilatory Response (HVR) (Ainslie et al., 2013; J. A.L. Calbet et al., 2003; José A.L. Calbet & Lundby, 2009; C. A. Gallagher et al., 2014; Peacock, 1998). Peripheral chemoreceptors forming the carotid body are generally stimulated by Carbon Dioxide (CO2) at sea level, whereas at high altitude a drop in PaO2 below 60 mmHg is the main signal causing increased ventilation (José A.L. Calbet & Lundby, 2009). Hyperventilation augments PaO2 by both eliminating CO2 and renewing the alveolar gas, as well as left-shifting the O2 dissociation curve of the haemoglobin, allowing SaO2 to be greater for a given PaO2 (José A.L. Calbet & Lundby, 2009; Lundby et al., 2006) (Fig.4). For these reasons, during exercise at a given absolute intensity, ventilation (Ve) is exaggerated in acute hypoxia compared to normoxia (Fig 4.), causing the fraction of the whole-body VO2 that must be used to sustain mechanical work for Ve to be greater at altitude than at sea level.

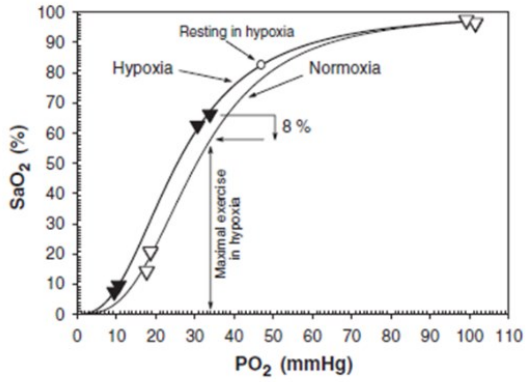

FIG. 3. Impact of hypoxia–hyperventilation on the hemoglobin dissociation curve. Effect of severe acute hypoxia ($F_{IO_2} = 0.105$) on the O_2 dissociation curve of the Hb during exercise in normoxia (white triangles; fine line) and hypoxia (black triangles; thick line). Note the left shift caused by hyperventilation and its impact on Sa_{O_2} at maximal exercise in hypoxia. Points on the graph represent the mean arterial or femoral venous values for each condition in nine subjects. (P_{O_2} values corrected for blood temperature) (Calbet et al., 2003a).

Figure 4 Taken from Calbet et al. 2009; Impact of hypoxia-hyperventilation on the haemoglobin dissociation curve, which is left-shifted in order to allow SaO2 to be greater for a given PaO2

The second important mechanism that takes place to ensure Oxygen supply is an increase in HR for same normoxic absolute WL in hypoxia (Fig. 5), that reflects itself in increased Cardiac Output (CO), due to unchanged (or only slightly decreased) Stroke Volume (SV) at acute exposure if compared to sea level values (José A.L. Calbet & Lundby, 2009; Mallet et al., 2023; Peacock, 1998; R. S.Mazzeo, 2006). Increased submaximal HR results from both increased sympathetic activity and partial parasympathetic withdrawal detected upon immediate HA exposure (R. S.Mazzeo, 2006): in fact, diminished PaO2 detected by the peripheral chemoreceptors results in catecholamine secretion and sympathetic activation, which stimulate the cardiac B-adrenergic receptors (C. A. Gallagher et al., 2014). Improved cardiovascular function comprises also enhanced vasodilation at systemic level and particularly to the exercising muscles, in order to guarantee proper oxygen supply through an increased blood flow (Dinenno, 2016; Lundby et al., 2006)

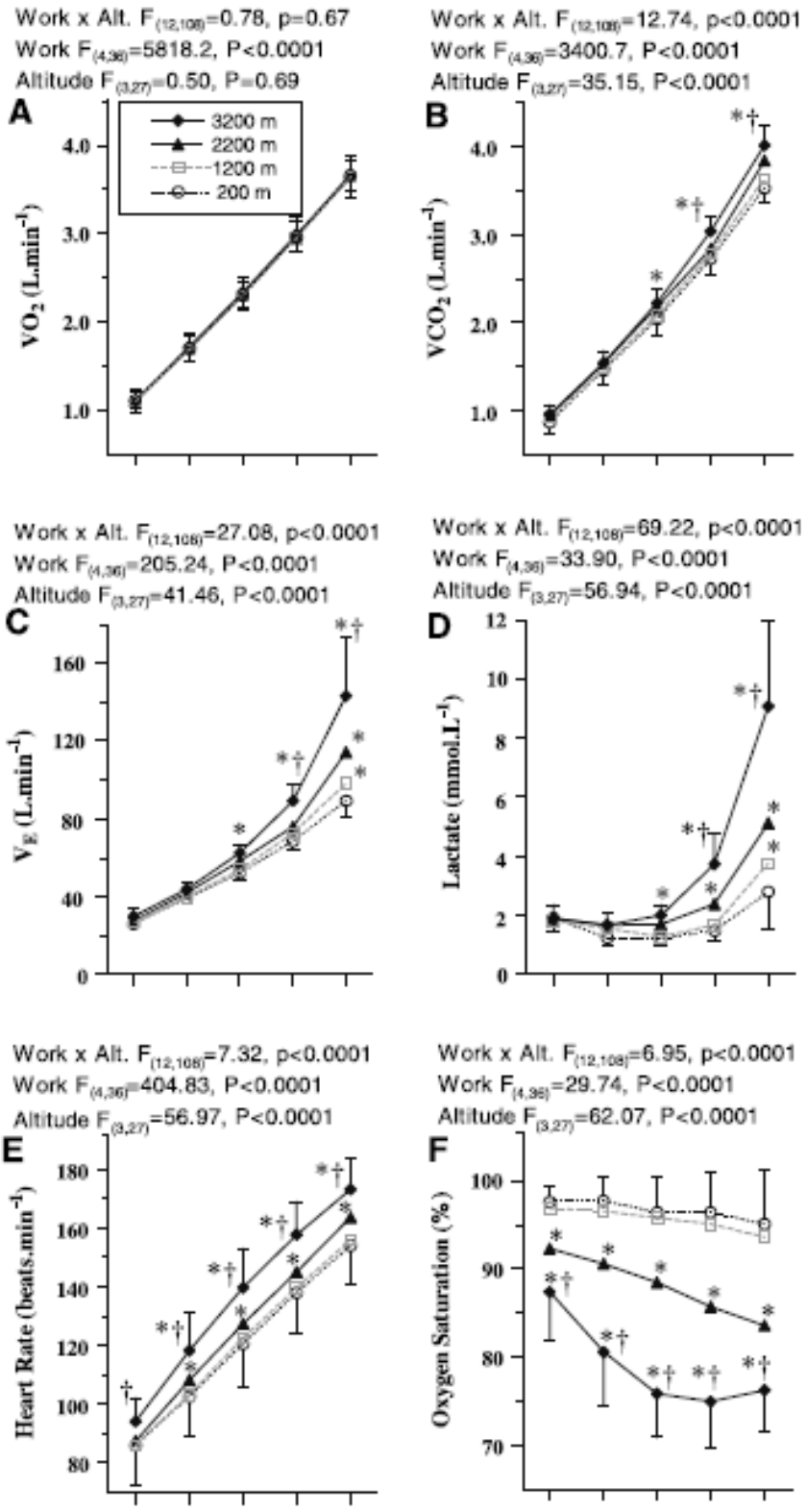

Figure 5 Taken from Clark et al., 2007; Submaximal VO2 (a), VCO2 (b), Ve (c), Lactate (d), HR and Oxygen saturation (f) at four simulated moderate altitudes in well trained subjects.

The two previous mentioned compensatory mechanisms activated to counteract reduced PaO2 during exercise come together with several other acute responses strictly related to hypoxia alone, but that may be advantageous for exercise practice. Firstly, haemoconcentration due to enhanced diuresis when acutely exposed to HA may increase arterial oxygenation (S. A. Gallagher & Hackett, 2004; Peacock, 1998). Furthermore, despite overall induced systemic vasodilation, hypoxia results in an intrinsic generalized pulmonary arteriolar vasoconstriction and a modest rise in pulmonary artery pressure that is independent of increased cardiac output (S. A. Gallagher & Hackett, 2004; Peacock, 1998). At first, this mechanism may serve to improve ventilation-perfusion (V/Q) matching by redistributing blood flow to best ventilated areas of the lungs; however, this rise in pulmonary artery pressure is also one of the main causes of High Altitude Pulmonary Oedema (HAPE). Finally, despite reduced oxygen availability, cerebral circulation is always maintained both at rest and during exercise through hypoxia-induced cerebral vasodilation (S. A. Gallagher & Hackett, 2004).

As explained, several mechanisms take place in order to ensure adequate Oxygen delivery for a given absolute WL, despite this requires exercising at much higher relative intensities in hypoxia, thus modifying substrate metabolism and increasing lactate production. A quick overview of physiological responses to acute high altitude exposure is presented in Fig. 6.

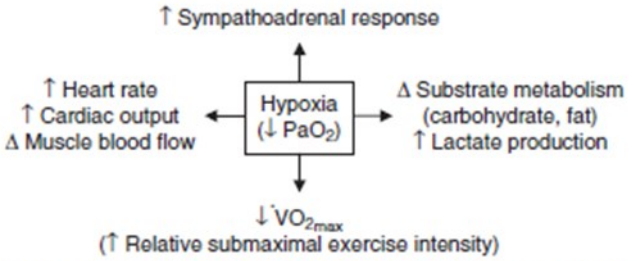

Fig. 4. A summary of key adaptations associated with exposure to high altitude. The primary environmental stimulus inducing these changes is the reduction in oxygen arterial pressure (PaO_2). This disruption in homeostasis elicits sympathoadrenal responses, which affect many physiological and metabolic variables. $\dot{V}O_{2max}$ = maximum oxygen consumption; ↑ indicates increase; ↓ indicates decrease; Δ indicates change.

Figure 6 Taken from Mazzeo et al., 2006: A summary of key adaptations associated with acute HA exposure.

1.3.2 THE CASE FOR MAXIMAL HR REDUCTION

When analysing oxygen consumption's changes from sea level values, it is clear that two different approaches have to be considered for maximal (i.e. reduced $VO2_{max}$ and maximal sustainable workload) and submaximal (i.e. same VO2 at each given workload, but different relative intensity) responses. Something similar happens also when looking at HR adjustments: in fact, we have extensively described how HR increases for same absolute WL in hypoxia in order to guarantee requested oxygen supplies, but a decrease in maximal Heart Rate (HRmax) is well documented both in acute and chronic hypoxic exposure (Grataloup et al., 2007; Mollard, Woorons, Letournel, Cornolo, et al., 2007; Mourot, 2018; Naeije, 2010). In a recent work, Mourot L. (Mourot, 2018) described a 1.7 bpm reduction for every 1000m of altitude gain and interestingly attempted to put together all the possible mechanisms responsible for this reduction.

First of all, chronic reduction in CaO2 may cause a lowering in cardiac B-receptors numbers, with consequent blunted HR responses for a similar (maximal) stimulus. This may happen i) with chronic HA exposure (thus not being a cause of acute HR reduction), ii) as a consequence of the repetition of long duration exercises by subjects who exhibit EIAH: this could induce a situation similar to a chronic exposure to hypoxia, interfering also with acute hypoxic effect on HR (Benoit et

al., 2003; Grataloup et al., 2007; Mollard, Woorons, Letournel, Lamberto, et al., 2007b).

Secondly, Benoit et al. (Benoit et al., 2003) suggested a direct effect of hypoxia on the cardiac electrophysiological properties, with a modification of repolarization length and transmission time on the Atrio-ventricular node responsible for HR reductions already with acute exposure. Grataloup and colleagues (Grataloup et al., 2007) suggested that the highest the exercise induced hypoxemia superimposed on HA related oxygen deficiency, the greatest the modifications of cardiac properties.

Another contributor, likely the major one, seems to be the change in the activity of the autonomic nervous system (ANS). If both an increase in parasympathetic activity with a densitization of the adrenergic pathway are responsible for HR reductions in chronic exposure (Grataloup et al., 2007; Mourot, 2018), only increased parasympathetic activity might be responsible for the blunting of the cardiac chronotropic function during acute hypoxia. As previously described for B-receptors density, also ANS responses to acute hypoxia could be influenced by repeatedly performing long duration exercises in subjects developing EIAH (Grataloup et al., 2007; Mollard, Woorons, Letournel, Cornolo, et al., 2007).

Also central factors may play a role in HR_{max} reductions both in acute and chronic exposure. In fact, it has been proposed that a "central governor" (Noakes et al., 2001) situated in the central nervous system (CNS) strictly controls the oxygen tension in the coronary vascular bed, regulating maximal myocardial work in order to protect this tissue from ischemia. Moreover, reduced maximal external work may be strictly related to reduced HR_{max} in hypoxia (Mourot, 2018).

Finally, it has been proposed that maximal skeletal muscle VO2 dictates maximal cardiac output in hypoxia: arterial hypoxemia may induce a decrease in this parameter, which in turn determines a similar decrease in cardiac output due to decreased HR and similar SV if compared to sea level values (Mourot, 2018).
Interestingly, as for $VO2_{max}$, also HR_{max} decrease in acute hypoxia has been shown to be related to subjects' training status, with a greater decrease for trained subjects, even though no clear explanation for this phenomenon is present (Mollard, Woorons, Letournel, Cornolo, et al., 2007) (Fig.7).

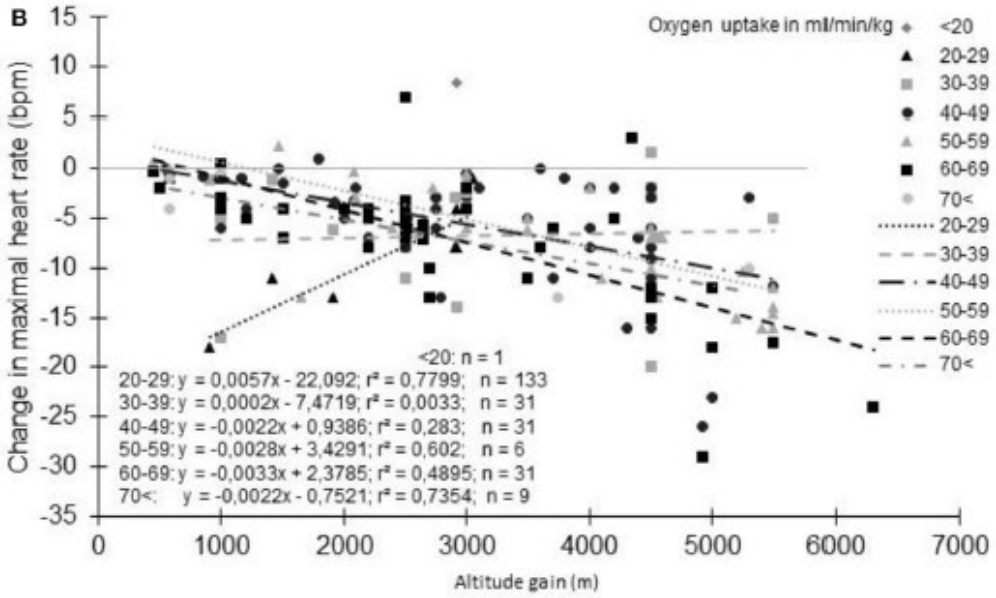

Figure 7 Taken from Mourot et al., 2018; Decrement of maximal heart rate with altitude: Effect of physical fitness.

1.3.3 CHRONIC HIGH ALTITUDE EXPOSURE AND ACCLIMATIZATION

Figure 8 illustrates the importance of separately considering acute and chronic HA exposure in determining hypoxic induced physiological responses of the exercising body; in fact, adjustments that happen within minutes may regress with acclimatization, in favour of long-term adaptations occurring only after days or months of permanent stay at high altitude (Mallet et al., 2023).

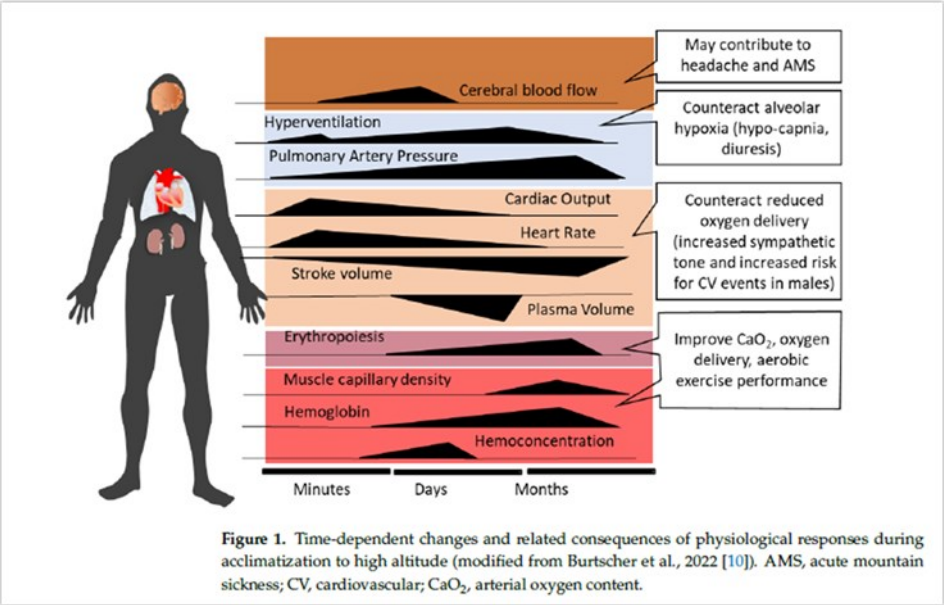

Figure 1. Time-dependent changes and related consequences of physiological responses during acclimatization to high altitude (modified from Burtscher et al., 2022 [10]). AMS, acute mountain sickness; CV, cardiovascular; CaO_2, arterial oxygen content.

Figure 8 Taken from Mallet et al., 2023; Time-dependent changes and related consequences of physiological responses during acclimatization to high altitude.

As very first haemoconcentration due to hypoxic induced diuresis subsides, HIF-1 related upregulation of erythropoietin (EPO) sustains the increases in Haemoglobin (Hb) concentration and consequently the increase in arterial blood O2 concentration (CaO2) (≈1-2 weeks of exposure). This is followed also by another HIF-1 initiated response, represented by the increase in muscle capillarity (Mallet et al., 2023).

Furthermore, compared to acute exposure, submaximal exercising Ve remains at the same level with acclimatization (José A.L. Calbet & Lundby, 2009), while maximal alveolar minute ventilation even increases up to 10 days after exposure before reaching a plateau due to sensitization of the peripheral chemoreceptors (José A.L. Calbet & Lundby, 2009; Dempsey, A.; Forster, 1982)

On the opposite side, with acclimatization to altitude CO for a given power output is reduced due to both decreased SV and blunted HR response (i.e. attenuation in cardiac responsiveness to B-adrenergic stimulation with acclimatization), this causing net O2 delivery during submaximal exercise remaining unchanged despite increased CaO2 if compared to acute exposure (Mallet et al., 2023). SV reduction is partially due to loss of plasma volume starting immediately and resolving within

weeks of HA exposure, but also to an increased after-load placed upon the heart as systemic vascular resistance augment following hypoxic-induced sympathetic nerve over-activation (S. A. Gallagher & Hackett, 2004; R. S.Mazzeo, 2006).

These long-term adaptations allow for a lesser reduction in performance at High Altitude, but still both $VO2_{max}$ and performance remain significantly impaired compared to sea level. Reduced maximal Cardiac Output and redistribution of blood flow to non-exercising tissues may contribute to this phenomena (R. S.Mazzeo, 2006).

1.4 THE ROLE OF BAROMETRIC PRESSURE: NORMOBARIA VS HYPOBARIA

The last part of this entirely hypoxic-dedicated session is necessary to better understand not only the results of this Thesis, but also the controversy that can be found in literature regarding hypoxia related changes in some parameters.

Systemic hypoxia is caused by a reduction in the partial pressure of inspired oxygen (PiO2), which can be achieved by both a decrease in barometric pressure (PB) leading to hypobaric hypoxia (HH ; FiO2: 20.9%; PB< 760 mmHg) or by a decrease in the fraction of inspired Oxygen (FiO2), without a change in PB, creating the so called normobaric hypoxia (NH; FiO2 < 20%; PB =760 mmHg) (Grégoire P. Millet et al., 2012; Netzer et al., 2017; Richard & Koehle, 2012; Savourey et al., 2003). Since a decrease in PiO2 was thought to be the only factor relating to the physiological responses to hypoxia, these two methods have been used interchangeably, both in medicine and sport (Grégoire P. Millet et al., 2012). However, ever-growing body of evidence challenges this assumption, suggesting an independent role of PB in determining differences in physiological responses (Grégoire P. Millet & Debevec, 2020). This differences have been firstly linked to ventilatory aspects, fluid balance, Nitric Oxide metabolism (NO) and Acute Mountain Sickness (AMS) prevalence (Grégoire P. Millet et al., 2012), being subsequently integrated also with information regarding possible other differential responses also for pulse saturation (SpO2), sleep quality, systemic oxidative stress, postural stability and (as a consequence of all above) performance (Grégoire P.

Millet & Debevec, 2020). Considering the data that will be presented thereafter, only NH vs HH changes in submaximal and maximal cardio-respiratory responses will be discussed, with a particular focus on oxygen consumption (VO2), ventilatory data (i.e. ventilation (Ve), tidal volume (Vt) and breathing frequency (Rf)), pulse oxygen saturation (SpO2) and HR.

An interesting recent review by Treml and colleagues (Treml et al., 2020) suggests that $VO2_{max}$ is reduced to a larger extent in NH if compared to HH (Effect Size (ES): 1.19), and this is coupled with increased Ve_{max} in HH and decreased Ve_{max} in NH if compared to normoxic values. Small to medium ES were detected for SpO2 (ES: 0.27) and HRmax (ES: 0.49) differences between conditions, which were both decreased by hypoxic exposure (see chapter 1.3.3) (Fig. 9)

Figure 2 depicts the salient findings of this focused review in regard to divergent oxygen uptake and ventilation responses in hypo- and normobaric hypoxia.

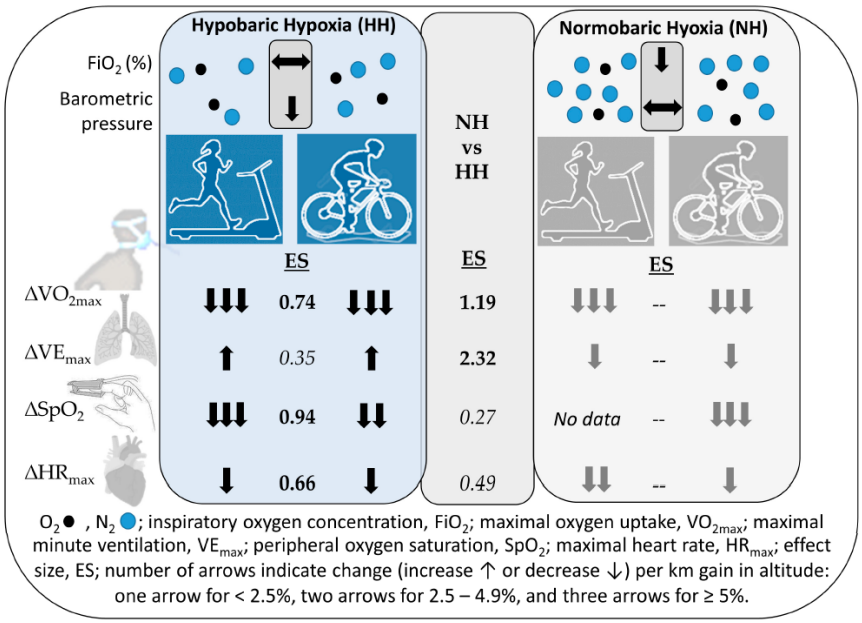

Figure 2. Schematic presentation of the key findings.

Figure 9 Taken from Treml et al., 2020; Main differences in VO2max, Vemax, SpO2 and HRmax between normobaric (NH) and hypobaric (HH) hypoxic exposure.

Larger VEmax responses in acute HH compared to NH (at comparable simulated altitude levels) may be expected due to the lower air density in HH, but also the fact that NH induced greater $VO2_{max}$ decreases and thus reduced exercise performance

may be partially responsible for different Vemax results (Mollard, Woorons, Letournel, Lamberto, et al., 2007a).

Differently from maximal values, at both rest (Savourey et al., 2003) and submaximal exercising intensities (Basualto-Alarcón et al., 2012; Coppel et al., 2015; Faiss et al., 2013; Grégoire P. Millet & Debevec, 2020), Ve is generally reduced in HH if compared to NH (but in both cases increased Ve for a given WL is detected if compared to normoxic conditions): as for Vemax, this could be simply linked to a slight reduction in air density under hypobaric conditions that justifies a lower ventilation rate for the same work load of the respiratory muscles (Basualto-Alarcón et al., 2012). However, Faiss and colleagues (Faiss et al., 2013) suggested also a possible connection between lower minute ventilation and decreased NO bioavailability in HH if compared to NH, whereas others claimed that hypobaria per se could provoke higher hypoxic pulmonary vasoconstriction, resulting in changes of alveolar dead space and altering ventilation–perfusion ratio and consequently ventilation (Grégoire P. Millet & Debevec, 2020).

Submaximal HR responses seem to be similarly affected by both HH and NH (Coppel et al., 2015), even though some inconsistencies exist, with some studies showing slightly increased submaximal HR in HH if compared to NH (Netzer et al., 2017; Savourey et al., 2003), and other showing no differences between the 2 conditions (Faiss et al., 2013).

This inconsistencies persist also for SpO2, which seems to be more reduced in HH for short term exposure (Coppel et al., 2015), but present different outcomes as exposure time augments (Coppel et al., 2015; Netzer et al., 2017). Finally, the probability to develop AMS seems to be higher in HH (Coppel et al., 2015), as well as performance decrements more accentuated in this condition (Beidleman et al., 2014; Saugy et al., 2016).

As highlighted, the duration of the hypoxic exposure impacts the obtained results, making it difficult to perform an overall comparison between studies. However, in light of the projects presented in this Thesis, which have all been carried out in NH, it is crucial to analyse the outcomes while considering the actual experimental condition, to avoid misinterpreting study outcomes.

HIGH ALTITUDE AND FREEZING TEMPERATURES: A CASE FOR COMBINED STRESSORS EXPOSURE

2.1 TYPE OF STRESSORS INTERACTIONS: ADDITIVE, SYNERGISTIC OR ANTAGONIST

Up to this moment hypoxia has been treated as a single environmental stressor, and as extensively presented in the previous section, great literature interest on the effect of this stressor on several cardio-respiratory, hemodynamic and transcriptional factors has been developed throughout the last decades. This has allowed for a better understanding of the risks associated with hypoxic exposure, but also of its positive adaptations that can be leveraged for performance enhancement at sea level. However, High Altitude environment is characterized by an 'extreme' nature, as it often displays the simultaneous presence of numerous stressful factors (Lloyd & Havenith, 2016): alpinists trying to reach the highest summits of the world encounter freezing temperatures, harsh terrains as well as significant cognitive stress, but also athletes of specific winter sports (i.e. Ski-mountaineers, Cross-country skiers and Biathletes) find themselves training or competing while facing several, if not all, previous mentioned challenges altogether. Also, as stated at the beginning of this work, many people reach altitudes ≥2000 m asl every year for recreation, rising the interest in better understanding the potential risks related to combined stressors exposure. So the problem is that in real-world, multiple stressors generally co-exist (Tipton, 2012), but a dearth of interaction specific studies is present in literature since such approaches can be experimentally, temporally and practically challenging (Lloyd & Havenith, 2016).

In order to deal with this phenomenon, Lloyd and Havenith (Lloyd & Havenith, 2016) proposed a multifactorial approach based on the principle that combined effect of 2 or more stressors can be categorised by their net effect under 3 classifications: additive, when the effect of combined stressor exposure on one outcome is identical to the sum of the individual effect of each stressor on that parameter (not resulting in a statistically significant interaction); synergistic or hyper-additive, when the net combined effect is significantly higher than the

additive effect; and finally antagonistic, when the net effect is significantly less than the additive one (Fig.10).

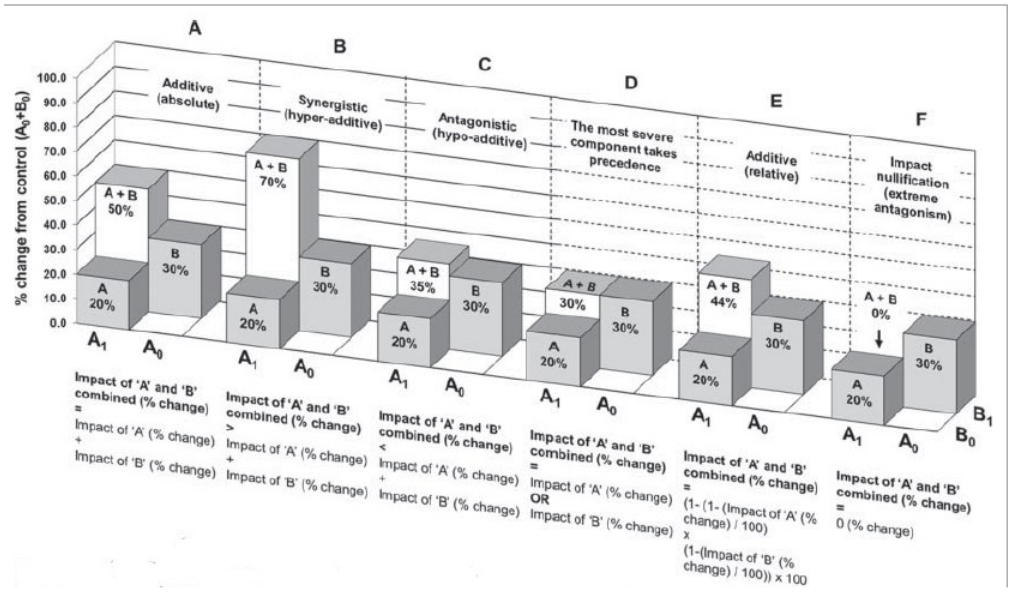

Figure 10 Taken from Lloyd and Havenith, 2016; Types of interactions in human physiology and performance. (A, B and C) show the 3 types of interaction (additive, synergistic, antagonistic) that can occur when combining independent stressors. (D, E and F) show examples of 3 interaction sub-types (see text).

Several interpretations of this types of interaction have also been given by the authors: additive effects may result from combining stressors with independent mechanisms, because for one stressor to influence the expression of another (generating a synergistic interaction), the 2 factors must share a common pathway of action (mechanism of action theory). Additive (or relative additive) effects usually generate when combining stressors that taken individually have a mild effect on the considered variable/outcome (Fig.10 A-B-E). However, the authors claims that when very severe and simultaneous strains are present, the 'most severe one takes precedence', resulting in a net effect that is considerably lower (if not completely nullified) than the additive one: this infers the presence of a maximum threshold for the deterioration of the considered parameter, probably in order to avoid extreme homeostasis perturbance and thus incurring in life-threatening risks for the organism (Fig.10 C-D-F).

The two most prominent stressors at altitude are cold ambient temperatures and low levels of oxygen (hypoxia): however, to better understand the possible interaction effect of these two stressors on human physiological responses while exercising, a brief look at cold stressor alone is necessary.

2.2 THE COLD

The impact of cold environments on individuals ranges from mild degradations in performance to tissue damage (frostbite, non-freezing cold injury), to life-threatening pathological conditions (hypothermia).
Hypothermia is defined by a core body temperature below 36°C, with increasing severity as it drops further from this threshold (Table 1) (Taylor et al., 2008)

Table 1 Taken from Taylor et al., 2008; Core and skin temperatures related to clinically life-threatening conditions.

Table 20.1 Clinically significant core and skin temperatures (°C)

Core temperatures	
36.5–37.0	Normothermia
36–33	Mild hypothermia
33–25	Moderate hypothermia
<25	Profound hypothermia
<24	Possible death without rewarming
<22	Profound clinical hypothermia
Skin temperatures	
28–33	Thermal comfort
25–28	Cool through to discomfort (cold)
20	Impaired dexterity
15	Pain
10	Loss of skin sensation
5	Non-freezing cold injury (time dependent)
<0.55	Freezing cold injury (frostbite)

The Autonomic Nervous System (ANS) controls responses to stressors and resting states of the body throughout daily activity, and its components (i.e., sympathetic (SNS) and parasympathetic (PNS) branches) are activated at different capacities according to the body's requests aimed at maintaining homeostasis. The cold

stressor represents a challenging situation for the body, and several adjustments take place to keep core temperature within its optimal range (≈37°C).

Specifically, to preserve heat when exposed to really cold temperatures, the human physiological systems implement two main strategies: one for heat conservation (i.e., vasoconstriction) and the other for heat production (i.e., shivering and non-shivering thermogenesis) (M. Burtscher et al., 2018). Both these strategies require increased sympathetic activation paired with partial parasympathetic withdrawal. In fact, reductions in skin blood flow mediated by the SNS represent the first line of heat conservation (Taylor et al., 2008), with the aim of increasing the insulation of the body by reducing the thermal gradient between the skin and the environment (Castellani & Tipton, 2016; Mugele et al., 2021). Vasoconstriction begins when skin temperature falls below 35°C and becomes maximal at about 31° C (Castellani & Tipton, 2016). These elevations in vascular tone are elicited via adrenergic receptor activation, through a direct effect of the lower skin temperature on blood vessel diameter, and also by an elevation in plasma noradrenaline concentration (Castellani & Tipton, 2016) Vasoconstriction occurs both in response to reflex and local cooling of skin, as well as a decrease in core temperature (Castellani 2016). As a result of the higher peripheral resistance, systolic (SBP) and diastolic (DBP) blood pressure increase. However, although seemingly counterintuitive for the anticipated increased overall SNS activity in acute cold exposure, resting heart rate responses depend on the type of cold exposure, but are not generally altered much with whole-body cold exposure (Ikäheimo, 2018; Raiko et al., 2021; Robinson & Haymes, 1990), showing unaltered or even slightly reduced values, independently from the presence of facial cooling. This avoids an excessive increase in the rate pressure product (systolic blood pressure * heart rate), considered a surrogate marker for myocardial oxygen demand and whose high values are related to increased cardiovascular strain.

The second line of defence relies instead on metabolic heat production through shivering and non-shivering thermogenesis. Shivering is a form of involuntary skeletal muscle activation, consisting of repeated muscle contractions during which most of the metabolic energy expended is liberated as heat (efficiency near 0) (Castellani & Tipton, 2016; Taylor et al., 2008). Sustained shivering determines a

3-to-5-fold increase in metabolic rate, and it can still occur during exercise, even though normally only for low relative exercising intensities.

Non-shivering thermogenesis pertains instead to both voluntarily modifying behaviour (i.e., increasing physical activity) or heat production generated from brown adipose tissue, that becomes active upon cold exposure. This last mechanism is triggered mainly by mild cold and can increase resting energy expenditure to an extent of ≈30%, without inducing the large discomfort determined by shivering thermogenesis (Brychta & Chen, 2017). In fact, cold-induced sympathetic over-activation is responsible for beta-3 adrenoreceptor stimulation, situated within fat cells and deputed to fat oxidation and lipolysis (Simmons, 2012). Specifically, in white and in brown adipose tissue the cold-sensing receptor TRPM8 seems to initiate increased mitochondrial thermogenesis by up-regulating mitochondrial UCPs (e.g., UCP1) (Roth et al., 2021). In brown adipose tissue, UCP1 uncouples mitochondrial oxygen consumption from oxidative phosphorylation resulting in heat production, which is an important means to regulate body temperature in acute cold exposure (M. Burtscher et al., 2018).

As previously described, life-threatening risks of cold exposure are linked to core temperature, without any specific related external ambient temperature. This is because ‘a man in the cold is not necessarily a cold man’ (Castellani & Tipton, 2016). In fact, similar outside temperatures may generate very different core temperature responses when considering, for example, resting vs exercising conditions. Also, proper clothing insulation may create a relatively warm ‘micro-environment’, maintaining core and muscle temperature within normal ranges. Moreover, same outside temperature but changes in wind or humidity conditions differently affect the effort to maintain thermal balance, following variations in the heat exchange processes between the body and the environment (conduction, convection, and radiation) (Castellani & Tipton, 2016; Taylor et al., 2008). Thus, when discussing cold effect on exercising responses and performance, a clear distinction has to be made between A) studies evaluating a decrease in core and, consequently, muscle temperature, but with subjects breathing normal-temperature air (i.e. through cold water immersion) , B) studies considering subjects exercising while breathing cold air, but with preserved core-temperature ranges and C) studies

combining A+B in their experimental design. This implies a distinction between systemic and bronchial-related physiological responses to cold exposure.

2.2.1 EXERCISE AND REDUCED CORE TEMPERATURE

Systemic responses to reduced core body temperature while exercising comprise mainly cardiovascular, muscular and metabolic adjustments.

As previously described, cold-induced peripheral vasoconstriction determines a rise in mean arterial pressure (BP) and stroke volume, with a consequent reduction in exercising HR (Castellani & Tipton, 2016; Doubt, 1991; Mugele et al., 2021; Taylor et al., 2008), which is more evident than HR changes at rest: in fact, HR_{max} has been shown to be lowered by 10 to 30 beats· when the deep body temperature is lowered by 0.5 to 2.0 °C, whereas submaximal exercising HR seems to be ≈15% lower at any given power output or VO2 during cold exposure if compared to temperate conditions (Castellani & Tipton, 2016; Doubt, 1991). Since overall sympathetic nervous activity actually increases during cold exposure, HR_{max} reductions have been linked to a reduced sympathetic drive on the heart as well as in intrinsic changes in heart mechanics (Castellani & Tipton, 2016; Mugele et al., 2021). However, this displacement of blood from the peripheral/limb circulation to the central circulation determines an increase in pulmonary artery pressure and blood volume, negatively impacting upon respiratory mechanics by reducing lung volume and increasing the work of breathing (WOB). Moreover, if deep muscle temperature is below normal exercising levels (≈39°C optimum), muscle blood flow at a given workload is reduced relative to warmer conditions in order to avoid heat dissipation (Castellani & Tipton, 2016; Doubt, 1991; Taylor et al., 2008). This may actually happen only when considering really low exercising intensities at extreme rigid ambient temperature (i.e. near the summit of Mount Everest, with average ambient temperature being −36°C and exercise practice extremely limited by low oxygen availability): since most of the passive body insulation comes from both cutaneous and skeletal muscles' beds vasoconstriction, if vasodilation requested from the muscle in order to sustain an external Workload determines an heat loss that is higher than heat production generated from that exercise, a

protective mechanisms for thermal homeostasis induces muscle vasoconstriction in order to maintain body insulation (Doubt, 1991; Taylor et al., 2008). In addition to a decrease in muscle blood flow, cold has also been proposed to likely induce a decrease in muscle unloading of oxygen from haemoglobin due to a leftward shift in the oxygen-haemoglobin dissociation curve (Castellani & Tipton, 2016), as proved also by a lower muscle oxygenation seen in resting human ankle dorsiflexor muscles cooled from the skin surface if compared to thermoneutral conditions (Yanagisawa et al., 2007).

As a consequence of all previous-mentioned physiological adaptations to reduction in internal temperature, a cooled muscle has also a decreased capacity to generate force expressed on cross-sectional area (Castellani & Tipton, 2016; Doubt, 1991; Wakabayashi et al., 2015). In addition to this, antagonists muscles activity has been suggested to be increased when exercising in the cold (i.e. braking effect), this being even worsened when exercising intensity is very low and shivering of these muscles interferes with the control of motor activity (Doubt, 1991; Wakabayashi et al., 2015).

Finally, at metabolic level a shift from aerobic to anaerobic metabolism while exercising in the cold has been stated (Castellani & Tipton, 2016; Doubt, 1991; Taylor et al., 2008): the reasons for this change are still unclear, but cold-induced vasoconstriction of peripheral adipose tissue may decrease lipid mobilisation (Doubt, 1991). This may account also for higher lactate levels and greater rates of muscle glycogen depletion at same submaximal workload if compared to temperate conditions. Also, the simultaneous presence of shivering in antagonist muscles may worsen this already highly compromised metabolic state, due to heavy reliance of this mechanism on carbo metabolism.

2.2.2 BRONCHIAL RESPONSIVENESS TO COLD AIR-BREATHING

Even when maintaining core temperature in cold conditions through sufficient exercise intensity and appropriate clothing, preventing the inhalation of dry-cold air while exercising can be quite challenging. The primary effect of cold air on the respiratory system is to decrease minute ventilation and chemosensitivity (M. Burtscher et al., 2018). Moreover, a growing body of literature indicates ambient

temperatures below -15°C to result in cold air-induced airway constriction during and especially after exercise (Dillard et al., 2005; Kennedy et al., 2019a; Kennedy & Faulhaber, 2018; Sandsund et al., 1997). This phenomena is particularly pronounced in individuals already experiencing exercise-induced bronchoconstriction (EIB) under normal temperature conditions, but its prevalence seems to be much higher in the cold, suggesting that temperature itself may trigger EIB in individuals who otherwise exhibit normal airway responses to exercise (Kennedy et al., 2019a, 2020; Kennedy & Faulhaber, 2018; Sandsund et al., 1997).

Ventilating heavily cold air worsen EIB due to both increased water loss from the airway (osmotic effect) during, and rapid and extreme airway rewarming (thermal effect) after exercise cessation if compared to thermoneutral ambient temperatures (Anderson & Daviskas, 2000a; Bonini, 2018; Parsons et al., 2013). Interestingly, a correlation between EIB prevalence and the repetition of high-intensity training sessions in the cold among winter athletes is well established (Kennedy & Faulhaber, 2018; Sue-Chu, 2012), with an incidence of EIB >50% in elite cross country skiers (Bergeron et al., 2012). However, it is worth noting that bronchoconstriction in these athletes may follow a different pathway compared to asthmatic individuals (Heaton et al., 1983; Sue-Chu, 2012).

For a more in-depth exploration of this process and its impact on ventilatory responses during exercise, refer to Project 2 in this Thesis.

2.3 COLD, $VO2_{max}$ AND PERFORMANCE

Given the challenges highlighted in previous discussions on physiological responses, several authors have sought to understand the impact of the cold on $VO2_{max}$ and performance deterioration. However, conflicting results have emerged, primarily due to a lack of consideration for various testing modalities, such as evaluated ambient temperature and chosen clothing. This oversight inevitably led to compare results obtained in varying degrees of both core and muscle temperature decreases, along with cold air-induced airway constriction.

Summarizing, when considering an effective decrease in muscle temperature (Tm), peak torque of knee extension in both isometric and dynamic conditions has been shown to be reduced (Wakabayashi et al., 2015). This reduction in muscle fibres force generation may be responsible for reduced exercising efficiency, explaining both a decrease in maximal exercising capacity and an increase in submaximal strain when ambient temperature decreases. Also, for high-to-maximal exercising intensities and colder air exposures (i.e. -15°C), more severe constriction of the bronchial tree may also result in a diminished $VO2_{max}$ (Oksa et al., 2004). This reduction has been quantified in a 5% decrease in - 20°C and 0°C compared to +20◦C (Castellani & Tipton, 2016; Quirion et al., 1989), but a real paucity of data does not admit definitive conclusions.

Furthermore, aside from reduced exercising efficiency, increases in oxygen uptake at submaximal level may be also due to persistence of shivering during exercise, to an increase in muscle tonus in the absence of over shivering, or to non-shivering thermogenesis according to the considered experimental design (Doubt, 1991).

As previously highlighted, when attention is driven to real performance changes, even more conflicting outcomes are present, as summarized in Table 2. In conclusion, it can be affirmed that more systematic data are needed to clarify whether cold air impacts long-duration aerobic performance or not.

Table 2 Taken from Castellani et al., 2016; Summary of Human Studies Examining the Effect of Cold Exposure on Aerobic Exercise Performance

Reference	Sample size	Exercise mode	Intensity	Environments	Findings/results
Patton and Vogel (186)	Eight males	CE 60 RPM	75%-80% VO_{2max} 30 h at each temperature before exercise	20 and −20°C RH 20%-40% AV 0.5 m s^{-1}	TTE decreased 38% from 20 to −20°C
Galloway and Maughan (94)	Eight males	CE 60-70 RPM	70% VO_{2max} to exhaustion	4, 11, 21, and 31°C air RH 70% AV 0.7 m s^{-1}	VO_2 was higher at 4 and 11°C vs. 21°C TTE highest at 11°C (93.5 ± 6.2 min) with 15% reduction at 4°C
Sandsund et al. (205)	Eight males	TM 6% grade	55% VO_{2max} for 10 min 60%-95% VO_{2max} 4 × 5 min 95% VO_{2max} for 10 min (Salbutamol) 85% VO_{2max} for 5 min (Salbutamol)	−15°C air with/without Salbutamol 23°C/RH 52% AV 1.5 m s^{-1}	TTE shorter at −15°C compared to 23°C VO_2 was higher at −15°C Salbutamol increased FEV_1 after exercise
Parkin et al. (184)	Eight males	CE 80RPM	70% VO_{2max} until exhaustion	3, 20, and 40°C RH <50%	TTE highest at 3°C
Carling et al. (34)	Nine males	Professional Soccer Match	Running speeds 0.0-14.3 km/h 14.4-19.7 km/h ≥19.8 km/h	≤5°C, 6-10°C, 11-20°C, and ≥21°C	Shortest distances ran in ≥21°C (118.7 ± 6.9 m) Greater distances covered per minute in ≤5°C (9.1 ± 3.8 m)
Sandsund et al. (204)	Nine males	TM 6% grade	60% VO_{2max} for 10 min then 67%-91% VO_{2max} 4 × 5 min Followed by VO_{2max} and TTE	−14 and −9°C −4 and 1°C 10 and 20°C AV 5 m s^{-1}	TTE highest at −4 and 1°C No significant differences in VO_{2max}
Renberg et al. (198)	Nine women		71% VO_{2max} for 10 min then 76%-89% VO_{2max} 4 × 5 min Followed by VO_{2max} and TTE	−14 and −9°C −4 and 1°C 10 and 20°C AV 5 m s^{-1}	No difference in TTE among different air temperatures

Abbreviations: CE, cycle ergometer, TM, treadmill; TTE, time to exhaustion; RH, relative humidity; VO_{2max}, maximum oxygen uptake; MST, mean skin temperature; FEV_1, forced expiratory volume in 1 s; AV, air velocity.

2.4 COLD-HYPOXIC EXPOSURE: WHAT DO WE KNOW?

With both an overlook on hypoxic and cold induced effect on physiological responses at maximal and submaximal exercising intensities as well as on performance, at this point a question naturally arises: What do we know about combined stressors exposure on these aspects? The answer, as already anticipated, is that limited information is available (Mugele et al., 2021; Tipton, 2012).

Given that both cold and hypoxia cause cardiovascular, respiratory and metabolic adjustments to maintain homeostasis, one stressor has the potential to affect multiple pathways relating to the adjustments induced from the other stressor, generating one of the three previous mentioned type of interactions (i.e. additive, synergistic or antagonistic).

Recently, an interesting descriptive review by Mugele and colleagues (Mugele et al., 2021) tried to gather all together the available information on cold and hypoxic

effect on cardiovascular, pulmonary and thermoregulatory aspects, describing their possible interaction based on the multifactorial approach proposed by Lloyd et al. (Lloyd & Havenith, 2016) (Fig. 11). Thus, in the following paragraph, a brief overview of all available information regarding cold-hypoxic exposure on thermoregulation, cardiovascular responses and central and peripheral fatigue will be provided.

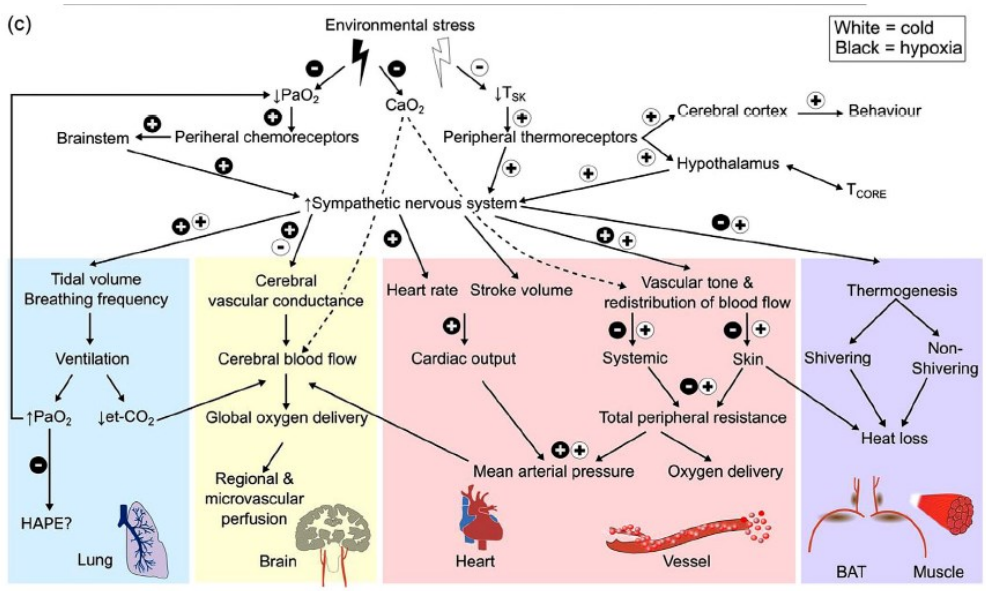

Figure 11 Taken from Mugele et al, 2021; Individual and potential synergistic and/or antagonistic responses to the cold (white) and hypoxia (black) on the cardiovascular, pulmonary and thermoregulatory system. The symbols indicate an attenuation (−) or augmentation (+), and a dashed line indicates a direct influence on a system.

The great majority of studies dealing with cold-hypoxic exposure focused on their possible interaction on thermoregulatory aspects. This is because it has initially been supposed that a competition may occur between maintaining oxygen delivery within distinct vascular beds (hypoxia-induced vasodilatation) and cold-induced vasoconstriction to avoid heat dissipation (Mugele et al., 2021). This has been confirmed by several studies showing that in hypoxic conditions, the cutaneous vasoconstrictor response to cold seems attenuated, this leading to higher skin temperature and more pronounced heat dissipation. As a consequence, core temperature falls more rapidly and to a lower absolute temperature (Keramidas et al., 2014, 2019). All together, these detrimental effects of hypoxia on

thermoregulatory aspect in the cold have been proposed to expedite shivering onset time, overruling the hypothesis of a change in central thermo-effector threshold (i.e. altered afferent thermosensitivity or efferent drive) in cold-hypoxic conditions (Arnold et al., 2021). Interestingly, even though the functioning of the 'thermal sensitivity system' does not seem to be affected by hypoxia, processing speed of cold perception has been proposed to be lowered (Golja et al., 2004; Keramidas et al., 2019; Malanda et al., 2008), predisposing individuals to higher cold-injury risks when combined stressor exposure is present.

Cardiovascular conflicts between adjustments elicited by cold and hypoxia have also been studied in the literature, but overall responses are less understood than those related to thermoregulation. Both stressors induce an increase in blood pressure, which has been proposed to be mediated by neural sympathetic hyperactivity (Li et al., 2009). However, if on one side hypoxia causes profound peripheral chemoreceptor-mediated sympathetic hyperactivity, with an elevation in heart rate and cardiac output, but reduced total peripheral resistances, whole body cooling elicits BP increases associated to overall peripheral vasoconstriction, decreased HR but slightly increased CO (Mugele et al., 2021). Robinson and colleagues (Robinson & Haymes, 1990) proposed that increased CO from hypoxia combined with increased peripheral resistances from cold-induced cutaneous vasoconstriction may lead to increased cardiac work and thus energy requirement in cold-hypoxic exposure. Furthermore, decreased CaO2 in hypoxia, combined to decreased local muscle oxygen availability assessed by near infrared spectroscopy in the cold (Horiuchi, Handa-Kirihara, et al., 2019), suggested that insufficient oxygen delivery to active muscles is the cause of the demonstrated additive effect of cold and hypoxia in further decreasing economical speed while walking in the presence of combined stressors if compared to single stressor alone (Horiuchi, Handa-Kirihara, et al., 2019). Also, cold-hypoxic interaction in maintaining blood flow in specific districts of the body (i.e. cerebral circulation) even when exercise is performed and most of the blood needs to be directed to exercising muscles is still unclear (Mugele et al., 2021). For a more in-depth look at possible cold-hypoxic interaction effects on ANS responses, refer to Table 3.

Finally, some authors have also tried to understand central and peripheral fatigue responses in combined cold-hypoxic exposure: in fact, as extensively explained above, cold exposure can reduce a muscle's mechanical response to a given electrophysiological excitation or descending voluntary drive (Lloyd et al., 2015) due to the reduced contractile function of a cooled muscle; this aspect, combined to increased co-activation of the agonist-antagonist pair, as well as reduced muscle blood flow, reduces aerobic mechanical efficiency and accelerate the onset of fatigue (Lloyd et al., 2015). On its side, hypoxia is well known to increase muscle fatigue during prolonged exercise at the same normoxic absolute workload due to a shift of the relative exercise intensity, higher muscle fibre recruitment, and thereby increased intramuscular metabolic disturbance (G. Y. Millet et al., 2009). Based on these assumptions, Lloyd and colleagues hypothesized a synergistic effect of cold and hypoxia on fatigue development in both finger flexors and leg extensors (Lloyd et al., 2015; Lloyd & Havenith, 2016). However, they finally determined only additive or partial additive effect of the two environments on fatigue, proposing that cold and hypoxia may influence this aspect through sufficiently independent cellular mechanisms that do not lead to synergistic effects. Also, these authors hypothesized the presence of a maximum threshold for performance deterioration that does not allow synergistic effects to happen.

Overall, it is clear that some insights into cold-hypoxic physiological responses have recently tried to be made in literature, but still conflictual results are present, with some topic being still completely unknown (i.e. whole body exercise performance and submaximal vs maximal physiological responses). This consideration is what drove my interest in studying the combination of cold and hypoxia from a new perspective.

Table 3 Possible Cold-Hypoxic interaction effects on ANS responses.

	MACROCIRCULATION		MICROCIRCULATION				VENTILATORY RESPONSES	THERMOREGULATORY RESPONSES
	CO	BP	NON-ACRAL SKIN	MUSCLE	LUNG	CBF		
COLD	↑↔ due to: **↑↔SV ↔ HR,** parasympathetically mediated	↑ due to: **↑↑ TPR,** sympathetically mediated (α1 e α2 receptors)	↓↓↓ , sympathetically mediated (α1 e α2 receptors)+ increased sensitivity to NE+ inhibition NO signal	**↓↔,** sympathetically mediated (α receptors), insulation effect, **but ↑ if shivering is present**	↓, locally mediated by inflammatory factors	↑, sympathetically mediated (α2 receptors)	**↔ or ↑when shivering** is present for ↑ metabolic demands	↓↔ core temp; ↓skin temp
HYPOXIA	↑ due to: **↑ HR but ↔SV,** sympathetically(B2 receptors) + cardiac vagal withdrawal from high Ve and pulmonary stretch receptors activation	↓ immediately**, but than** ↑ due to: **↑↑ HR,** (see left)**, higher than ↓↓TPR**	**↑ in thermoneutral conditions or short exp,** NO-dependent mechanism, **but ↓ in prolonged exp** due to centrally mediated sympathoexitation	**↑,** sympathetically mediated (B-adrenergic blockade) + NO release + attenuated NE sensitivity	**↓↓,** Hypoxic pulmonary vasoconstriction, locally mediated (ROS)	**↑,** sympathetically mediated, directly regulated by local CaO2 content	**↑↑,** chemoreceptor stimulation of sympathetic nervous system	**↑↔** shivering and vasoconstriction threshold?
NET EFFECT	**?**	**↔**	**↑ RELATIVE ADDITIVE**	**↑ RELATIVE ADDITIVE**	**ADDITIVE?**	**? CEILING EFFECT?**	**↑ RELATIVE ADDITIVE**	**↑ RELATIVE ADDITIVE**

CO, Cardiac Output; SV, Stroke Volume; HR, Heart Rate; TPR, Total Peripheral Resistances; CBF, Cerebral Blood Flow; NO, Nitric Oxide; CaO2, arterial concentration of Oxygen; NE , Norepinephrine; Exp, cold exposure. Responses during cold or hypoxic stimuli are in comparison with thermoneutral normoxic, with increased (↑), decreased (↓), or no change (↔). Same principle is applied to the net Cold-hypoxic effect, but an unknown (?) response is added when interaction effect is difficult to predict. Partially taken from (Wait et al., 2023).

SEX DIFFERENCES IN HIGH ALTITUDE EXERCISE PHYSIOLOGY

3.1 THE SEX DATA GAP IN SPORT SCIENCE RESEARCH

Despite the decreasing sex gap in sport and exercise participation, females are significantly underrepresented within sport and exercise science research. Cowley and colleagues (Cowley et al., 2021) demonstrated that between 2014 and 2020, female accounted only for 34% of total participants within articles published on six between the most famous sport and exercise science journals, and only 6% of total publications were conducted exclusively on females. As if that weren't enough, when studies have included both sexes, only one third have factored sex into their analysis (Ansdell et al., 2020), whereas the others simply tested women in the low hormone phase of the cycle (i.e. follicular phase or hormone-free week of oral contraceptive (OC) users), assuming a physiological profile similar to that of men. This perpetuates the assumption that physiological responses of women are the same as those of men, but the fact is that 'Women are not small men' (Sims & Heather, 2018), as we will describe in the following paragraphs.

The reason for this sex disparity within scientific literature resides in the complexities associated with the menstrual cycle, often cited as being too difficult to study (Sims & Heather, 2018). Across each cycle (lasting 28-32 days), oestrogen and progesterone levels fluctuates determining 3 main phases: a follicular phase (∼12–14 days; low levels of oestrogens and progesterone), ovulation (∼1 day, preceded by an oestrogen surge) and a luteal phase (∼12–14 days; high levels of oestrogens and progesterone) (Fig.12). These hormones can have different target organs and promote different physiological end points, which can actually affect exercising responses and adaptations, as well as athletic performance (Carmichael et al., 2021). Thus, to ensure accurate detection of correct responses, study designs involving women must meticulously consider the menstrual cycle phase of participants (Schmalenberger et al., 2021): this entails careful planning to avoid amalgamating results obtained from subjects with different hormonal profiles, as well as distinguishing between normally menstruating individuals and those using oral contraceptives is essential. However, I clearly understand the difficulties

related to previous affirmations, specifically when complex study designs are present: recent scientific literature helps practitioners to deal with these problems (Schmalenberger et al., 2021; Su et al., 2017; Wideman et al., 2013), but also common sense has to be used, especially understanding the magnitude impact of the menstrual cycle on the investigated variable if compared, for example, to the intervention applied during the study.

Despite all these considerations, up-to-date literature research highlights several major sex differences within physiological systems, which will be presented in the following paragraph.

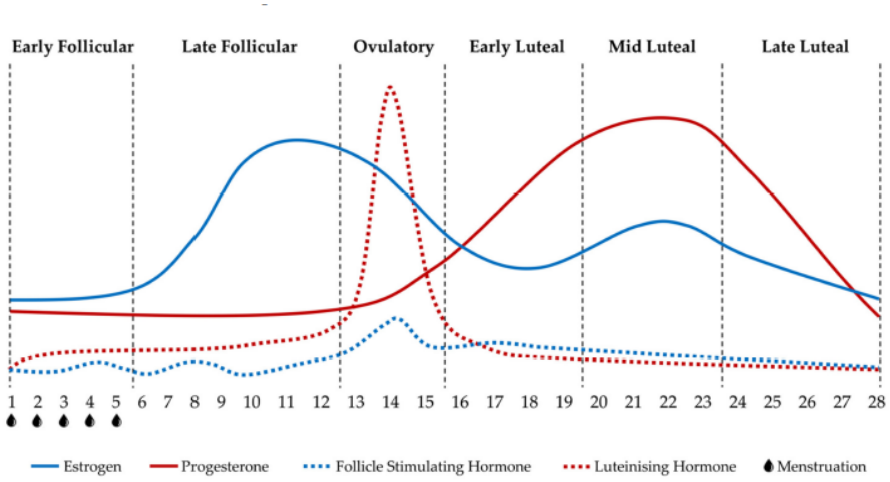

Figure 1. Hormonal events and phases in a eumenorrheic 28-day menstrual cycle. Adapted from McNulty et al. [5] and Farage et al. [6].

Figure 12 Taken from Carmicael et al. 2021; Hormonal events and consequent menstrual cycle phases.

3.2 EFFECT OF BIOLOGICAL SEX ON THE OXYGEN CASCADE: NORMOXIC AND HYPOXIC CONSIDERATIONS

A description of overall sex differences within physiological systems involved in determining exercise responses is far beyond the scope of this work. However, a brief overview of the up to date literature on the most important processes belonging to the oxygen cascade pathway that might be affected by biological sex is important to understand the scientific basis that led me to the development of study 3 presented thereafter. The pathway for O2 from the atmosphere to the mitochondria can be subdivided into a series of step that, if not properly working, may represent

a potential limitation to O2 flux (David R. Bassett & Howley, 2000; Paolo B. Dominelli, Ph.D1, Chad C. Wiggins, Ph.D2, Tuhin K. Roy, Ph.D, Ph.D, M.D.2, Timothy W. Secomb, Ph.D3, 4, Timothy B. Curry, Ph.D, M.D.2, 2021): respiratory aspects (minute ventilation, O2 diffusion capacity), oxygen carrying capacity (maximal Cardiac Output (CO) and circulatory O2 delivery), and skeletal muscles properties (muscle O2 diffusion, utilization and ATP turnover capacity) (Fig 12). Notably, high altitude exposure and consequent reduced oxygen availability affect several aspects of the oxygen cascade, possibly leading to oxygen delivery limitations otherwise not present in normoxic conditions.

A brief overview of possible sex differences influencing the Oxygen cascade pathway under hypoxic conditions is presented in figure 13.

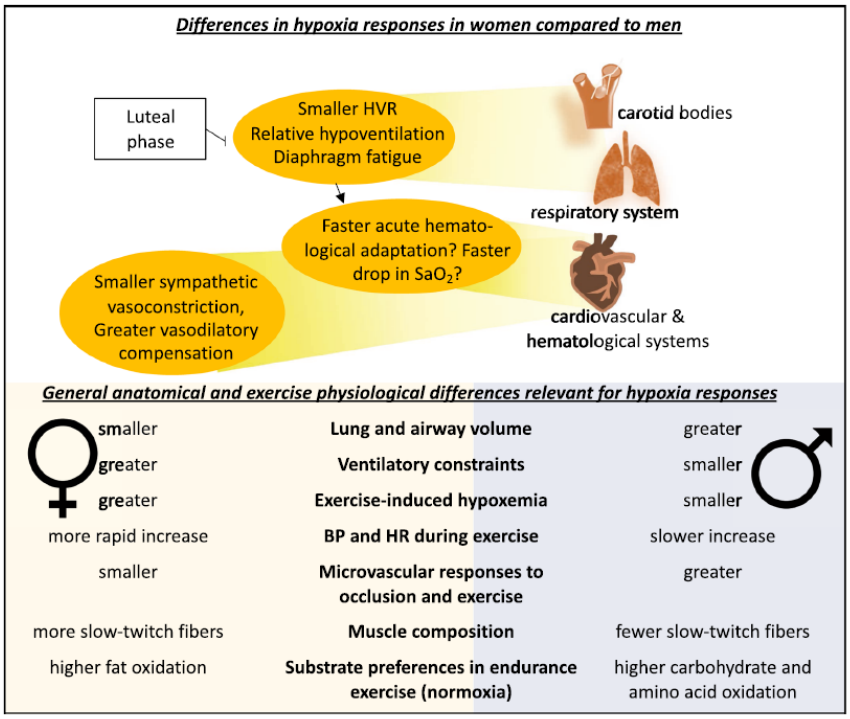

Fig. 3 Mechanisms of sex-related differences in response to hypoxia. *HVR* hypoxic ventilatory response, SaO_2 oxygen saturation, *BP* blood pressure, *HR* heart rate. References: Lung and airway volume [37, 38, 44], Ventilatory constraints [22, 23, 33], Exercise-induced hypoxemia [27, 28, 33], BP and HR during exercise [57–59], Microvascular responses to occlusion and exercise [122, 127], Muscle composition [130], Substrate preferences in endurance exercise [129]

Figure 13 Taken from Raberin et al., 2023; Mechanisms of sex-related differences in response to hypoxia.

3.2.1 RESPIRATORY SYSTEM

Females have smaller lungs and airway than men, even when matched for standing height (Dominelli et al., 2013). This causes greater work of breathing (WOB) for a given minute ventilation (Ve) if compared to men (Dominelli et al., 2019; Santisteban et al., 2022), accompanied also by greater absolute VO2 of respiratory muscles at elevated Ve (>55 L/min) (Dominelli, Render, et al., 2015). In fact, 14% of whole-body $VO2_{max}$ at maximal exercise intensity, as well as a higher portion of maximal Cardiac Output (CO), is dedicated to respiratory muscles in women, against only 9% measured in (Dominelli, Render, et al., 2015). However, in their favor, it seems that women exhibit greater resistance to diaphragm fatigue (Archiza et al., 2021; Geary et al., 2019), this being linked to both increased fatigue resistance intrinsic properties of skeletal muscles in female subjects, as well as amplified recruitment of accessory respiratory muscles other than diaphragm to sustain required minute ventilation (Ansdell et al., 2020; Archiza et al., 2021; Dominelli et al., 2019).

Interestingly, even when matched for lung size, females present smaller cross-sectional areas of the trachea and large-conducting airways, a phenomenon that is known as respiratory dysanapsis (A. William Sheel et al., 2016). Some authors claim that this anatomical feature causes women to be more likely to reach their maximum capacity to generate expired flow during exercise, a concept known as Expiratory Flow Limitations (EFL) (Dominelli et al., 2019). Supporting the theory of greater flow limitations in women rather than a change in the mechanical properties of the lungs is also the fact that only the resistive component of the WOB is higher in females subjects for Ve>60 L/min, whereas no differences in the elastic components have been found between sexes (Dominelli et al., 2019). However, when larger sample sizes have been considered, there is not a clear sex-based difference in the prevalence of expiratory flow limitations during heavy exercise in healthy young (< 45 years) adults (Archiza et al., 2021); thus, further studies on this aspect are necessary.

These anatomical differences may also indicate that women experience greater limitations in diffusion capacity for oxygen from the alveoli to arterial blood

(Santisteban et al., 2022): in fact, diffusion is strictly related to available surface area for gas exchange to happen (lung size), as well as number of capillaries perfusing the alveoli for O2 transfer from lungs to the blood: Bouwsema and colleagues (Bouwsema et al., 2017) found that women did exhibit consistently lower membrane diffusing capacity and capillary blood volume if compared to height-matched men, but these sex differences were eliminated when the alveolar volume was accounted for. These findings are confirmed also by Olfert et al. (Olfert et al., 2004), who described no differences in O2 arterial partial pressure of Oxygen (PaO2) neither in normoxic or hypoxic conditions between men and women, as well as no differences in ventilation to perfusion (V/Q) mismatch or diffusion limitation when comparing height, age, fitness, and lung volume matched men and women.

Highlighted sex differences in previous mentioned respiratory properties may become preponderant at High Altitude: in fact, the ventilatory response to exercise emerges as one of the most sex-sensitive factors that may modify reactions to hypoxia between men and women (Raberin et al., 2023).

First of all, in chapter 1.3.1, we highlighted the importance of hypoxic ventilatory response (HVR) as an acute physiological adaptation to HA in order to counteract reduced Oxygen availability. A recent study on a large cohort reported a blunted HVR in women even after correction for body surface area (Goldberg et al., 2017), with this relative hypoventilation being explained mainly as an interfering effect of female sex hormones, whose receptors are located in the carotid body, which contains the primary chemoreceptors monitoring blood oxygen levels. Apart from reduced chemosensitivity, hypoventilation may also be due to mechanical constraints such as expiratory flow limitations.

Regarding diffusion properties, only one study has been conducted specifically accounting for sex differences on this aspect in hypoxic conditions (Olfert et al., 2004): the authors showed that women did not display higher limitations neither in normoxia or hypoxia, but further investigations are warranted especially considering hypoxic induced pulmonary vasoconstriction and the attenuated pulmonary artery vasoreactivity in hypoxia when circulating oestrogen level is elevated (Raberin et al., 2023). Interestingly, a recent work by Horiuchi and

colleagues (Horiuchi, Kirihara, et al., 2019) determined that the relative contribution of ventilatory aspects on SpO2 decrease from normoxia to hypoxia was significantly higher in women (4.1%) if compared to men (1.7%), suggesting that these results may be partially related to differences in pulmonary O2 diffusion capacity, that influences SpO2 reductions while exercising.

3.2.2 EXERCISE INDUCED HYPOXEMIA (EIH) AND HYPOXIC RESPONSES: THE ROLE OF SEX

Overall, it is still not clear whether sex differences in respiratory components may have an influence on the incidence of exercise-induced hypoxemia (EIH) at sea level (Dominelli & Sheel, 2019). The possible physiological mechanisms beyond EIH development have been recognized as i) relative hypoventilation, ii) diffusion limitations, iii) ventilation-perfusion (VA/Q) mismatch, and iiii) intrapulmonary arterial-venous anastomosis (IPAVA), or intra-cardiac right-to-left shunts (Dominelli & Sheel, 2019), all aspects that may be influenced by previously mentioned anatomical differences in the respiratory system between sexes. As mentioned in Chapter 1.2, women have been shown to exhibit EIH at lower relative intensities compared to men (Santisteban et al., 2022). This phenomenon appears to be related to relative alveolar hypoventilation, indicated by lower PAO2 levels, as there is minimal change in the alveolar-arterial oxygen difference (AaDO2) during submaximal exercise intensities (Dominelli and Sheel). Moreover, also moderately trained women seem to be affected by this phenomena, whereas EIAH has been shown only in highly trained men at near-maximal exercising intensities (Archiza et al., 2021). It has been suggested that in some untrained women, the presence of mechanical ventilatory constraints (Dominelli & Sheel, 2019), but also differences with respect to pulmonary diffusing capacity during exercise (Bouwsema et al., 2017; Smith et al., 2015), especially when diffusion is expressed relative to cardiac output**,** results in the development of EIH. These aspects would also explain the fact that at a given $VO2_{max}$, women have, on average, a lower PaO2 if compared to aerobic fitness-matched men.

Given that individuals presenting EIH at sea level seem to manifest a larger drop in $VO2_{max}$ in acute hypoxia, recent scientific literature tried to clarify the relationship between these two phenomena in female subjects. Interestingly, Harms and

colleagues (Harms et al., 1998) suggest that within females acutely exposed to hypoxia, for every 1% reduction in SaO2 below resting levels, $VO2_{max}$ reduction is between two and four times greater than in men. However, Chaoman et al. (Robert F. Chapman et al., 2011) did not find any sex difference within national-class endurance athletes performing a 3000-m race at 2100m simulated altitude. A very recent study by Rberin et al. (Raberin et al., 2024) found greater EIH presence in women if compared to men, independently of fitness level. Interestingly, these women presented lower EFL prevalence and similar chemoreceptor sensitivity of their male counterparts. Moreover, the relationship between the severity of EIH and the normoxia-to-hypoxia drop in $VO2_{max}$ was found in men but not in women, suggesting that the responses to acute hypoxia in women with EIH are different from those in men. Finally, when considering trained women ($VO2_{max}$: 54 ml/kg/min) and untrained men (54 ml/kg/min), normoxia-to-hypoxia drop was higher in the latter group (-11 vs -16%, respectively).

Altogether, these findings underline that i) EFL prevalence is not sex-dependent but it arises from an imbalance between ventilatory demands and capacity, ii) the relationship between EIH and $VO2_{max}$ drop in hypoxia is not so evident in women, despite EIH prevalence is higher in females independently of fitness level and iii) further studies are needed to better elucidate women respiratory and performance responses to exercise at high-altitude.

3.2.3 O2 CARRYING CAPACITY

Cardiac and haemodynamic responses present well known differences between sexes, even though often when parameters are normalized for body surface area, these differences are reduced or even completely nullified (Ansdell et al., 2020). As an example, women at rest present similar or higher heart rate (HR) and lower absolute stroke volume (SV) and cardiac output (CO), but higher total peripheral resistances (TPR) compared with men, but when body surface area is considered, differences in SV and TPR seem to disappear. Also, men show higher systolic and diastolic pressure than women, at least before menopause occurs. Furthermore, divergent cardiovascular responses to dynamic exercise have been shown, even when men and women are matched for body size. In fact, it has been suggested that

females rely more on HR to meet the metabolic requests during exercise, whereas men rely more on preload and enhanced use of the Frank–Starling mechanism to increase CO (Higginbotham et al., 1984). As a consequence, during high-intensity exercise, women reach lower maximal stroke volumes compared to men, resulting in a lower maximal cardiac output as maximal heart rate is similar across sexes (Santisteban et al., 2022). As previously described, it is known that exercise at altitude (acute exposure) increases HR but not SV for the same exercise performed at sea level, but the literature is still scarce concerning HR, SV, and CO responses to hypoxic exercise in females versus males.

Oxygen transportation depends also from total haemoglobin mass, and women are reported to have mean haemoglobin levels of 12% lower than age and race matched men (Santisteban et al., 2022). If coupling lower Oxygen content for the same quantity of blood flow and overall lower blood volume relative to body size, it is easy to understand that women may be disadvantaged when oxygen availability is reduced at HA. However, recent works suggest that premenopausal women present lower sympathetic vasoconstrictor activity during hypoxic exercise compared to men, and therefore a greater compensatory vasodilation at peripheral level that may counteract for impaired convective properties (Raberin et al., 2023). Furthermore, despite overall reduce Hb mass in women, most of the results do not show any large impact of sex on erythropoietic adaptation, neither in acute or chronic terms (Raberin et al., 2023).

3.2.4 MUSCLE PROPERTIES

Males do not only possess a greater quantity of skeletal muscle than females, but considerable differences in the morphological composition of these muscles exist, with women showing greater proportional area of type I muscle fibers (Ansdell et al., 2020). From a metabolic perspective, this is linked to higher fat and less carbohydrate oxidation in women if compared to men (Tarnopolsky, 2008), with a consequent higher ATP resynthesis from oxidative phosphorylation during exercise. Furthermore, women present greater vasodilatory responses (Parker et al.,

2007) and higher density of capillaries per unit of skeletal muscle, this probably promoting greater muscle perfusion during exercise (Ansdell et al., 2020).

In hypoxia, a decreased reliance on free fatty acids and an increase in glucose dependence is well known, but still females tend to shift toward greater fat use and males toward greater carbohydrate use during hypoxic exercise (Sandoval & Matt, 2002).

3.3 SEX DIFFERENCES, $VO2_{max}$ AND PERFORMANCE: WHAT CONSEQUENCES AT HIGH ALTITUDE?

The overall interaction of aforementioned systems working to enhance the transport of oxygen from air to muscle for a given exercise intensity is what defines performance fatiguability (Amann & Calbet, 2008). Considering the fact that whole-body exercising performance depends both on oxygen transport (convective factors) and delivery (diffusive factors), female present reduced oxygen availability at muscular level due to smaller lung volumes, lower haemoglobin concentrations and overall disadvantages in O2 convective properties, but greater metabolic properties of the skeletal muscles and consequently enhanced diffusive capacities when compared to men counterparts. Despite this, male subjects still present a 10-12% advantage in all whole body exercise distance events, as well as even truly elite women have $VO2_{max}$ values ~10% lower than those seen in men of similar elite status when expressed as mL/kg/min (Santisteban et al., 2022). The explanation to this phenomena resides in central hemodynamic factors being commonly considered the actual limitation of Oxygen delivery and consequently $VO2_{max}$ (Santisteban et al., 2022). In fact, sex performance gap has been shown to disappear when completing tasks that are less dependent on the cardiopulmonary system and more related to diffusive properties at muscular level (i.e. single leg endurance exercise) (Ansdell et al., 2020; Santisteban et al., 2022).

These differences in convective and diffusive components of $VO2_{max}$ are responsible also for $VO2_{max}$ reductions in hypoxia: as accurately described in figure 14 by Raberin and colleagues (Raberin et al., 2023), female individuals seem more centrally but less peripherally limited than men when exercising in hypoxia, but

being the relative importance of the two components imbalanced in determining $VO2_{max}$ values during whole body exercise, the decrease in this parameter is expected to be higher in females subjects. Interestingly, as for normoxic conditions, Shepard and colleagues (SHEPHARD et al., 1988) demonstrated that the differences between males and females in maximal oxygen consumption in hypoxia is reduced when the volume of active muscles is reduced and peripheral limitations become predominant in determining endurance performance.

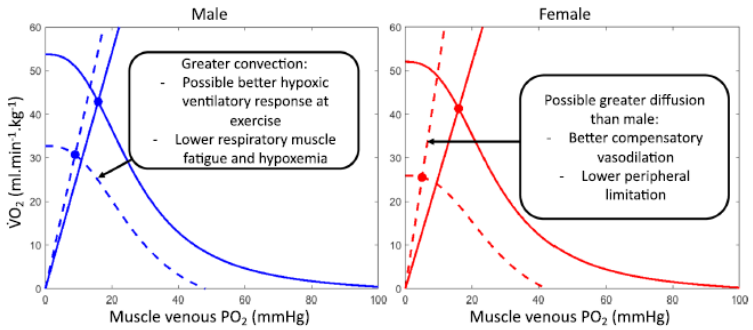

Fig. 1 Schematic representation of altitude-related changes in convective (calculated from $\dot{V}O_2$ = cardiac output × difference in arteriovenous O_2 content, sigmoid line, [236]) and diffusive (calculated from $\dot{V}O_2$ = diffusion coefficient × mixed venous O_2 pressure, straight line, [236]) components of VO_{2max} in one male and female individual matched for sea-level VO_{2max}. The full line is in normoxia, the dotted line is in hypoxia (5260 m). The individual datasets are from [237]. Cardiac output was computed with pulse wave contouring analysis. The convective component was reduced to a larger extent in female individual due to a higher altitude-induced hypoxemia and lower hemoglobin concentration, while no clear differences in cardiac hemodynamic responses were noted. Due to a higher compensatory vasodilation and lower sympathetic vasoconstrictor activity, the diffusive component of VO_{2max} was improved in female individuals in hypoxia. Therefore, female individuals seem more centrally but less peripherally limited than men when exercising in hypoxia. $\dot{V}O_2$ oxygen uptake, PO_2 oxygen pressure

Figure 14 Taken from Raberin et al., 2023; Representation of altitude-related changes in convective and diffusive components of $VO2_{max}$ in one male and female matched for sea-level $VO2_{max}$.

3.4 SEX-RELATED RISKS TO HIGH ALTITUDE EXPOSURE: AMS PREVALENCE

Considering the increasing number of female subjects involved in HA activities not only for performance purposes but also for recreational interests, it is important to understand whether sex-related risks are present when oxygen availability is reduced in order to implement specific acclimatization strategies that may overcome these problems. Acute mountain sickness is the mildest but also most common pathological representation at HA, presenting itself with symptoms like

dizziness, headache, nausea and vomiting, loss of appetite and shortness of breath with physical exertion. A recent meta-analysis performed by Hou and colleagues (Hou et al., 2019) demonstrated that the prevalence of AMS is 1.24 times higher in women than in men, regardless of race or age. On the contrary (Derstine et al., 2023), reported similar incidence of AMS in men and women. However, possible mechanisms explaining this phenomenon remain largely unexplored (J. Burtscher et al., 2023). Some authors proposed that women may present higher intracranial hypertension when exposed to HA due both increased vascular permeability (oestrogen-related upregulation of vascular endothelial growth factor) and fluid retention (oestrogen-related effect on anti-diuretic hormone), this hypertension leading to higher headache and AMS related symptoms (Hou et al., 2019). Moreover, greater AMS development in women could also be related to an initial more severe systemic hypoxemia due to the previously mentioned higher cardiac and ventilatory constraints in response to a hypoxic stimulus (J. Burtscher et al., 2023). Several studies (M. Burtscher et al., 2008; Camacho-Cardenosa et al., 2022; Richalet et al., 2012) reported a relationship between oxygen desaturation within 20 and 30 mins of rest or while performing an exercise bout after being acutely exposed to hypoxia. Interestingly, one of the most plausible causes of higher acute reductions in SaO2 is a blunted HVR, but the relationship between HVR and AMS development is quite debated (Camacho-Cardenosa et al., 2022; Richalet et al., 2012). In fact, the biphasic pattern of the ventilatory response to hypoxia is not given adequate consideration when hypoxic testing is done only for a few minutes as during HVR determination in some studies (just 5-10 mins of recording after acute exposure) (M. Burtscher et al., 2008): this is because after a marked increase in minute ventilation during the first minutes of hypoxia, there is a decrease in ventilation, which is mainly due to the decrease of the central chemoreceptor drive (Ainslie et al., 2013). However, the contribution of the central and the peripheral chemoreflexes may vary between subjects, and thus, also the ventilatory decline after 20–30 minutes of exposure may differ between individuals and may not be in agreement with acute ventilatory response. As previously mentioned (chapter 3.2.1), an interfering effect of female sex hormones, whose receptors are located in the carotid body, could cause an inadequate ventilatory response to hypoxia, leading

to a higher decrease in blood SaO2, and thus causing cerebral blood flow to increase in order to guarantee oxygen requests: this adjustment comes with the risk of excessively increasing intracranial pressure, leading to increased AMS related symptoms in women.

If not properly treated, AMS may worsen in life-threatening conditions such as High Altitude cerebral or pulmonary oedema: thus, understanding the sex-related mechanisms involved in AMS development may be useful in preventing its manifestation, as well as developing the best strategies for acclimatization for both men and women.

GENERAL AIM AND OVERVIEW OF THE THESIS

The general aim of this doctoral thesis was to enhance our comprehension of acute physiological responses to exercise under conditions of reduced oxygen availability, with a specific focus on two aspects that are often underrepresented in sports science research: exposure to combined cold and hypoxic stressors, and the exploration of sex differences in exercise physiology under hypoxic conditions. Advancing our understanding of these dimensions is crucial for effectively addressing the challenges associated with real-world high-altitude exposure. In fact, such exposure is frequently characterized by the simultaneous presence of various stressors (Tipton, 2012), which may impact physiological responses to exercise differently when experienced individually or in combination (Lloyd & Havenith, 2016).

Additionally, delving into the realm of sex differences in physiological responses to exercise under diverse environmental conditions aims to challenge the notion that 'women are just small men' (Sims & Heather, 2018). This underscores the importance of developing tailored interventions to ensure the safest high-altitude exposure for both sexes, with a focus on optimizing success rates for both performance and recreational purposes.

To achieve these objectives, this doctoral thesis presents and discusses findings from two distinct data collections and presented as three different studies. The first focuses exclusively on trained male subjects exercising in a cold-hypoxic environment, while the second involves both healthy trained males and females engaging in exercise under normobaric hypoxic conditions. In all projects, particular emphasis is placed on evaluating physiological responses to exercise, as they offer valuable insights into the impact of combined stressors exposure and biological sex on whole-body homeostasis during high-altitude exercise.

Furthermore, special attention is given to respiratory responses throughout and after exercise, as they appear to play a pivotal role in determining exercise limitations, especially when considering cold exposure (Kennedy et al., 2019a) or female subjects (Ansdell et al., 2020; Raberin et al., 2023).

More specifically, Study 1 entitled **'*Independent, additive and interactive effects of acute normobaric hypoxia and cold on submaximal and maximal endurance exercise.'*** aims at understanding maximal, submaximal and lactate threshold responses when exposed to cold and hypoxic conditions, both independently and combined, in a male cohort.

Study 2, derived from the same data collection of study 1 and entitled *'**Freezing hypoxia and exercise: impact of concurrent stressors on ventilatory responses, lung function, and respiratory muscle work.**'* investigates cold-hypoxic exposure effect on post-exercise lung function and respiratory muscles fatigue, as well as the relationship between these outcomes and ventilatory responses during exercise.

Finally, study 3, entitled ***'Sex differences in cardiac and respiratory responses during exercise under hypoxic conditions.'*** compared ventilatory responses, diffusion capacity of the lungs and circulatory aspects while exercising at SL and HA in trained men and women.

STUDY 1 - INDEPENDENT, ADDITIVE AND INTERACTIVE EFFECTS OF ACUTE NORMOBARIC HYPOXIA AND COLD ON SUBMAXIMAL AND MAXIMAL ENDURANCE EXERCISE.

ABSTRACT

Purpose: To evaluate the independent and combined effects of hypoxia (FiO2=13.5%) and cold (-20°C) on physiological and perceptual responses to endurance exercise.

Methods: 14 trained male subjects ($\dot{V}O_{2max}$: 64±5mL/kg/min) randomly performed a discontinuous maximal incremental test to exhaustion on a motorized treadmill under four environmental conditions: Normothermic-Normoxia (N), Normothermic-Hypoxia (H), Cold-Normoxia (C) and Cold-Hypoxia (CH). Performance and physiological and perceptual responses throughout exercise were evaluated.

Results: Maximal WorkLoad (WL) and WL at lactate threshold (LT) were reduced in C (-2.3% and -3.5%) and H (-18.0% and -21.7%) compared to N, with no interactive (p=0.25 and 0.81) but additive effect in CH (-21.5% and -24.6%). Similarly, HR_{max} and Ve_{max} were reduced in C (-3.2% and -14.6%) and H (-5.0% and -7%), showing additive effects in CH (-7.7% and -16.6%). At LT, additive effect of C (-2.8%) and H (-3.8%) on HR reduction in CH (-5.7%) was maintained, whereas an interactive effect (p=0.007) of the two stressors combined was noted on Ve (C:-3.1%, H:+5.5%, CH:-10.9%). [La] curve shifted on the left in CH, displaying an interaction effect between the 2 stressors on this parameter. Finally, RPE at LT was exclusively reduced by hypoxia (p<0.001), whereas TS_{max} is synergistically reduced by cold and hypoxia (interaction p=0.047).

Conclusion: If compared to single stress exposure, exercise performance and physiological and perceptual variables undergo additive or synergistic effects when cold and hypoxia are combined. These results provide new insight into human physiological responses to extreme environments.

INTRODUCTION

Real-world extreme environments often combine multiple environmental stressors, thereby making their overall effects on the individuals less predictable. High altitude is characterized by this 'extreme' nature, as it often displays the simultaneous presence of numerous stressful factors (e.g. hypobaric hypoxia and cold) (Lloyd & Havenith, 2016). However, despite the independent effect of hypoxia (Fulco et al., 1998; R. S.Mazzeo, 2006) and cold (Castellani & Tipton, 2016; Oksa et al., 2004; Stensrud et al., 2007; Taylor et al., 2008) has been well studied in literature, scarce knowledge is present on the combined effect of the two environmental conditions on human physiology and performance, especially when considering endurance exercise (Bortolan et al., 2021). In fact, currently only 2 studies (Lloyd et al., 2015, 2016) have examined the individual and combined effects of cold and hypoxia on performance at altitude, but none of them investigated physiological and mechanical work responses during whole body dynamic endurance exercises like cycling or running. To date research on single stress exposure suggests that many of the key physiological strains associated with thermal cold and hypoxia are precursors of detrimental effects on exercise capacity; it is well known that, as altitude increases, the systemic reduction in arterial O_2 content strains the cardiovascular system's ability to meet the required O_2 delivery to active musculature (Amann et al., 2006; Fulco et al., 1998), causing a linear decrease in maximal oxygen uptake ($\dot{V}O_{2max}$) corresponding to ≈6.3% per 1000 m increasing altitude in endurance trained athletes up to 3000 m (Wehrlin & Hallén, 2006). However, despite great differences in relative exercise intensity, submaximal oxygen uptake at a specific external workload is similar at sea level and altitude (Fulco et al., 1998; Wehrlin & Hallén, 2006). Higher controversy exists on $\dot{V}O_{2max}$ changes in the cold: Oksa et al. (2004) and Quirion et al. (1989) reported a 5% decrease in $\dot{V}O_{2max}$ at -20°C if compared to +20°C, whereas Renberg et al. (2014) and Sandsund et al. (2012) claim no changes in ambient temperatures between -14 and +20, and Therminaris et al. (1989) found a 13% increase in $\dot{V}O_{2peak}$ at -2°C if compared to +24°C. These results suggest that $\dot{V}O_{2max}$ values may be affected in the cold for ambient temperatures lower than -15°C, and the proposed

reason for this decrease are the cold-induced local vasoconstriction that reduces venous washout of metabolic by-products in the active muscles (Oksa et al., 2004; Quirion et al., 1989), reduced ventilation due to cold-induced bronchus constriction (Kennedy et al., 2019b) or cooling-induced neuromuscular changes like decreased maximal force production or slower nerve conduction and muscle contraction velocity (Oksa, 2002). More agreement exists in relation to higher $\dot{V}O_2$ at submaximal exercise intensities in the cold (Oksa et al., 2004; Quirion et al., 1989; Therminarias, 1992; Therminarias et al., 1989) due to both a reduction in the mechanical efficiency of working muscles and to the shivering produced by muscles not involved in muscular exercise(Oksa, 2002; Therminarias, 1992).

As $\dot{V}O_{2max}$, also aerobic performance is consequently affected by environmental condition. The state of art regarding maximal incremental test in hypoxia shows a 10 to 13% decrease in peak power output (PPO) or maximal aerobic velocity (VAM) at altitudes between 2500 and 3500m if compared to sea level (Faulhaber et al., 2021; Friedmann et al., 2005; Friedmann et al., 2004; Lorenz et al., 2006; Ofner et al., 2014; Weckbach et al., 2019), and the same happens at the intensities associated with the lactate thresholds, with a reduction ranging from 12 to 19% considering different detecting methods (Faulhaber et al., 2021; Weckbach et al., 2019). Similarly, Quirion et al.(1989) found a 22% reduction in maximal WorkLoad (WL) and Oksa et al.(2004) a 9% decrease in running performance time when exposed to -20°C if compared to +20°C. Concerning WL at Lactate Threshold (LT), the same distinction between moderate (>-15°C) and severe (<-15°C) cold previously mentioned for $\dot{V}O_{2max}$ should be considered: in fact, Morrissey et al. (2019) found a 22% higher WL and Sandsund et al. (2012) a 10% increase in running speed at LT within -4 and 1°C if compared to 20°C (suggesting this as the optimal ambient temperature range for aerobic endurance performance), whereas Renberg and colleagues (2014) found no differences in PO at -14°C if compared to +20°C in women. However, no information on mechanical work variation at LT when exposed to severe cold (i.e. -20°C) is available.

Both $\dot{V}O_{2max}$ and consequent aerobic performance reductions are linked to environmental induced changes in physiological responses to exercise, although the magnitude and mechanism of action for these changes are in many cases still

unclear. For the purposes of this study, only responses related to acute environmental stressor exposure will be considered. HR_{max} has been shown to be reduced (Fornasiero et al., 2018; Grataloup et al., 2007; Mourot, 2018; Ofner et al., 2014) when acutely exposed to hypoxic environments, the magnitude of this reduction being better explained by the altitude gain between normoxic and hypoxic incremental tests rather than by absolute altitude per se (i.e. 1.7 bpm per 1000m gain in altitude (Garvican-Lewis et al., 2015), which corresponds to ≈3/4% reduction in HR_{max} for altitudes of 3500 m asl (Fornasiero et al., 2018; Ofner et al., 2014)). Changes in cardiac electrophysiological properties (Benoit et al., 2003; Mourot, 2018) and a reduced central drive on the heart as a protective mechanism from myocardial ischemia (Noakes et al., 2001) have been addressed as possible mechanisms for HR_{max} reductions with acute hypoxic exposure. At submaximal exercise intensities, for the same external workload, HR in hypoxia is increased in order to meet exercising muscles oxygen requests (Clark et al., 2007). However, when considering workload in relative terms, HR in normoxia and hypoxia is similar (Ofner et al., 2014): this may explain why, despite absolute HR at lactate threshold seems to be reduced in hypoxia, when it is expressed as a percentage of maximal values in the respective conditions it shows no differences from sea level values (Friedmann et al., 2004, 2005). In the cold, HR_{max} reduction has been addressed as primarily responsible for the reduced $\dot{V}O_{2max}$, decreasing from 10 to 30 bpm when deep body temperature is lowered by 0.5 to 2.0◦C (Castellani & Tipton, 2016). Specifically, a percentage decrease ranging from -2.5 to -5.5% has been found for ambient temperatures between -14 and -20°C if compared to +20°C (Oksa et al., 2004; Renberg et al., 2014; Sandsund et al., 2012). Submaximal HR changes in the cold is more controversial, with some studies showing a reduction (Sandsund et al., 2012) and other no changes (Renberg et al., 2014) for ambient temperatures lower than -14°C if compared to thermoneutral conditions. Cold induced peripheral vasoconstriction that results in an elevation of blood pressure, increased central blood volume and higher stroke volume (Doubt, 1991; Gisolfi & Wenger, 1984) seems to be responsible for a parasympathetically mediated reduction in HR (Doubt, 1991; Sandsund et al., 2012; Taylor et al., 2008).

The lactate-power output/velocity curve is left shifted in hypoxia (Clark et al., 2007; Friedmann et al., 2005; Ofner et al., 2014), testifying greater reliance on anaerobic metabolism when comparing a same absolute exercise intensity. However, Ofner et al. (2014) found completely the same pattern of the curve and no significant difference in lactate concentration between normoxia and hypoxia in relative terms (i.e. same lactate concentration per watt in both environments). Furthermore, despite anaerobic threshold concepts are very popular to prescribe intensity zones for endurance training, scientific literature dealing with this topic in hypoxia is scarce, and some authors questioned the validity of these concepts at high attitude (Faulhaber et al., 2021). Lactate production [La] and clearance at rest and during exercise is influenced also by ambient but especially muscle temperatures, and magnitude and direction of this influence depend on the entity of cold (Therminarias, 1992). Blomstrand et al. (1984) and No et al. (2016) suggested that higher levels of [La] are reached when muscle temperatures are low, due to a cold-induced change in muscle fibre recruitment from types 1 to 2 and a consequent greater reliance on anaerobic metabolism in this situation (Blomstrand & Essén-Gustavsson, 1987), along with other factors contributing to fatigue, e.g. low levels of ATP and PCr (phosphocreatine). This would suggest that the net efficiency of exercise in the cold is lower than under normal conditions. However, Renberg et al. (2014) found no differences in blood lactate concentration at LT between -14 and +20°C and Quirion et al. (1989) suggested that the anaerobic threshold corresponding to a lactate concentration of 4 mmol at -20°C is not significantly different compared to the threshold measured at +20°C.

Finally, also minute ventilation (Ve) is affected by acute hypoxic exposure, which results exaggerated compared to normoxia during exercise at a given absolute intensity. This allows arterial O_2 (PaO_2) to increase, despite the fact that the alveolar-to-arterial O_2 pressure difference is increased during exercise (José A.L. Calbet & Lundby, 2009). However, this phenomena may be muffled or reversed when considering normobaric hypoxia (NH) due to greater air viscosity if compared to hypobaric conditions, especially at maximal exercise intensities (≈2.5% decrease in Ve_{max} per 1000m of altitude gain in NH (Treml et al., 2020)). However, Friedmann et al. (2005) showed no differences in Ve at LT and Ofner et al. (2014)

found similar ventilation in relative terms between normoxia and normobaric hypoxia. Also cold seems to have an influence on Ve (Oksa et al., 2004) since ventilating heavily cold air (<-15°C, Kennedy et al., 2020) may induce a bronchus constriction (induced by the contraction of bronchial smooth muscles), diminishing the amount of air that can be ventilated both at maximal and submaximal exercise intensities (Anderson & Daviskas, 2000b).

In the complex situation of combined cold and hypoxic environments, the effect of one stressor on performance, physiological and perceptual adjustments may be subject to change, simply due to the presence of the other independent stressor. Such differential influences can occur in three basic forms: additive, antagonistic, and synergistic (Lloyd & Havenith, 2016), and each term defines a fundamental concept of inter-parameter interactions. Thus, the aim of this study is to provide further information regarding maximal, submaximal and lactate threshold responses when exposed to cold and hypoxic conditions, both independently and combined, taking into account the multifactorial approach proposed by Lloyd et al. (Lloyd & Havenith, 2016). This should be helpful in better understanding the characteristics of interactions as well as their role in the operation of dynamic systems. On the basis of previous research (Lloyd et al., 2015, 2016), it was hypothesized that combined environmental stressor exposure will induce additive rather than synergistic effects on several physiological and perceptual parameters.

MATERIALS AND METHODS

Subjects

Fourteen trained (De Pauw et al., 2013) male subjects volunteered for this study (age: 27.3±3.4 , $\dot{V}O_{2max}$: 64±5.2 mL/min/kg, BMI:22.4±1.7 kg/m^2). All subjects were non-smokers, free of any systemic or chronic illness, and not taking medications. They were asked to refrain from intense physical activity on the day before and from drinking any alcohol and caffeinated beverages the day of the test. Furthermore, a nutrition diary was provided for writing down the meals of the day before and of the actual day of the first visit, in order to replicate as much as possible those meals also previous to the following sessions.

Thirteen subjects completed all experimental sessions, whereas one subject completed 4 out of 5 sessions. All study protocols were approved by the local ethics Committee (University of Verona- Project N. 4105CESC) and conformed to the Declaration of Helsinki. Before data collection, all participants were properly informed about the experimental procedures and gave their written informed consent for the measurements.

Study Design

Each participant visited the laboratory on 5 different occasions (once pre-test and four main tests) within the same time of the day and completed the protocol within a 6-week period. The pre-test defined subjects' $\dot{V}O_{2max}$ and individual Maximal Ascensional Velocity (VAM) through an incremental test to exhaustion on a motorized treadmill (slope: 25%, starting speed 2.0 km/h increased by 0.7 km/h every 3 min). Cardiorespiratory measures were collected continuously with breath-by-breath method using an automated open-circuit gas analysis system (Quark PFT Ergo, Cosmed Srl, Rome, Italy) and HR was recorded continuously during the test by a HR monitor incorporated into the gas analysis system. The results were used to define individual running speed in the exercise protocols for the four main tests. The main tests were randomly performed in an environmental chamber in one of the following conditions: Normothermic Normoxia (N: 18°C, 20.9% FiO2), Normothermic Hypoxia (H; 18°C, 13.5% FiO2), Cold Normoxia (C: -20°C, 20.9% FiO2) and Cold Hypoxia (CH: -20°C, 13.5% FiO2). The hypoxic environment was created through the manipulation of the FiO2 by means of an oxygen dilution system based on the Vacuum Pressure Swing Adsorption principle (B-Cat, Tiel, The Netherlands). FiO2 was set at 13.5% to simulate an altitude ≈3500 m a.s.l. Each session consisted of a 30-min resting period already exposed to the specific environmental condition, followed by a 10-min warm up phase (2 km/h, slope 25%) and a submaximal to maximal test of 4-min intervals at increasing velocities, interspersed by 2 minutes of passive recovery performed in standing position on the treadmill using handrail support. The 30-minute resting period is necessary to ensure that first short-term physiological responses to the hypoxic environment occur (Duffin, 2007), but was repeated within all conditions in order to guarantee subject's blindness to the experimental session. For the submaximal to maximal

test, treadmill inclination was kept constant at 25% (Fornasiero et al., 2018) whereas test's speed started from 30% of individual VAM (measured at pretest) and increased by 10% every interval until exhaustion. During cold conditions, participants wore extreme cold weather technical clothing individually chosen by each subject (including winter sport jacket/sweater, trousers, gloves and hat or band, with the only instruction of not covering the mouth with any scarf or neck warmer; estimated clothing insulation in the cold: 1.50 l_{cl}(clo)), which remained identical for C and CH trials. Moreover, during exercise subjects were allowed to undress and during resting periods to wear additional clothing if they started to feel uncomfortable with their own clothes. Since in real life situations people generally adjust their clothing in order to not feel hot or cold, with this study we aimed at being as much ecological as possible letting the subjects choose their own clothes throughout the test: as a consequence we expect that, if any impact of cold on exercise performance and physiological parameters is present, it might be primarily related to airways limitations rather than to core temperature changes.

Throughout rest, exercise, and recovery phases, beat-to beat heart rate (HR) was continuously recorded using a Polar RS800CX HR monitor (Polar, Kempele, Finland). Pulse oxygen saturation (SpO2) was continuously recorded during exercise by ear pulse oximetry (Nonin Medical, Minneapolis, MN) at a sampling frequency of 1.0 Hz. Due to the extreme cold conditions, it was not possible to collect cardio-respiratory measures through the automated open-circuit gas analysis system. However, during resting conditions and the last 40 seconds of each exercise intensity (when steady state of $\dot{V}O_2$ was assumed to be reached), ventilatory data was collected using a flowmeter connected to a measuring system build on purpose for this project from our engineers. The flowmeter used was that of the Quark PFT system and it was calibrated with a 3-L syringe following exactly the instructions of the open-circuit gas analysis system.

The individual RPE was assessed using the CR100 Scale at the end of each exercise intensity (Borg & Borg, 2002), together with thermal sensation (TS) using a 9-point scale (from -4 [very cold] to +4 [very hot]) (Arens et al., 2006). To measure blood lactate, a blood sample was collected from the earlobe after the first minute of recovery at the end of each exercise intensity and 3, 5 and 7 min after test

conclusion. The lactate analyser (Biosen C-line, EKF Diagnostics GmbH, Barleben, Germany) was calibrated according to the manufacturer's instructions.

Data Analysis

Maximal workload (WL_{max}) achieved at athlete's exhaustion during all incremental tests was determined according to the following equation: WL_{max}(km/h)= speed last stage completed (km/h)+ [t(s)/step duration(s) * step increment(km/h)], where t is the time of the uncompleted stage (Kuipers F T J; Keizer, H A; Geurten, P; van Kranenburg, G, 1985).

Lactate thresholds were determined throughout three different descriptors (4mMol, Dmax and DmaxMOD (Fabre et al., 2010) thanks to the customised Lactate-E-excel worksheet (Newell et al., 2007): DmaxMOD results were finally chosen for discussion in order to overcome Dmax method underestimation of HR and to reduce the problems related to individual variability when considering fixed lactate concentration method (4mMol) instead of individual lactate kinetics for each subject (Fabre et al., 2010). The DmaxMOD is a modified Dmax method, identified as the point on the third order polynomial curve that yielded the maximal perpendicular distance to the straight line formed by the point preceding an increase of lactate concentration greater than 0.4 mmol/L and the final lactate point (Bishop et al., 1998).

Ventilatory data were processed and analysed with MATLAB 7.0 (The MathWorks, Inc., Natick, MA, USA), using a customized code to calculate minute ventilation (Ve), respiratory frequency (Rf) and tidal volume (Vt). Mean values of HR, Ve, Rf, Vt and SpO2 were averaged over the last 40 seconds of each submaximal exercise intensity until 80% of individual VAM (the last point in which we had data for all our subjects also in H and CH conditions). For maximal data, peak values registered during the last or the last but one stages were considered (since in some cases subjects completed less than 2 minutes during the last stage and given the 2-min recovery phase between stages, at the end of the test some parameters were still rising).

Statistical Analysis

Values presented are expressed as mean ± standard deviations (SD). All the data were tested for their normal distribution (Shapiro–Wilk test). When normality was not met, data were log transformed. [La], Thermal Sensation (TS) and SpO2 values at maximal level and WL, HR, RPE and ventilatory data at both maximal and threshold level were compared using a two-way ANOVA for repeated measures (RM), with ‘‘temp’’ (+18°C and -20°C) and ‘‘FiO2’’ (normoxia and hypoxia) as factors. When an interaction effect (temp*FiO2) was found, Sidak post hoc test was used for specific comparisons (Cunha et al., 2015). Moreover, [La], HR, RPE, TS, SpO2 and ventilatory data at each submaximal exercise intensity until 80%VAM were compared using a three-way RM ANOVA, with ‘‘temp’’ (+18°C and -20°C), ‘‘FiO2’’ (normoxia and hypoxia) and ‘’intensity’’ (INT30%, INT40%, INT50%, INT60%, INT70%, INT80%) as factors. When ‘temp*FiO2’ or ‘temp*FiO2*intensity’ interaction effects were found, Sidak post hoc test was used for specific comparisons (Cunha et al., 2015). Partial eta squared was calculated for each factor, individually and combined. Statistical analysis was completed using a statistical software (SPSS Inc, Chicago, Illinois, USA). The level of statistical significance was set at $p<0.05$. Interpretation of partial eta squared values was conducted as follows: $\eta^2<0.01$: negligible effect; $0.01\leq\eta^2<0.06$: small effect; $0.06\leq\eta^2<0.14$: moderate effect; $\eta^2\geq0.14$: large effect**.**

RESULTS

Maximal WL, physiological and perceptual parameters

Complete results from two-way RM ANOVA are reported in **Table 1**. [La] and RPE at maximal exercise were not different between environmental conditions. Main effects of hypoxia and cold were found for HR_{max}, WL_{max}, Ve_{max} and Vt_{max}, which resulted lower in H and C compared to N, with no further significant reduction in CH. Oppositely, no individual but ‘temp*FiO2’ interaction effect was seen on Rf_{max}, which was significantly higher in CH than in C and H alone. SpO2min was lower in hypoxic conditions, with no effect of cold. Finally, an

interaction ‘temp*FiO2’ effect was seen in TS_{max}, that was lower in the cold if compared to normothermic conditions, but was further reduced in CH if compared to C alone. Visual representation of maximal WL (Fig.1a), HR (Fig.1b), RPE (Fig.1c) and Ve (Fig.1d) in the four environmental conditions is reported below.

[La], WL, HR, RPE and ventilatory data at the lactate threshold

Mean and SD of [La], WL, HR, RPE and ventilatory data detected with the DmaxMOD method are presented in **Table 2.** WL and HR at threshold intensity were reduced both by cold and hypoxia, with no ‘temp*FiO2’ interaction effect, whereas RPE was reduced by hypoxia alone, with no effect of cold nor interaction. Also, HR and WL expressed as a percentage of maximal values in the respective conditions showed differences between normoxia and hypoxia. There were no differences in [La] concentration at LT in the four conditions. Vt showed a general decreasing effect of both cold temperature and FiO2 without interaction, whereas ‘temp*FiO2’ interaction was found for Ve and Rf, showing lower Ve in CH than C and H alone, but higher Rf than N only in H. Visual representation of WL (Fig.1e), HR (Fig.1f), RPE (Fig.1g) and Ve (Fig.1h) at LT in the four environmental conditions is reported below.

Table 1. Maximal values registered in the four experimental conditions.

MAXIMAL VALUES																						
		N			H			C			CH			FiO2	η^2		temp	η^2		inter	η^2	
		mean		sd	mean		sd	mean		sd	mean		sd									
La	mMol	11.6	±	1.9	11.6	±	2.6	11.4	±	2.1	11.8	±	2.6	0.71	0.01	small	0.98	0.00	neg.	0.63	0.02	small
WL	(km/h)	6.8	±	0.6	5.5	±	0.5	6.6 $	±	0.6	5.3 &#	±	0.5	**<0.001***	0.98	large	**0.001***	0.70	large	0.25	0.11	mod
HR	bpm	190	±	7	181	±	10	184	±	9	176	±	8	**<0.001***	0.91	large	**0.001***	0.83	large	0.68	0.02	small
Ve	L/min	163.3	±	21	152	±	20.6	139.5 $	±	20.7	136.2 #	±	25.6	**0.017***	0.39	large	**<0.001**	0.71	large	0.12	0.19	large
Rf	bpm	64	±	11	62	±	11	61	±	11	65 **&#**	±	13	0.53	0.03	small	0.95	0.00	neg.	**0.013***	0.41	large
Vt	L/min	2.82	±	0.4	2.6	±	0.5	2.6	±	0.6	2.3	±	0.5	**0.002***	0.54	large	**<0.001***	0.88	large	0.63	0.04	small
RPE		97.7	±	3.1	96	±	4.5	96.8	±	3.2	96.0	±	6.1	0.06	0.26	large	0.77	0.01	neg.	0.67	0.02	small
TS		2.9	±	1.1	2.8	±	1.1	1.3 **$**	±	1.4	0.3 **&#**	±	1.5	**0.007***	0.47	large	**<0.001***	0.89	large	**0.047***	0.29	large
SpO2	%	94.4	±	2.9	75.1	±	3.5	93.6	±	5.1	76.3	±	5.2	**<0.001***	0.95	large	0.78	0.01	neg.	0.20	0.13	mod

Maximal values for: [La]: lactate; WL: workload; HR: heart rate; Ve: ventilation; Rf: respiratory frequency; Vt: tidal volume; RPE: rate of perceived exertion; TS: Thermal Sensation; SpO2: minimum Pulse Oxygen Saturation at the end of exercise. N: Normothermic Normoxia (18°C, 20.9% FiO2), H: Normothermic Hypoxia (18°C, 13.5% FiO2), C: Cold Normoxia (-20°C, 20.9% FiO2) and CH: Cold Hypoxia (-20°C, 13.5% FiO2). FiO2: general effect of fraction of inspired oxygen; temp: general effect of ambient temperature; inter: 'temp*FiO2' interaction effect. When a 'temp*FiO2' interaction effect was found, it has been reported as follow: $: C≠N; &: CH≠C; #: CH≠H. *$p<0.05$. $\eta^2<0.01$: negligible effect (neg); $0.01\leq\eta^2<0.06$: small effect; $0.06\leq\eta^2<0.14$: moderate effect (mod); $\eta^2\geq0.14$: large effect**.**

Table 2. Threshold values identified through the DmaxMOD in the four environmental conditions.

THRESHOLD VALUES																						
		N			H			C			CH			FiO2	η^2		temp	η^2		inter	η^2	
		mean		sd	mean		sd	mean		sd	mean		sd									
La	mMol	4.1	±	0.5	4.2	±	1.0	3.8	±	0.5	4.2	±	0.8	0.24	0.11	mod	0.47	0.04	small	0.39	0.06	small
WL	km/h	5.4	±	0.5	4.2	±	0.4	5.2	±	0.5	4.0	±	0.4	**<0.001***	0.96	large	**0.013***	0.42	large	0.81	0.01	neg
HR	bpm	167	±	8	160	±	10	162	±	9	157	±	10	**0.004***	0.51	large	**<0.001***	0.66	large	0.62	0.02	small
VE	L/min	97	±	15	102	±	13	94	±	15	86	&# ±	15	0.52	0.04	small	**0.008***	0.46	large	**0.007***	0.47	large
Rf	bpm	40	±	6	46 §	±	8	43	±	7	45	±	9	**0.014***	0.41	large	0.47	0.05	small	**0.032***	0.33	large
Vt	L/min	2.5	±	0.4	2.3	±	0.5	2.2	±	0.5	2.0	±	0.5	**0.007**	0.47	large	**<0.001**	0.80	large	0.389	0.06	mod
RPE		66	±	5	58	±	8	65	±	7	56	±	7	**<0.001***	0.74	large	0.45	0.05	small	0.67	0.02	small
WL%max	%	79	±	4	76	±	4	78	±	3	76	±	4	**0.006***	0.48	large	0.77	0.01	neg	0.49	0.04	small
HR%max	%	83	±	2	87 §	±	2	84	±	2	88 &#	±	2	**<0.001**	0.85	large	0.06	0.27	large	0.60	0.02	small

DmaxMOD: modified Dmax method for LT determination; [La]: blood lactate; WL: Workload; HR: heart rate; Ve: ventilation; Rf: respiratory frequency; Vt: tidal volume: RPE: rate of perceived exertion; WL%max: WL expressed as a percentage of maximal values in the respective conditions; HR%: HR expressed as a percentage of maximal values in the respective conditions. N: Normothermic Normoxia (18°C, 20.9% FiO2), H: Normothermic Hypoxia (18°C, 13.5% FiO2), C: Cold Normoxia (-20°C, 20.9% FiO2) and CH: Cold Hypoxia (-20°C, 13.5% FiO2). FiO2: general effect of fraction of inspired oxygen; temp: general effect of ambient temperature; inter: 'temp*FiO2' interaction effect. When a 'temp*FiO2' interaction effect was found, it has been reported as follow: §: H≠N; &: CH≠C; #: CH≠H. *$p<0.05$. $\eta^2<0.01$: negligible effect (neg); $0.01\leq\eta^2<0.06$: small effect; $0.06\leq\eta^2<0.14$: moderate effect (mod); $\eta^2\geq0.14$: large effect (large).

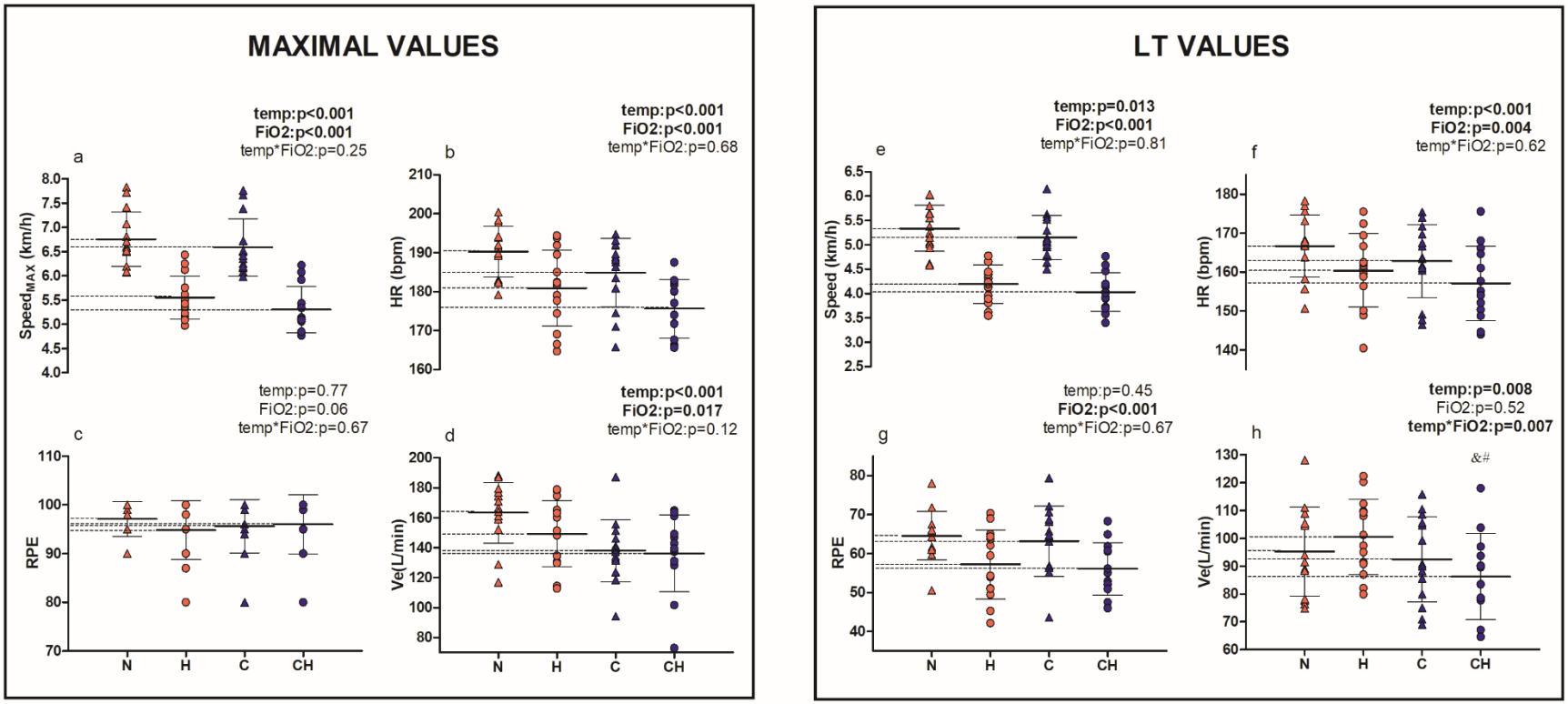

Fig. 1. WL, HR, RPE and Ve at maximal (MAX) and lactate threshold (LT) intensities. N: normothermic normoxia, red triangles; H: normothermic hypoxia, red circles; C: cold normoxia, blue triangles; CH: cold-hypoxia, blue circles. Symbols indicate individual values, dark continuous line represents the mean. Dotted lines underline the differences between the means. &: CH≠C; #: CH≠H

Submaximal physiological and perceptual adjustments

Mean and SD data for submaximal exercise intensities as well as complete results from three-way RM ANOVA are reported in Figure 2. Given the nature of a maximal incremental test to exhaustion, a general effect of 'intensity' was detected for each of the examined variables except for SpO2 **(Fig.2e)**. [La] accumulation **(fig.2A)** was generally increased by both cold and hypoxia, showing a 'temp*FiO2' but not a 'temp*FiO2*intensity' interaction: post hoc comparison showed that overall [La] curve was left-shifted only in CH if compared to H alone, but not in C if compared to N. HR **(Fig.2b)** was always higher in the hypoxic conditions, but at INT80 it was also higher in H if compared to CH condition ('temp*FiO2*intensity' interaction effect). RPE **(Fig.2c)** showed a general effect of hypoxia but also a 'temp*FiO2*intensity' interaction: post hoc comparison revealed that in H and CH subjects had higher RPE than temperature matched normoxic conditions (N and C, respectively) for each exercise intensity, but also that RPE at INT40, INT50 and INT60 was higher in C if compared to N. TS **(Fig.2d)** was lower in the cold regardless of present FiO2 at each 4-min submaximal exercise intensity. As for maximal values, SpO2 during submaximal exercise in normobaric hypoxia was significantly lower than in normoxia, with no further effect of environmental temperature or exercise intensity **(Fig.2e)**. General effects of hypoxia and cold were seen on ventilatory parameters: Ve and Rf **(Fig.2f and Fig.2h)** were higher in the hypoxic conditions if compared to the normoxic ones, whereas a general effect of cold on Ve **(Fig.2f)** and Vt **(Fig.2g)** was found. A 'temp*FiO2*intensity' interaction effect on Ve revealed that this parameter decreases significantly at high exercise intensities in the cold only when also hypoxia is present (Ve in CH is lower than in H alone at INT70 and 80, but Ve in C is similar to N).

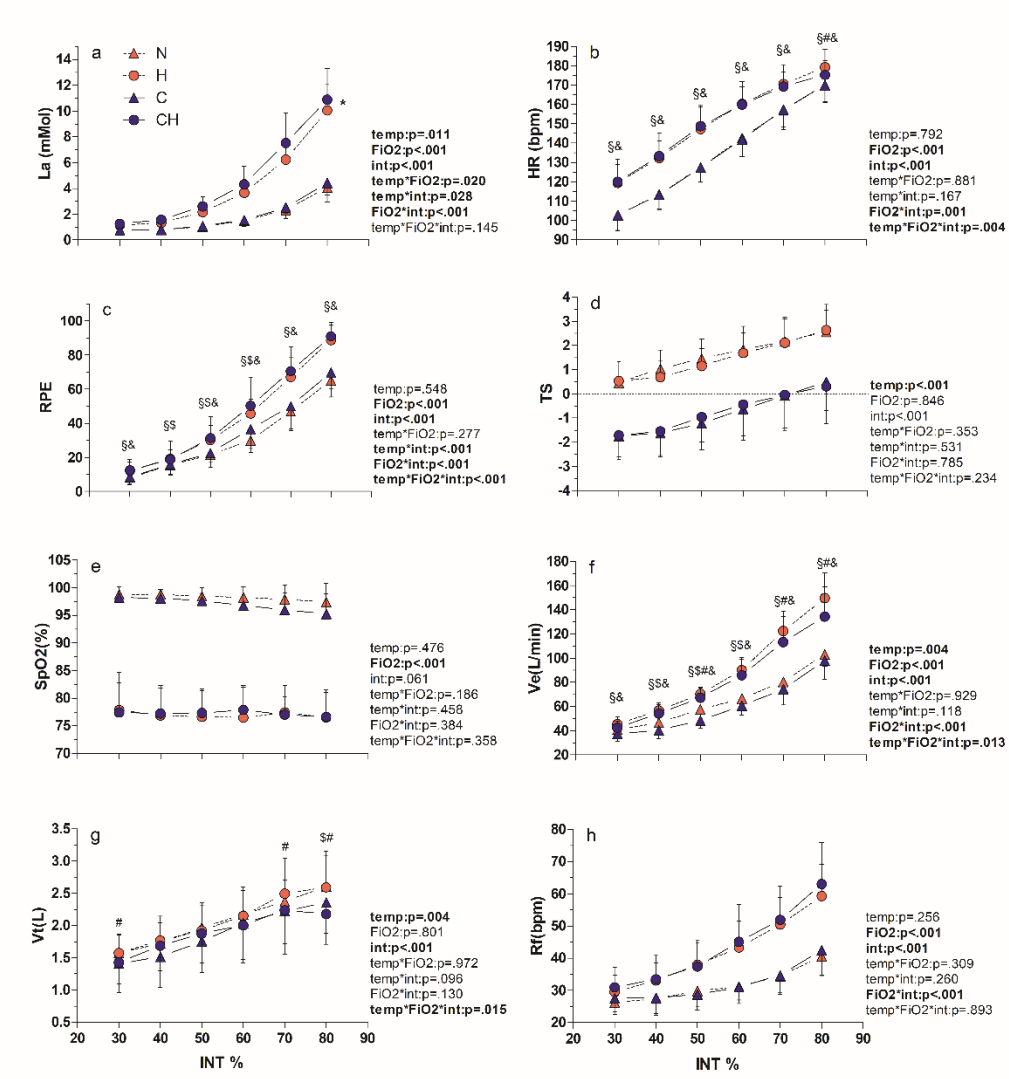

Fig.2. Mean submaximal values in the four environmental conditions. Lactate [La], Heart Rate (HR), RPE, Thermal Sensation (TS), Pulse Oxygen Saturation (SpO2), Ventilation (Ve), Tidal Volume (Vt), Respiratory Frequency (Rf) during an incremental treadmill test performed in normothermic normoxia (N, red triangles), normothermic hypoxia (H, FiO2 13.5%;red circles), cold normoxia (C,-20°C; blue triangles) and cold-hypoxia (CH, blue circles). Data are reported until 80% of individual maximal ascensional velocity. Values are presented as means ± standard deviations. When a 'temp*FiO2*intensity' interaction effect was found, it has been reported as follow: §H≠N; $C≠N; &CH≠C; #CH≠H. When a 'temp*FiO2' interaction effect was found, it has been reported as follow: * overall CH curve≠overall H curve.

DISCUSSION

Even though the independent effects of cold and hypoxia on performance and physiological and perceptual adjustments to exercise have been widely described in literature (Castellani & Tipton, 2016; R. S.Mazzeo, 2006), their combined influence on these aspects have been only investigated on segmental exercising tasks (i.e., Knee extensors and finger flexors); (Lloyd et al., 2015, 2016)). Indeed, to the best of our knowledge, this is the first study examining whole body endurance exercise responses when combinedly exposed to hypoxia and cold ambient temperature. The key findings of this study were that performance (WL_{max} and WL at LT) and most physiological variables (HR_{max} and HR at LT, Ve_{max} and Vt) show an additive rather than interactive effect (Lloyd & Havenith, 2016) in the CH condition, i.e., the decrease of above-mentioned variables by the combination of cold and hypoxia was equal to the sum of the effects exerted by the two environmental stressors alone. [La] levels throughout the test displayed an overall synergistic effect of cold and hypoxia on this parameter. Moreover, Ve at LT was characterized by synergistic effect of the two conditions, decreasing significantly in CH. Finally, RPE at lactate threshold intensity is exclusively reduced by hypoxia (i.e., exclusive effect), whereas TS_{max} is synergistically reduced by cold and hypoxia.

MAXIMAL AND LT WORKLOAD

It is already well documented that exercise performance for endurance-type efforts deteriorates in hypoxia (Amann et al., 2006; Doubt, 1991; Fornasiero et al., 2018; Goodall et al., 2022), whereas it is still controversial if this occurs in response to a cold stimulus (Castellani & Tipton, 2016; Castellani & Young, 2016), primarily due to different tested ambient temperatures. In our study, we found a 18% reduction in maximal exercise capacity in H, higher than the 10 to 13% decrease previously found for altitudes between 2500 and 3500 m (Faulhaber et al., 2021; Friedmann et al., 2004, 2005; Lorenz et al., 2006; Ofner et al., 2014; Weckbach et al., 2019). However, WL reduction (-21%) at LT in H is in line with previous defined reduction in PO at LT in hypoxia (≈11 to 19% (Faulhaber et al., 2021; Weckbach et al., 2019)), especially if considering greater simulated altitude in this study if compared to the others (3500 vs 3000 and 2650m asl). In C, our data show a 2.3%

reduction in WL_{max}, much lower than the 20% decrease found by Quirion et al. (1989) at -20°C. Also WL at LT is decreased in C (-3.5%): previous research demonstrated greater WL at LT within the so defined optimal ambient temperature (from -4 to 0°C , if properly dressed (Morrissey et al., 2019; Sandsund et al., 2012)) if compared to higher or lower ambient temperatures. On the other hand, others claim no differences in WL at LT or VT between +20°C and -14°C (Renberg et al., 2014; Therminarias, 1992; Therminarias et al., 1989)**.** Our results partially disagree with these findings, and this may be due to the much lower ambient temperature tested in this study.

A novel finding was that combining cold and hypoxia induced an additive rather than a synergistic effect (Lloyd & Havenith, 2016), further compromising WL at maximal (-21.5%) and LT level (-24.6%) in CH. These observations pertain to workload, as we did not have the possibility to measure actual $\dot{V}O_2$ due to constraints in the use of measurement tools in the cold. If on the one hand the main cause for WL_{max} reduction in H is linked to $\dot{V}O_{2max}$ reduction (Wehrlin & Hallén, 2006), on the other hand, maximal exercise capacity in the cold might be impaired by both $\dot{V}O_{2max}$ reduction (Oksa et al., 2004; Quirion et al., 1989), as well as by cold induced bronchoconstriction and reduced mechanical efficiency (Castellani & Tipton, 2016; Sandsund et al., 2012). In fact, muscle cooling impairs most functional properties, including a reduction in both the shortening and lengthening velocity of the muscle and in the capacity of power expression in agonist muscle groups (Renberg et al., 2014; Wiggen et al., 2013). The reason for additive and not synergistic effect on WL in CH may be explained as follows: firstly, evidences in exercise capacity impairments in the cold are not clear and thus the low impact magnitude of this stressor on performance is what may dictate the ‘additive’ and not ‘synergistic’ type of interaction expressed between these two stressors (Lloyd et al., 2016). Moreover, the physiological mechanisms leading to WL changes in hypoxic and cold environments could not always share a common pathway of action (PaO2 reduction in hypoxia vs bronchoconstriction and reduced mechanical efficiency in the cold), and as proposed by previous authors, interactive effects probably arise only when combining stressor that are mechanistically similar (Broadbent, 1963; Lloyd et al., 2016). In the opinion of the authors, since our

subjects were well dressed and did not feel cold at the end of exercise, muscle temperature and blood flow were probably preserved, remaining bronchoconstriction the principal cold-induced effect that led to WLmax reductions in our cold trials; for this reason, the 'mild stressor effect' of cold remains the preferred explanation for additive rather than interactive effects between our stressors.

MAXIMAL AND SUBMAXIMAL PHYSIOLOGICAL RESPONSES

Heart Rate

Similarly to previous published data (Fornasiero et al., 2018; Grataloup et al., 2007; Mourot, 2018; Ofner et al., 2014), we showed a decrease in HR_{max} when acutely exposed to H and this reduction (-10 bpm at 3500 m) appears to be slightly higher than the proposed average decrease of 1.7 bpm per 1000m gain in altitude (Garvican-Lewis et al., 2015). However, Mollard et al. (Mollard, Woorons, Letournel, Lamberto, et al., 2007b) demonstrated that at 3500m of altitude HR decreased by 11 bpm in trained ($\dot{V}O_{2max}$>60 ml/kg/min, as for our subjects) and by 5 bpm in untrained ($\dot{V}O_{2max}$<50 ml/kg/min) subjects, proving an effect of training status on HR reduction in hypoxia (Richalet R; Antezana, A -M, 1992; Richalet, 1988). The explanations for the reduction in HR_{max} when acutely exposed to hypoxia include i) a change in cardiac electrophysiological properties (i.e. increased duration of repolarization length and slower atrio-ventricular conduction) (Benoit et al., 2003; Grataloup et al., 2007; Mourot, 2018), ii) a decrease in muscle $\dot{V}O_{2max}$ due to arterial hypoxemia that leads to reduced cardiac output (CO) (Benoit et al., 2003) and iii) a decrease in exercise effort due to reduced oxygen content that is perceived by the central nervous system, causing accelerated development of muscle fatigue: this implies an increase in inhibitory afferent signals and a reduced central drive on the heart as a protective mechanism from myocardial ischemia (Noakes et al., 2001).

More debated is the topic on HR at LT in hypoxia: it is often assumed that training with the same HR in normoxia and hypoxia would result in equivalent training intensities in these environments (Brosnan et al., 2000; Ofner et al., 2014). However, Friedmann et al. (2005;2004) showed a reduction in HR at LT (detected with different methods) ranging from -3 to -4% at 2500 m if compared to sea level

values: similarly, we found a 3.8% reduction in HR at LT at 3500 for our subjects, suggesting that HR reductions at LT in H may be characterized by a ceiling effect as altitude increases. Interestingly, as opposed to Friedmann et al. (2004), also HR expressed as a percentage of maximal values in the respective conditions showed differences between normoxia and hypoxia. This point is of paramount importance for training prescription to prevent overtraining, for performance to target best pacing strategy, but also for the design of scientific studies in order to guarantee same relative exercise intensity in studies confronting normoxic and hypoxic environments.

We also found a general effect of cold on maximal HR, which decreased on average by 3.2% in C if compared to N, in line to the ≈4% decrease that has been found for ambient temperatures between -14 and -20°C if compared to +20°C (Oksa et al., 2004; Renberg et al., 2014; Sandsund et al., 2012). No effect of cold on submaximal HR when considering same external workload was found, but HR at LT was reduced by 2.8% in C. Our result disagrees with Therminaris et al. (1989), who determined that HR at -2°C was reduced up to moderate exercise intensities, remaining unchanged at LT: the magnitude of cold (-20 vs -2°C) could be addressed as a possible explanation for these differences. The reasons for HR reductions in the cold are still controversial but have been mainly related to cold induced peripheral vasoconstriction that increases central blood volume and consequently stroke volume (Doubt, 1991; Gisolfi & Wenger, 1984), implying a reduced sympathetic drive and a change in heart mechanics (Castellani & Tipton, 2016; Doubt, 1991; Sandsund et al., 2012). However, also lower external work performed both at maximal and LT intensities could play a major role in this reduction (Castellani & Tipton, 2016).

The CH condition induced an additive decreasing effect (Lloyd & Havenith, 2016) of C and H on HR_{max} (H: -5.0%, C:-3.2%, CH -7.7% if compared to N, respectively) and a relative additive effect on HR at LT (H:-3.81%, C:-2.80%, CH:-5.69%). A relative additive effect displays a situation in which the combination effect of two stressors on one variable is lower than the sum of independent effect, but to an extent that do not induce a real antagonistic effect between the 2 stressors: this is probably due to the fact that C and H alone can be considered as mild stressors that

operate on the heart with partial independent mechanisms (see above) (Broadbent, 1963; Lloyd et al., 2015, 2016) showing just a tendency towards the 'worst strain take precedence principle' on exercising HR in CH.

Lactate

There were no differences in $[La]_{max}$ between environmental conditions, suggesting that our subjects reached exhaustion in all conditions despite reduced WL_{max} in H, C and CH. [La] was significantly higher in hypoxic environments from the beginning to the highest exercise intensities (Fig.2a). This result was expectable (Clark et al., 2007; Friedmann et al., 2005), considering that the absolute workload of the protocol was the same for the 4 sessions. However, [La] at LT was not different between normoxic and hypoxic conditions (Table 2). No differences in [La] accumulation throughout the test (Fig.2a) nor at LT intensity (Table 2) were seen between C and N conditions: the influence of cold exposure on blood lactate response has been debated in literature, suggesting that it depends on several factors, such as intensity and duration of cold exposure. Therminaris et al. (1989) found greater [La] levels below LT and lower [La] levels above LT at -2°C ambient temperatures if compared to +20°C, and Blomstrand et al. (1984) suggested that most subjects at reduced muscle temperature attain higher muscle lactate concentrations for same exercising workload (Blomstrand et al., 1984), as well as show delayed but higher peak blood lactate concentration at the end of exercise (Blomstrand & Essén-Gustavsson, 1987), indicating a lower flux of lactate from muscle to blood in this condition. This increase in blood and muscle lactate concentration at low muscle temperatures suggests a greater reliance on anaerobic metabolism: cold exposure, as well as muscular exercise, stimulates the sympathoadrenal system, increasing plasma catecholamine concentrations that are responsible for increased muscle glycogenolysis (Himms-Hagen, 1972). Moreover, reduced local blood flow could lead to decreased oxygen delivery during exercise (with consequently higher reliance on anaerobic metabolism) and delayed muscle lactate release from the muscle, further enhancing the accumulation of lactate in the muscle (Blomstrand & Essén-Gustavsson, 1987; Castellani & Tipton, 2016). The reason why we did not find any difference in [La] accumulation throughout the test

in C if compared to N may be related to the fact that our subjects were allowed to wear the clothes they preferred, thus never causing a decrease in muscle temperature to an extent that determined a change in lactate metabolism. More controversial are the results concerning hypoxic trials, in which [La] curve was left-shifted in CH if compared to H, suggesting an effect of cold on [La] metabolism only when combined to the hypoxic stressor. Furthermore, [La] at LT in CH was similar to the other conditions (Table 2), but corresponded to the lowest exercising WL (additive effect between C and H), confirming that in CH higher values of La are expected when considering same WL as both H or C. Further studies are needed to better clarify this aspect, accurately measuring muscle temperature, blood flow and PaO_2 in cold-hypoxic environments.

Ventilation

Ve_{max} (H=-6.9%, C=-14.6%, CH=-16.6%) and Vt_{max} (H:-7.05%; C:-9.57%, CH:-18.65%) decreased from N with both a general effect of hypoxia ($p=0.017$ and 0.002) and cold (both $p<0.01$), displaying a partial (i.e. Ve) or complete (i.e. Vt) additive effect in CH without any statistical interaction. Conversely, Rf_{max} was similar to N in CH (despite the lowest WL_{max}), but was significantly higher than in H (+5.7%) and C (+7.0%) alone.

Ve_{max} in normobaric hypoxia has been shown to be slightly decreased if compared to hypobaric hypoxic conditions (at comparable simulated altitude level) (Treml et al., 2020), probably due to distinct breathing patterns related to changes in air density that differently affect the central motor drive (Amann & Dempsey, 2016). However, the lower Ve_{max} in hypoxia at reduced exercise performance in our study has to be emphasized. Conversely, ventilating heavily cold air induces bronchus constriction (Oksa et al., 2004), diminishing the amount of air that can be ventilated (Anderson & Daviskas, 2000b): in fact, the rapid recruitment of the smaller airways into the heating and humidifying process cause a quicker water loss, creating a greater osmotic gradient than that related to warm air breathing. Bronchoconstriction leads to the so-called dynamic hyperinflation mechanism: some air remains trapped in the lungs, causing a temporary increase in end expiratory lung volume above its baseline level, and reducing tidal volume and

consequently ventilation, since not properly compensated by increased respiratory frequency (Stickland et al., 2022). Furthermore, Kennedy et al. (Kennedy et al., 2020) claim that exercise induced bronchoconstriction could be greater at temperature colder than -15°C if compared to 0°C, which is exactly the case for the proposed study. Interestingly, in CH there is a complete additive reduction effect of H and C on Vt, but just a relative additive reduction effect on Ve, since probably Rf_{max} was increased to overcome cold induced Vt reductions. However, when considering same relative submaximal exercise intensity at LT, Ve is lower in CH than in H and C alone, remaining similar to N in the other experimental conditions (H:-6%; C:-3%; CH:-11% if compared to N). This is linked to a complete additive effect of H (-9%) and C (-10%) on Vt in CH (-19%) (as it happens at maximal level), combined to a nullification (Lloyd & Havenith, 2016) effect of the 2 environments on Rf increase from N (H: +16%, C:+8%, CH:+13%), possibly also related to reduced exercising WL in this condition. Finally, considering submaximal exercising WLs, both Vt and Ve (Fig. 2g and 2f) seem to be affected by the presence of cold. All these information confirm the effect of extreme cold on respiratory mechanics while exercising, but also the necessity to deepen knowledge on the interaction role of hypoxia and cold on Ve, Vt and Rf at both maximal and submaximal intensities. Further studies on ventilatory responses, cold induced bronchoconstriction and exercise performance in CH should be implemented.

MAXIMAL AND SUBMAXIMAL PERCEPTUAL RESPONSES.

RPE

No differences were seen in RPE_{max}, further confirming (together with $[La]_{max}$) that our subjects reached maximal effort in all experimental sessions. RPE at submaximal exercise was higher in hypoxic conditions (due to same absolute but different relative intensity), with no differences in CH if compared to H alone. More controversial is RPE reduction at LT in hypoxia: Aliverti et al. (2011) explained that both leg and breathing RPE are higher in hypoxia at same leg power output to normoxia (due to different relative exercise intensities), but breathing RPE is the same in normoxia and hypoxia when considering equal Respiratory muscles power

output (PO) at similar ventilation rates. Interestingly, reduced RPE values at LT has been given by our subject in H, mainly due to decreased leg PO in this condition, but a further reduction in RPE was seen in CH, the only condition in which Ve at LT was lower than in the other experimental sessions. This confirms the idea that the lower the ventilatory response (and thus respiratory muscle PO), the lower the perceived exertion despite similar relative exercise intensity (Nicolò et al., 2015). The mechanisms underlying this phenomena should be further studied, especially considering the valuable importance of RPE as a monitoring tool for training when physiological parameters are not so reliable (i.e. at high altitude).

TS

At maximal exercise, TS in CH resulted lower than in C alone, implying an effect of hypoxia in the perception of cold that could be related to the reduced mechanical work performed at the end of exercise. In fact, TS at submaximal level (and same exercising WL) showed only an effect of ambient temperature, being lower in cold than normothermic conditions, with no further effect of hypoxia. The interaction effect of cold and hypoxia on the perception of thermal stimuli has been extensively debated in literature (Golja et al., 2004; Keramidas et al., 2019; Malanda et al., 2008; Massey et al., 2015), with the most quoted idea being that acute exposure to normobaric hypoxia in combination with whole body cooling results in vasoconstriction at warmer skin temperature compared to a normoxic condition (Massey et al., 2015); however, decreased neural processing and/or decreased nerve conduction speed in the sensor-to-effector pathway (Malanda et al., 2008) alters subjects thermal perception and comfort, consequently attenuating thermoregulatory behaviour during cold exposure at altitude (Golja et al., 2004). It is possible that at submaximal exercise intensities, our subjects perceived the same TS value in C and CH, even though in the latter condition the thermal stimulus for the body was worse. Also greater relative exercise intensity (and consequently heat production) in CH associated with same perceived TS as C alone underlines impaired thermal perception, that requires further attention in future studies for both safety and performance reasons.

LIMITATIONS

For this study, we could not record any metabolic data in the cold conditions due to the technical impossibility of using the breath-by-breath metabolimeter at temperatures below zero (manufacturer instructions temperature range: 10-40°C). However, going deeper into exercise physiology in the cold is warranted, and future studies should consider the use of Douglas bags for Oxygen consumption measurements. Moreover, some results (i.e, ventilation) may have been affected by the fact that we studied acute exposure to normobaric hypoxia, which is known to induce some different physiological adjustments with respect to hypobaric exposure. In addition, we do not have any information regarding subjects core or skin temperature, which would have allowed us to exclude any role of reduced overall body's temperature in measured outcomes. Finally, repeating the same incremental test in the four conditions causes lower exercise durations in hypoxia and this may also affect some results: a piece of advice for the future is the use of matched exercise intensity protocols between normoxic and hypoxic conditions, that should partially overcome this problem and allow easier comparison of measured variables between conditions.

CONCLUSION AND FUTURE PERSPECTIVE

The combination of cold (-20°C) and hypoxia (≈3500 m) exerted additive rather than synergistic effects on exercise performance, decreasing LT and maximal exercising workload to an extent that is equal to the sum of the two stimuli alone. Both exclusive effects of hypoxia (i.e. maximal and submaximal SpO, submaximal Rf and RPE at LT) and cold (i.e. submaximal Vt and TS) and different additive (i.e. maximal and LT HR, Ve $_{max}$) and synergistic (i.e. TS_{max} and Ve at LT) effects of the two stressors were found on the investigated maximal and submaximal physiological and perceptual variables. Future studies should i) better understand the magnitude of influence of cold induced bronchoconstriction on exercise performance, especially when combined to acute hypoxic ventilatory response and ii) consider combined cold and hypoxic effect on performance when also a significant reduction in core and muscle temperature is expected, accurately measuring these parameters. These results provide new insight into human

responses to exercise in cold and hypoxic environments, highlighting the need for careful consideration of independent and combined stressor impact on considered variables for optimal exercise intensity prescription and training load monitoring in athletes training/competing in hypoxic and/or cold environments.

STUDY 2 - FREEZING HYPOXIA AND EXERCISE: IMPACT OF CONCURRENT STRESSORS ON VENTILATORY RESPONSES, LUNG FUNCTION, AND RESPIRATORY MUSCLE WORK.

ABSTRACT

Purpose: Cold temperatures(<-15°C) increase exercise-induced-bronchoconstriction (EIB), while hypoxia exacerbates respiratory muscle work. This study aimed to determine the combined effects of cold and hypoxia on lung function (LF) and respiratory muscle work, and their relationship to ventilatory responses during high-intensity exercise.

Methods: 14 trained male runners (VO_{2max}:64±5 mL/kg/min) randomly performed an incremental test to exhaustion (CPET) followed by an 8-min exercise bronchoprovocation test (EBPT) under four environmental conditions: Normothermic(18°C) Normoxia(FiO2:20.9%) and Hypoxia(FiO2:13.5%), and Cold(-20°C) Normoxia and Hypoxia. Ventilatory responses during exercise, maximal inspiratory pressure (MIP) and expiratory pressure (MEP) before (PRE) and immediately after (POST) the CPET, and LF PRE and POST-EBPT were evaluated.

Results: FEV1, FEV1/FVC, PEF, FEF25-27% and MEF50% were affected by cold exposure, with no further cold-hypoxic effect. No significant PRE to POST exercise change in MIP and MEP was found, independently of environmental conditions. Greater LF impairments in C and CH were associated with the lowest peak ventilatory responses during exercise.

Conclusions: Post-exercise LF impairments associated with decreased peak ventilatory responses in the cold were found, with no additional hypoxic effect. No effect of environmental conditions on respiratory muscle work was detected, but future studies should focus on longer exercising tasks to better evaluate this aspect.

INTRODUCTION

Nowadays more and more people are venturing into the mountains, especially considering the increasing popularity of Mountaineering, Skyrunning, and Ski-Mountaineering as both recreational and competitive sports (Permanent Secretariat of the Alpine Convention, 2010). These activities generally take place at High Altitude (HA), where a reduction in barometric pressure causes partial pressure of inspired oxygen in ambient air (Pi_{O2}) to be lowered, leading to a cascade reduction of alveolar (PAO2) and arterial (PaO2) oxygen pressure (S. A. Gallagher & Hackett, 2004). Apart from reduced oxygen availability, HA exposure is often associated with really cold temperatures, but despite their independent effect on several exercising responses has been widely investigated in literature (Castellani & Tipton, 2016; Fulco et al., 1998; Oksa et al., 2004; R. S.Mazzeo, 2006; Stensrud & Carlsen, 2008; Taylor et al., 2008), the combined effect of the two stressors has been poorly studied (M. Burtscher et al., 2018; Mugele et al., 2021; Tipton, 2012) and to the best of our knowledge, only few investigations delved with whole-body dynamic exercise responses to cold-hypoxic exposures (Callovini et al., 2023; Fornasiero et al., 2020; K. Hinde et al., 2018). Specifically, types of interaction effects (Lloyd & Havenith, 2016) between cold and hypoxia on several exercise-related physiological responses have been investigated and presented in a previous manuscript by our research group (Callovini et al., 2023): interestingly, in this study both maximal workload (WL_{max}) and ventilation ($\dot{V}E_{max}$) exhibited complete additive reduction effect in the cold-hypoxic environment, suggesting that ventilatory constraints may play a central role in determining performance changes in both singular and combined stressor exposure. To avoid/postpone these ventilatory limitations, airway calibre during exercise has to be maintained or even increased, this being also confirmed by a post-exercise increase in some spirometry measures of expiratory flow in healthy (Andrew William Sheel & Romer, 2012) and pathological (Tucker et al., 2017) subjects. However, this adjustment sometimes fails to happen due to the narrowing of intrathoracic airways as a consequence of bronchoconstriction of bronchiolar smooth muscle and/or inflammation during the hyperpnea of exercise. This phenomenon is referred to as exercise-induced bronchoconstriction (EIB), and it is known to occur not only

among subjects with asthma but also in some groups of subjects without asthma, such as elite athletes (Parsons et al., 2013). In fact, the necessity of humidifying large volumes of air in a short time (such as at elevated exercising intensities) causes water loss by evaporation from the airway surface, leading to both an osmotic and a thermal effect responsible for the airways narrowing (Anderson & Daviskas, 2000b; Bonini, 2018; Parsons et al., 2013). The osmotic effect refers to the transient increase in osmolarity of the airway surface liquid due to dehydration consequent to water loss to humidify inspired air, which favors the release of mediators such as histamine (a potent bronchoconstrictor) from mast cell degranulation: these mediators are responsible at first for smooth muscle relaxation (i.e., first few minutes of exercise) but then cause its contraction in patients presenting EIB. The thermal effect is linked instead to rapid airway rewarming after they have been cooled during exercise, which results in airway narrowing due to reactive hyperemia of the bronchial vasculature and consequent increased vascular permeability and mucosal oedema, exacerbating bronchoconstriction after the end of exercise (Parsons et al., 2013). Interestingly, chronic airway inflammation also plays an important role in the pathogenesis of EIB in asthmatic subjects, leading to mucus production, smooth muscle hypertrophy, and bronchospasm: however, the role or significance of inflammation in the pathogenesis of EIB in subjects without asthma is unclear: (Bougault et al., 2009) compared pulmonary function, airway responsiveness (AHR) and inflammation of cold-air athletes with healthy and asthmatic controls, underling that the former did not have higher eosinophil and neutrophile count than healthy controls, and both groups presented lower levels of inflammatory cell count than the asthmatic group. However, the majority of elite athletes showed evidence of bronchial epithelial damage that could possibly contribute to the development of airway hyperresponsiveness. It is, therefore, possible that cold-air athletes develop AHR and asthma through a mechanism somewhat different from the classical forms of asthma.

Given EIB precursor mechanisms, it is reasonable to think that its prevalence may increase when exercising while inhaling cold ambient air (which is typically also dry), especially for those exercising intensities in which oral rather than nasal breathing is necessary to sustain ventilation requirements ($\dot{V}E$ above ≈ 35 L/min)

(Hanstock et al., 2020; McFadden et al., 1985). In fact, high-risk sports for EIB development have been shown to include long episodes of exercise greater than 5 to 8 minutes in certain environments such as cold, dry air (i.e. high altitude sports) (Gerow M, 2024).

Apart from airway calibre modulation, the pulmonary system responds to increased ventilatory demands by increasing VT (up to 50 or 60% of vital capacity, in order to allow proper alveolar ventilation, avoiding an excessive increase in intra-abdominal pressure) and respiratory frequency (Rf), which remains the primary mechanism accountable for increased ventilation at high exercise intensities (Gibson et al., 2002). This complex regulation system allows the diaphragm to operate near its optimal length for force generation, minimizing the work of breathing and postponing respiratory muscle fatigue development, defined as a loss in the capacity for developing force and/or velocity, which reverses by rest (Oueslati et al., 2018). Despite all these adjustments aimed at preserving diaphragmatic force output to guarantee proper ventilation, increased respiratory muscle work and consequent early fatigue development in hypoxia has been detected even when ventilatory muscles' load is similar to normoxic conditions (i.e. similar ventilation with different external loads) (Vogiatzis et al., 2007). Furthermore, hyperventilatory response stimulated by decreased PaO2 detected from central and peripheral chemoreceptors for the same absolute WL in hypoxia (A. William Sheel et al., 2010) may determine a pivotal role of increased respiratory muscle work in causing exercise limitations in this condition (Jerome A. Dempsey et al., 2006; Vergès et al., 2005).

As described above, environmental challenges, in addition to those imposed by the demands of exercise alone, provide additional physiological stress to the pulmonary system and the respiratory muscles and may consequently affect exercise responses as well as represent a risk to health. However, although the relationship between exercising ventilatory responses in the cold and post-exercise EIB prevalence has been studied in the literature (27, 30), scarce knowledge is present on the possible concurrent effect of lung function and respiratory muscle work impairments on ventilatory aspects during exercise in cold-hypoxic environments.

For these reasons, the aim of this study is twofold: 1) to investigate possible exacerbation effects of combined cold-hypoxic exposure on EIB and respiratory muscle work related to strenuous exercise practice in healthy runners and 2) to understand the relationship between exercising ventilatory responses and lung/respiratory muscles function at simulated freezing altitude. We hypothesized that an increased EIB prevalence combined with exaggerated respiratory muscle work in the cold-hypoxic condition could lead to additional detrimental effects on ventilatory responses during exercise, thus playing a pivotal role in determining further performance decrements when combined instead of singular environmental stressors are present.

MATERIALS AND METHODS

Subjects

Fourteen trained (De Pauw et al., 2013) male subjects volunteered for this study (Table 1). All participants were non-smokers, free of any systemic or chronic illness, and not taking medications. They all had a valid sports medical examination, with no contraindications reported regarding cardiac or respiratory aspects. 4 participants' baseline FEV1/FVC ratio was less than <0.75, which has been described by some authors (Kennedy et al., 2019b) as a predictive value for exercise-induced bronchoconstriction development, at least when the cold stimulus is superimposed to exercise. However, all subjects claimed to have never experienced symptoms of EIB and cough during low or moderate-intensity exercise in cold weather or adverse respiratory symptoms due to high-intensity exercise. During the initial visit, subjects were asked to record in a nutrition diary the diet followed on that day and the preceding one, and they were recommended to maintain it as closely as possible during subsequent sessions. Thirteen subjects completed all experimental sessions, whereas one subject completed 4 out of 5 sessions. All study protocols were approved by the local ethics Committee (University of Verona- Project N. 4105CESC) and conformed to the Declaration of Helsinki. Before data collection, all participants were properly informed about the experimental procedures and gave their written informed consent for the measurements.

Table 1 Subjects characteristics and baseline pulmonary function evaluation

Characteristic		mean		sd
Age	years	27.23	±	3.40
Height	cm	177.21	±	4.54
Weight	kg	70.21	±	5.25
BMI	kg/m^2	22.37	±	1.67
VO2max	ml/kg/min	64.00	±	5.20
HRmax	bpm	191	±	6
FVC	L	5.55	±	0.59
FVC	% predicted	101.93	±	9.91
FEV1	L/min	4.39	±	0.50
FEV1	% predicted	97.00	±	10.24
FEV1/FVC	ratio	79.12	±	4.97
FEV1/FVC	% predicted	94.57	±	5.23
FEF25-75	L/min	3.98	±	0.94
FEF25-75	% predicted	85.00	±	19.12

Data reported as mean ± SD, (overall n = 14). BMI, Body Mass Index; HRmax: maximal Heart Rate; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; FEF 25-75, forced expiratory flow at 25%–75%.

Preliminary Assessment and Experimental Design

Each participant underwent a total of five laboratory visits, including an initial assessment and four subsequent experimental trials, all scheduled at the same time of the day.

During the preliminary session, a baseline spirometry assessment was completed prior to an incremental test to exhaustion on a motorized treadmill (slope: 25%, starting speed 2.0 km/h increased by 0.7 km/h every 3 min), through which subjects' $\dot{V}O_{2max}$ and individual Maximal Ascensional Velocity were determined. Cardiorespiratory measures were collected continuously with a breath-by-breath method using an automated open-circuit gas analysis system (Quark PFT Ergo, Cosmed Srl, Rome, Italy), and HR was recorded continuously during the test by an

HR monitor incorporated into the gas analysis system. The results were used to define individual running speed in the exercise protocols for the four experimental trials.

All the four sessions following the preliminary one were performed in an environmental chamber where it was possible to vary ambient temperature and simulate high altitude exposure (i.e. normobaric hypoxia). The hypoxic environment was created through the manipulation of the FiO2 by means of an oxygen dilution system based on the Vacuum Pressure Swing Adsorption principle (B-Cat, Tiel, The Netherlands). FiO2 was set at 13.5% to simulate an altitude ≈3500 m a.s.l. The temperature was regulated using a special air conditioning and refrigeration system (Frigotherm Ferrari SRL, Lana, Italy) capable of adjusting the room to the desired temperatures. Relative Humidity was set at 40% in all conditions.

During these sessions, participants were randomly exposed to each of the following conditions: Normothermic Normoxia (N: 18°C, 20.9% FiO2), Normothermic Hypoxia (H; 18°C, 13.5% FiO2), Cold Normoxia (C: -20°C, 20.9% FiO2) and Cold Hypoxia (CH: -20°C, 13.5% FiO2). They were blind to FiO2 value but not to temperature conditions.

There was a minimum of 48 hours between baseline experimental sessions. All participants completed the protocol within a 6-week period between March and November so that no cold-acclimatization was present. Subjects were allowed to continue their normal exercise and activity patterns but were asked to refrain from intense physical activity on the day before and from drinking any alcohol and caffeinated beverages on the day of the test.

Experimental Trials

The main test sessions started with pre-trial spirometry and respiratory muscle pressure measurements outside of the chamber at a normal indoor ambient temperature of 20°C. Participants were then equipped with a heart rate strap (Polar, Kempele, Finland) and invited to wear appropriate clothing depending on the environmental temperature of that specific session. During cold conditions, participants wore individually chosen extreme cold weather technical clothing

(including winter sports jacket/sweater, trousers, gloves, and hat or band (estimated clothing insulation in the cold: 1.50 l_{cl}(clo)), which remained identical for C and CH trials; however, they were not allowed to cover the face or mouth in any manner (scarf, buff, hand) throughout the whole exercise session, but could dress or undress as they felt comfortable during both exercise and rest phases. To ensure the occurrence of first short-term physiological responses to the hypoxic environment, once subjects entered the chamber, they remained seated for a 30-min resting period, already exposed to the specific environmental condition (Duffin, 2007). This was repeated within all conditions in order to guarantee participants' blindness to ambient FiO2.

The exercise protocol consisted of two separate exercising blocks (Fig.1): the first started with a 10-min warm-up phase (2 km/h, slope 25%) followed by a submaximal to maximal test of 4-min intervals at increasing velocities (CPET), interspersed by 2 minutes of passive recovery performed in standing position on the treadmill using handrail support. Treadmill inclination was kept constant at 25% (Fornasiero et al., 2018), whereas the test's speed started from 30% of the individual maximal speed measured at pretest and increased by 10% every interval until exhaustion. Respiratory muscle pressure measurements were repeated inside the environmental chamber two minutes after exercise cessation. The second exercise block started 10 minutes after the conclusion of the CPET and consisted of an 8-minute exercise bronchoprovocation test (EBPT) (Gibson et al., 2002). In brief, for the first 4 minutes, participants were asked to slowly reach the speed of the last completed step of the previously performed CPET (≈90% of specific condition maximal Workload), and then they had to try to walk/run the last 4 minutes at that speed or, if not possible due to insurgence of fatigue, at the maximal sustainable speed. It has to be noted that for a bronchoprovocation test to be considered valid, an exercising intensity reaching at least 85% of maximal predicted HR or 60% of maximal ventilation (VE) and lasting from 4 to 6 minutes should be performed (Weiler et al., 2016). At the end of exercise, participants exited the environmental chamber to perform spirometry at 1, 3, 6, 10, and 15 minutes post-exercise (1-POST; 3-POST; 6-POST; 10-POST; 15-POST) in accordance with previous methods and at the same ambient temperature as pre-exercise trials (Kennedy et al.,

2020; Kennedy & Faulhaber, 2018). During this phase, participants were allowed to walk around slowly in order to provide a typical cool down found after exercise**.** Throughout rest, exercise, and recovery phases, beat-to-beat heart rate (HR) was continuously recorded using a Polar RS800CX HR monitor (Polar, Kempele, Finland) and pulse oxygen saturation (SpO2) through an ear pulse oximeter (Nonin Medical, Minneapolis, MN) with a sampling frequency of 1.0 Hz. Due to the extremely cold conditions, it was not possible to collect cardio-respiratory measures through the automated open-circuit gas analysis system. However, at rest, during the last 40 seconds of each exercise intensity (when a steady state of $\dot{V}O_2$ was assumed to be reached) in the CPET and between the 7th and 8th minute of the EBPT, ventilatory data were collected using a flowmeter connected to a measuring system build on purpose for this project from our engineers. The flowmeter used was that of the Quark PFT system and it was calibrated with a 3-L syringe following exactly the instructions of the open-circuit gas analysis system.

Figure 1 Overview of the exercise protocol during the 4 experimental sessions; MIP: maximal inspiratory pressure; MEP: maximal expiratory pressure; CPET: cardio pulmonary exercise test; EBPT: exercise broncho provocation test.

Lung function and Respiratory Muscle Strength

Lung function tests were performed using an ergospirometer (Quark PFT, COSMED, Rome, Italy) in accordance with the guidelines of the American Thoracic Society (ATS) and the European Respiratory Society (ERS) (Graham et al., 2019)**.** All tests were performed by trained personnel to ensure consistency of the procedure.

The main outcome measures were volume of air forcefully exhaled in 1 s (FEV1), forced vital capacity (FVC), the ratio of FEV1 to FVC (FEV1/FVC%), maximal expiratory flow at 50% of FVC (MEF50), the average forced expiratory flow during the mid (25–75%) portion of the FVC (FEF 25–75%) and peak expiratory flow (PEF) (Kennedy et al., 2019b, 2020; Kennedy & Faulhaber, 2018; Sandsund et al., 1997). All manoeuvres complied with the general acceptability criteria of the ERS. Also maximal voluntary inspiratory (MIP) and expiratory pressure (MEP) were measured before exercise and within 4 min of CPET completion in order to evaluate exercise-induced changes in respiratory muscle strength as an indication of respiratory muscle fatigue (Oueslati et al., 2018). Participants were asked to produce a maximal inspiration or expiration through a mouthpiece into an occluded non-deformable tube (Gibson et al., 2002). A small leak (1 mm diameter) was used to prevent glottis closure. The tube was attached to a negative and positive pressure gauge depending on the performed manoeuvre (MIP or MEP, respectively). Measurement of MIP was initiated at maximal expiratory lung volume and MEP at maximal inspiratory lung volume and lasted a minimum of three seconds. Maximal efforts were repeated at least 3 times with a minimum of 30 seconds between measures until there were at least two maximal values within 10% variance (McConnell et al., 1997), and the highest value was subsequently used for analysis (K. L. Hinde et al., 2020). Considering the high variability in performing these tests within the same subject, each participant had a comprehensive familiarisation with the maneuvers and received verbal encouragement to maintain a maximal effort throughout all sessions. All maneuvers were performed standing with subjects' backs leaning on the wall in order to avoid abdominal muscle contraction during trials.

Data Analysis

Maximal workload (WL_{max}) achieved at athlete's exhaustion during all incremental tests was determined according to the following equation: WL_{max}(km/h)= speed last stage completed (km/h)+ [t(s)/step duration(s) * speed increment(km/h)], where t is the time of the uncompleted stage (Kuipers F T J; Keizer, H A; Geurten, P; van Kranenburg, G, 1985).

Absolute maximum PRE-to-POST change in spirometry values (FVC, FEV1, FEV1/FVC, PEF, MEF50% and FEF25–75%) were calculated in raw units as well as maximum percentage change ((pre-exercise - minimum post exercise)/(pre-exercise value) x 100) based on previously published protocol (Stensrud et al., 2007). The minimum post-exercise value was selected considering the time point after exercise which presented the maximum delta from pre-trial values (i.e. each trial may present the lowest post-exercise value at a different time-point). Moreover, the absolute changes in spirometry measures from all post-trial time points - the pre-trial value were calculated, and then relative changes were derived as explained above (Kennedy et al., 2020). Absolute and relative changes from PRE to selected POST-exercise MIP and MEP were also calculated (K. Hinde et al., 2018).

Ventilatory data throughout all experimental sessions were processed and analysed with MATLAB 7.0 (The MathWorks, Inc., Natick, MA, USA), using a customized code to calculate minute ventilation ($\dot{V}E$), respiratory frequency (Rf) and tidal volume (VT). Maximal values of HR, $\dot{V}E$, Rf, VT and SpO2 were averaged over the last 40 seconds registered during the last or the last but one stages of the CPET (since in some cases subjects completed less than 2 minutes during the last stage and given the 2-min recovery phase between stages, at the end of the test some parameters were still rising), and during the last minute of the EBPT.

Statistical Analysis

Descriptive analysis was used to report the results (mean and Standard Error (SE)). All the data were tested for their normal distribution (Shapiro–Wilk test). In the first step, possible differences in spirometry and MIP and MEP pretrial values before being exposed to the environmental condition between experimental sessions were tested using a one-way RM ANOVA with 'condition' (1 vs. 2 vs. 3 vs. 4) as a within-subjects factor.

Generalized Estimating Equations (GEE) analysis was used to test the main effects of 'FiO2' (20.9% vs 13.5%) and 'temperature' (+18°C vs -20°C), as well as their "interaction", on maximum relative changes (using minimum POST-exercise value, independently of considered time-point) in spirometry (FVC, FEV1, FEV1/FVC,

PEF, MEF50% and FEF25–75%) and respiratory muscle strength (MIP and MEP) parameters. When an interaction effect (FiO2*temperature) was found, Sidak post hoc test was used for specific comparisons (Cunha et al., 2015). Subsequently, relative changes for spirometry outcomes at each post-exercise time-point were analyzed using GEE with 'FiO2', 'temperature', and 'time' (1-POST, 3-POST, 6-POST, 10-POST, and 15-POST) as within-subjects factors. When an interaction effect ('FiO2*temperature', 'FiO2*time', 'temperature*time' or 'FiO2*temperature*time) was found, Sidak post hoc test was used for specific comparisons. GEE was implemented to analyze data since 1 subject did not complete all experimental sessions, and another presented some missing data in post-exercise measurements in one of the experimental sessions. All data were analyzed using a standard statistical package (SPSS Inc, Chicago, Illinois, USA).

Finally, repeated measures correlations (https://lmarusich.shinyapps.io/shiny_rmcorr/) were used for determining the common within-individual association between maximal ($\dot{V}E_{max}$, VT_{max} and Rf_{max}) and EBPT ($\dot{V}E_{EBPT}$, VT_{EBPT}, Rf_{EBPT}) ventilatory responses and maximum relative changes in lung function (FVC, FEV1, FEV1/FVC, PEF, MEF50% and FEF25–75%) and MIP and MEP parameters.

The threshold for statistical significance was set at $p < 0.05$.

RESULTS

Exercising parameters

Main decreasing effects of hypoxia (all $p<0.05$) and cold (all $p<0.05$) without interaction were found for CPET maximal exercising time (T_{max}; N: 00:32:28±26, H: 00:25:16±22, C: 00:31:22±26, CH: 00:23:40±24 hours), HR_{max} (N: 190±7; H: 181±10; C: 184±9; CH: 176±8 bpm), $\dot{V}E_{max}$ (N: 163.3±21; H: 152±20.6; C: 139.5±20.7 ; CH: 136.2 ±25.6 L/min) and VT_{max} (N: 2.82±0.4; H: 2.6±0.5; C: 2.6±0.6; CH: 2.3±0.5 L/min). Oppositely, no general hypoxic or cold effect, but 'temp*FiO2' interaction effect ($p=0.013$) was seen on Rf_{max} (N: 64±11; H: 62±11; C: 61±11; CH: 65±13 bpm) which was significantly higher in CH than in C and H alone. SpO2min was lower in hypoxic conditions ($p<0.001$), with no effect of cold

nor interaction (N: 94.4±2.9; H: 75.1±3.5; C: 93.6±5.1; CH: 76.3±5.2%) (Callovini et al., 2023).

The results of the EBPT are presented in Table 2. The HR reached during the EBPT (HR_{EBPT}) was >95% of the respective environmental condition's HRmax, despite a slightly greater relative intensity reached in the hypoxic trials compared to the normoxic trials. Furthermore, as for HR_{max}, general decreasing effects of hypoxia and cold on HR_{EBPT} were found, with an additive rather than a synergistic effect of combined cold-hypoxic exposure on this parameter (Callovini et al., 2023).

Ventilatory responses at the end of the EBPT ($\dot{V}E_{EBPT}$, VT_{EBPT}, Rf_{EBPT}) were decreased in the cold conditions (general effect of 'temperature': $p<0.001$), but they did not show the general decreasing effect of 'hypoxia' that was detected at the end of the incremental test (Callovini et al., 2023). However, in all conditions, at least 80% of VE_{max} was reached at the end of EBPT. Interestingly, $\dot{V}E_{EBPT}$ expressed as a percentage of maximal values showed a general decreasing effect of 'cold' despite similar relative exercise intensity (see %max HR_{EBPT}). Despite no 'FiO2*temperature' interaction, as already seen for $\dot{V}E_{max}$ (Callovini et al., 2023), partial additive effect of cold and hypoxia on $\dot{V}E_{EBPT}$ in CH has been maintained (H: -2.2%, C: -18.2% ; CH: -19.6% if compared to N). This comes with a lack in increasing Rf (which was seen instead for Rf_{max} (Callovini et al., 2023)), with the lowest value registered for the CH condition.

Table 2 Exercising results of the EBPT test.

		N		H		C		CH		FiO2	temp	FiO2* temp
		mean	SE	mean	SE	mean	SE	mean	SE			
HR	bpm	184 ± 2		178 ± 2		180 ± 2		172 ± 2		**<0.001**	**<0.001**	0.371
HR	%max	96.4 ± 0.6		98.3 ± 0.4		97.1 ± 0.5		98.2 ± 0.6		**0.002**	0.458	0.336
Ve	L/min	140.8 ± 5.7		137.1 ± 4.9		116.1 ± 5.0		114.1 ± 5.4		0.350	**<0.001**	0.675
Ve	%max	88.3 ± 2.1		92.3 ± 2.0		84.3 ± 2.8		86.4 ± 3.2		0.133	**0.021**	0.620
Rf	bpm	57.3 ± 2.3		59.4 ± 3.2		54.1 ± 2.8		53.1 ± 2.3		0.771	**0.001**	0.276
Vt	ml/min	2451 ± 92		2390 ± 134		2196 ± 113		2179 ± 115		0.515	**<0.001**	0.707
RPE		96 ± 1		92 ± 2		90 # ± 2		95 & ± 1		0.507	0.415	**<0.001**
SpO2	%	95 ± 1		79 ± 1		95 ± 1		78 ± 1		**<0.001**	0.738	0.975

Data reported as mean ± SE. HR, Heart Rate; Ve, Ventilation; Rf, Respiratory Frequency; Vt, Tidal Volume; RPE, Rate of Perceived Exertion; SpO_2, arterial O_2 saturation recorded at the end of the Exercise Broncho Provocation Test (EBPT). %max HR_{EBPT} and Ve_{EBPT} represent HR and Ve reached at the end of EBPT expressed as percentages of maximal values measured at the end of CPET in respective condition. FiO2, Fraction of inspired Oxygen; temp, ambient temperature. N: 18°C, 20.9% FiO2; H; 18°C, 13.5% FiO2; C: -20°C, 20.9% FiO2; CH: -20°C, 13.5% FiO2. # C≠N; C≠CH. Bold characters represent statistical significance ($p<0.05$)

Lung function and respiratory muscle strength

Pre-Trial Values (see Table 1 supplemental material for overall absolute pre and minimum post values)

No differences in PRE values between conditions were found for MIP (p=0.577) and MEP (p=0.980). Similarly, no differences in PRE FVC (p=0.944), FEV1 (p=0.859), FEV1/FVC% (p=0.954), PEF (p=0.893), FEF 25-75% (p=0.900) and MEF 50% (p=0.939) were detected between N, H, C and CH conditions.

Maximum relative PRE to POST changes

The outcomes for maximal PRE-to-POST changes in lung function and respiratory muscle strength are shown in Table 3. Changes in MIP and MEP values showed no general effect of 'FiO2' or 'temperature', nor a 'FiO2*temperature' interaction (see Figure 2).

Furthermore, no general effect of 'FiO2' nor 'FiO2*temperature' interaction was found in maximum relative PRE to POST EBPT changes for any of the considered lung function variables. However, except for FVC, a general effect of 'temperature' was found in all other parameters (all $p<0.001$), whose relative decrease from PRE-exercise trial values was significantly higher in the cold conditions if compared to the normothermic ones. Considering a Δ% change in FEV1 >10% as an index of EIB (Anderson & Daviskas, 2000b), 3 subjects developed mild EIB in N (21% incidence), with only 2 of them developing mild EIB also in H (14% incidence). Moreover, the 3 subjects that developed EIB in N presented this phenomenon also in C and CH conditions, with 2 other subjects in C (total number of subjects developing EIB in C= 5, 36% incidence) and one in the CH condition (total number of subjects in CH:4, 29% incidence). However, in all experimental conditions mild EIB severity at most was detected (see Table 2 supplemental material).

Table 3 Maximal decrease for MIP and MEP measurements post incremental test and FVC, FEV1, FEV1/FVC, PEF, FEF25-75 and MEF 50 measurements post Exercise Broncho Provocation Test (EBPT) in each environmental condition. Delta changes are expressed as percent change from pre-test values.

		N		H		C		CH		FiO2	temp	FiO2* temp
		mean	SE	mean	SE	mean	SE	mean	SE			
MEP	%	-6.5	± 3.4	-2.7	± 3.5	-12.3	± 4.4	-7.9	± 4.7	0.188	0.168	0.928
MIP	%	-5.3	± 2.6	1.8	± 4.5	-7.5	± 4.1	-6.4	± 3.3	0.201	0.171	0.382
FVC	%	-7.2	± 1.7	-7.6	± 1.9	-6.1	± 1.2	-8.0	± 1.4	0.284	0.619	0.423
FEV1	%	-2.8	± 1.6	-3.7	± 1.6	-7.6	± 1.5	-7.6	± 1.9	0.667	**<0.001**	0.589
FEV1/FVC	%	1.8	± 0.7	1.2	± 1.0	-3.3	± 0.9	-1.5	± 1.2	0.468	**<0.001**	0.135
PEF	%	-5.9	± 2.4	-4.9	± 1.7	-9.5	± 1.8	-12.6	± 2.8	0.571	**<0.001**	0.243
FEF25-75	%	3.0	± 2.8	0.3	± 2.4	-11.6	± 2.6	-6.8	± 3.7	0.682	**<0.001**	0.197
MEF 50	%	1.5	± 3.0	-3.0	± 2.0	-10.4	± 2.5	-12.0	± 4.6	0.273	**<0.001**	0.532

Data reported as mean ± SE. MIP, maximal inspiratory pressure, MEP, maximal expiratory pressure; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 s; PEF, peak expiratory flow; FEF 25-75, forced expiratory flow at 25%–75%, MEF 50, mid expiratory flow at 50%. FiO2, Fraction of inspired Oxygen; temp, ambient temperature. N: 18°C, 20.9% FiO2; H; 18°C, 13.5% FiO2; C: -20°C, 20.9% FiO2; CH: -20°C, 13.5% FiO2. Bold characters represent statistical significance ($p<0.05$).

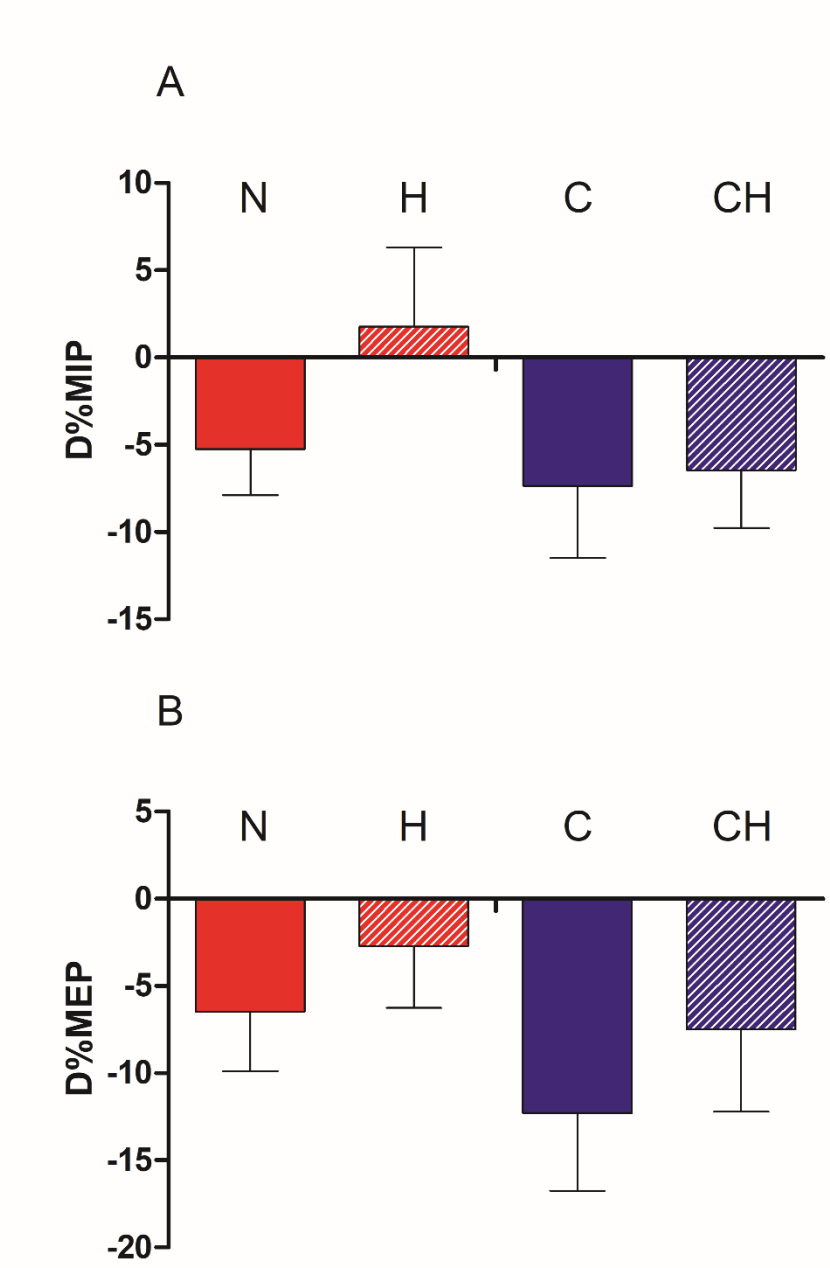

Figure 2 Acute MIP (Maximal Inspiratory Pressure, 2A) and MEP (Maximal Expiratory Pressure, 2B) changes expressed as % from pretrial values. Negative values showed a decrease from pretrial values. N: 18°C, 20.9% FiO2; H; 18°C, 13.5% FiO2; C: -20°C, 20.9% FiO2; CH: -20°C, 13.5% FiO2. Red columns: normothermia; Blue columns: cold; Plain columns: normoxia; Lined columns: hypoxia.

Relative changes at each post-exercise time point

A graphical representation of the results is presented in Figure 3. Considering FVC, no general 'FiO2' or 'temperature' effects on changes in this parameter were found, but a 'FiO2*time' interaction revealed that Δ% change in FVC was significantly higher at 3-POST if compared to 15-POST in the hypoxic conditions, regardless of temperature. For FEV1, a general effect of 'temperature' was detected, with Δ% changes being higher in cold conditions. However, a general effect of 'time' and a 'temperature*time' interaction effect revealed that this parameter tends to decrease from 1-POST to 10-POST regardless of environmental condition, starting to go back to normal values at 15-POST (in normothermic conditions: 1-POST≠10-POST; in the cold: 1-POST≠3-POST; 1-POST≠6-POST; 1-POST≠10-POST). Similarly, Δ% change in FEV1/FVC% showed a general effect of 'temperature', being significantly higher in the cold conditions, but also a general effect of 'time', which revealed that this parameter tends to decrease from 1-POST to 10-POST in all experimental conditions, without returning to normal values at 15-POST. The general effect of 'temperature' was maintained also for PEF, as well as the 'time' effect, showing a decrease in this parameter from 1-POST to 15-POST in all conditions. However, a 'FIO2*temperature*time' interaction effect showed that Δ% change in PEF is significantly higher in CH than in C alone at 3-POST exercise ($p=0.069$). Finally, also FEF 25-75% and MEF50% showed similar general effects of 'temperature' and 'time', with both parameters showing an increase immediately post-exercise cessation in all conditions (i.e.1-POST), tending to return to baseline values in normothermia or below this values in the cold at 15-POST.

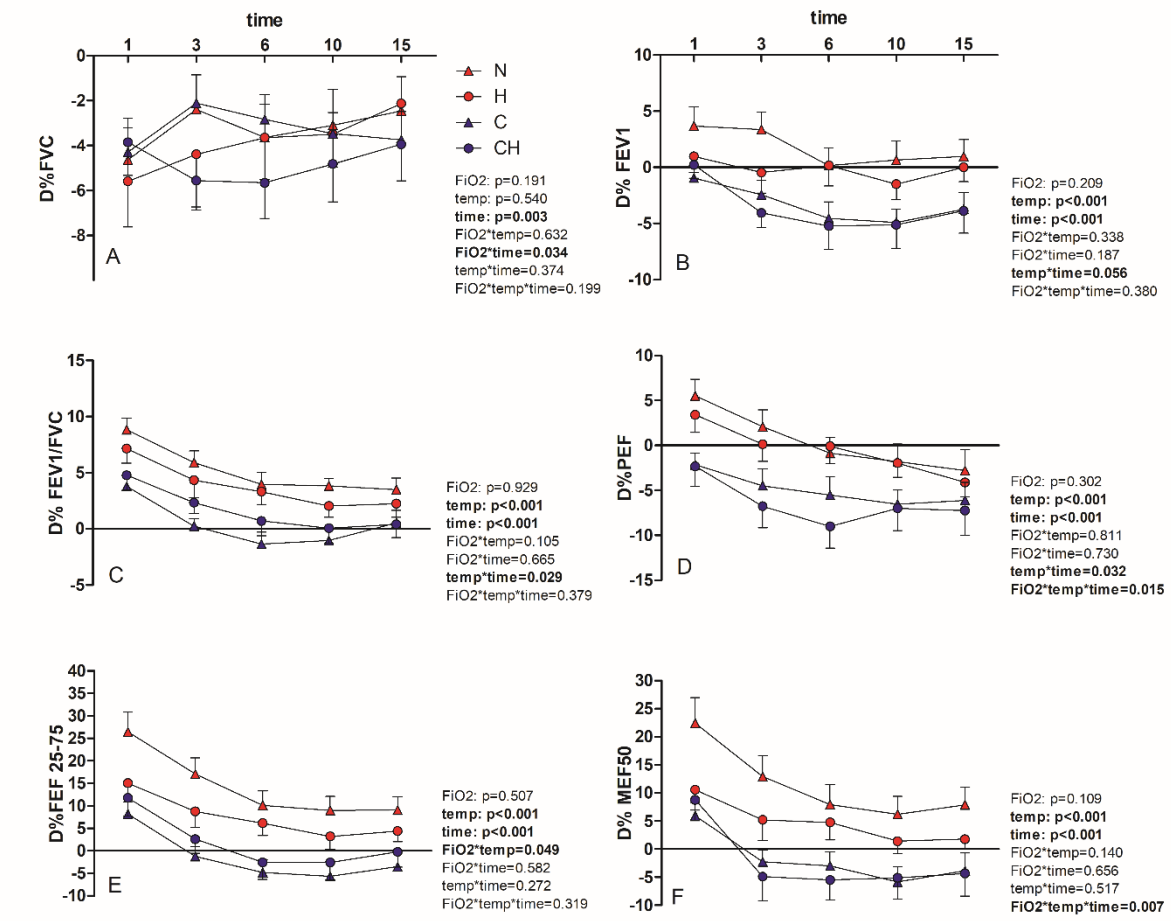

Figure 3 Acute lung function recovery variables at each post-exercise time point (1-POST, 3-POST, 6-POST, 10-POST, 15-POST) for FVC (A), FEV1 (B), FEV1/FVC (C), PEF (D), FEF25-75% (E) and MEF50% (F) expressed as % from pretrial values separately for normothermic normoxia (N, red triangles), normothermic hypoxia (H, FiO2 13.5%; red circles), cold normoxia (C,−20 °C; blue triangles) and cold-hypoxia (CH, blue circles). Negative values show a decrease from pretrial values. Values are presented as means±SE. FVC, forced vital capacity; FEV1, forced expiratory volume in 1 s; PEF, peak expiratory flow; FEF 25-75, forced expiratory flow at 25%–75%, MEF 50, mid expiratory flow at 50%.

Relationship between changes in LF, respiratory muscle strength and ventilatory data during exercise

The within-individual association between maximum PRE-to-POST exercise changes in MIP, MEP, FVC, FEV1, FEV1/FVC, PEF, FEF25-75 and MEF 50 and absolute ventilatory data during both incremental ($\dot{V}E_{max}$, VT_{max}, Rf_{max}) and EBPT ($\dot{V}E_{EBPT}$, VT_{EBPT}, Rf_{EBPT}) is presented in Table 4. The reader should be advised that when dealing with data presented as Δ% changes from pretrial values, they may display negative outcomes (i.e. -10% of PRE-test value); consequently, a positive correlation indicates that the greater is the decrease in the parameter, the lower is the value of absolute ventilatory responses during exercise; on the contrary, when lung function and respiratory muscle strength show an increase at POST exercise testing, the values of ventilatory data during exercise were also higher (Fig.4, showing the relationship between Δ% change in FEV1 and $\dot{V}E_{max}$ and $\dot{V}E_{EBPT}$ is explicative of this concept).

Δ% changes in MIP positively correlated with $\dot{V}E_{EBPT}$ ($r=0.325$, $p=0.041$), whereas no other significant correlations between respiratory muscle strength changes and ventilatory responses during exercise were found. A significant positive correlation (all $p<0.05$, descriptor: moderate ($0.35<r< 0.65$)) was also found between Δ% changes in FEV1, FEF 25-75%, MEF 50%, FEV1/FVC and PEF and $\dot{V}E_{EBPT}$, VT_{EBPT}, and $\dot{V}E_{max}$ (except for Δ%PEF).

Table 4 Results of repeated measure correlation analysis between MIP, MEP, FVC, FEV1, FEV1/FVC, PEF, FEF25-75 and MEF-50 expressed as % from pretrial values and ventilatory parameters at the end of incremental (Ve_{max},Vt_{max},Rf_{max}) and Exercise Broncho Provocation Test (Ve_{EBPT}, Vt_{EBPT}, Rf_{EBPT}).

		Vemax	Rfmax	Vtmax	VeEBPT	RfEBPT	VtEBPT
D% MEP	**rrm**	.259	.035	.224	.152	-.019	.201
	p	.098	.825	.154	0.342	.905	0.207
D% MIP	**rrm**	.219	.074	0.095	**.325**	.192	.161
	p	.170	.645	.556	**.041**	.234	0.32
D% FVC	**rrm**	-.072	**-.359**	.186	-.118	-.224	.030
	p	.653	**.021**	.243	0.47	0.166	0.856
D% FEV1	**rrm**	**0.425**	-.081	**.436**	**.571**	.301	**.377**
	p	**.006**	.613	**.004**	**0.001**	0.059	**0.016**
D% FEV1/FVC	**rrm**	**.447**	.194	.263	**.533**	.292	**.412**
	p	**.003**	.225	.097	**0.001**	0.067	**.008**
D% PEF	**rrm**	.239	-.142	**.355**	**.470**	**.315**	**.302**
	p	.133	.376	**.023**	**0.002**	**0.048**	**.058**
D% FEF25-75	**rrm**	**.329**	.076	.236	**.536**	.300	**.365**
	p	**.033**	.630	.133	**.001**	.057	**.019**
D% MEF50	**rrm**	**.497**	.188	**.308**	**.539**	**.330**	**.361**
	p	**.001**	.240	**.050**	**0.001**	**.038**	**.022**

MIP, maximal inspiratory pressure, MEP, maximal expiratory pressure; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 s; PEF, peak expiratory flow; FEF 25-75, forced expiratory flow at 25%–75%, MEF 50, mid expiratory flow at 50%. Ve_{max},Vt_{max},Rf_{max} and Ve_{EBPT}, Vt_{EBPT}, Rf_{EBPT} ; Ventilation, Tidal Volume and Respiratory Frequency at the end of maximal CPET and Exercise Broncho Provocation Test, respectively. Significant correlation have been presented in bold characters. Statistical significance has been set at $p<0.05$.

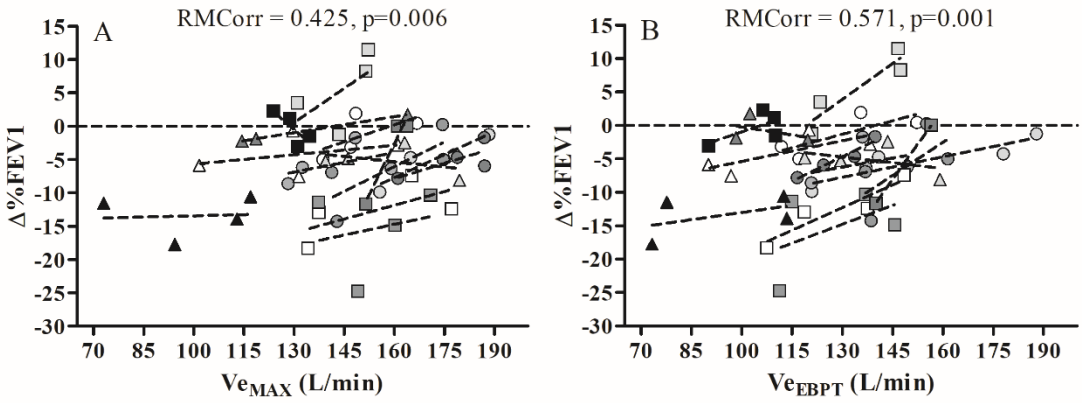

Figure 4 Relationship between PRE-to-POST exercise changes in forced expiratory volume over one second (FEV1%) and Ventilation at the end of CPET (Ve_{max}) and Exercise Broncho Provocation Test (Ve_{EBPT}). Same symbols represent the 4 experimental conditions for each subject, dotted lines represent singular subjects' correlation considering the 4 experimental trials.

DISCUSSION

Despite real-life high altitude exposure often occurring concurrently with extremely cold temperatures, studies on their combined effect on several physiological mechanisms that may limit exercise practice and/or pose health risks for practitioners are highly underrepresented in literature (Mugele et al., 2021; Tipton, 2012). Specifically, scarce information regarding the possible interactive effects of cold and hypoxia on impairments of lung function and respiratory mechanics is present. To the best of our knowledge, this is the first study evaluating together lung function and respiratory muscle strength in a cold-hypoxic environment, as well as their relationship to exercising ventilatory parameters during high-intensity exercise. The key findings of this study confirmed previously demonstrated (Kennedy et al., 2019b; Kennedy & Faulhaber, 2018) cold-induced impairment of lung function for temperatures lower than -15°C, with Δ% PRE-to-POST changes in FEV1, FEV1/FVC, PEF, FEF 25-75% and MEF 50% being significantly higher in the cold when compared to normothermic conditions, regardless of inspired FiO2. Conversely, no significant Δ% changes in MIP and MEP were found in any

of the presented experimental conditions. Within individuals correlation analysis revealed that the conditions with greater PRE-to-POST lung function and MEP impairments (i.e. C and CH) were the ones with the lowest peak ventilatory responses during exercise.

Lung function

Lung function results have been presented as both maximal relative changes from baseline values as well as changes at each post-exercise time-point based on previous data (Kennedy et al., 2020): maximum PRE to POST change in FEV1, FEV1/FVC, PEF, FEF 25-75% and MEF 50% presented a general effect of cold, being the maximal decrease in these parameters higher in C and CH conditions if compared to the normothermic ones. Furthermore, despite high inter-individual variability, single post-exercise time point data suggest that peak reduction happens between 6 to 10 minutes after exercise cessation, as previously demonstrated in the literature (Parsons et al., 2013). However, since the airway response is expressed as the percent fall in FEV1 from the baseline value, and EIB is diagnosed for decreases ≥10% (Anderson & Daviskas, 2000b), our results showed that the highest EIB incidence occurred in C (36%), but 3 out of 5 subjects already presented bronchoconstriction at the end of the N trial: given that our subjects do not exhibit the typical characteristics of asthmatics, one of the reasons for EIB development in normothermic conditions could be increased airway responsiveness to stimuli such as increased ventilation during exercise. This non-asthmatic airway responsiveness may be related to bronchial epithelial damage secondary to chronic cold-exposure exercise practice, especially considering that all subjects live in the north of Italy and some of them regularly practice winter sports. Unfortunately, we cannot draw definitive conclusions as we lack detailed information on the airway responsiveness of our sample (Bougault et al., 2009). If this were the case, the cold would represent an additional stimulus, but high ventilation during exercise in N would be sufficient to generate EIB in these subjects. Going deeper into FEV1 changes (see Table 2, supplemental material), it emerges that more subjects presented a post-exercise decrease in this parameter in the cold compared to the normothermic conditions. Simultaneously, flow-related measurements (FEF 25-75% and MEF 50%) showed

an increase immediately post-exercise in all conditions, probably due to exercise-induced airway shear stress and consequent bronchodilation, at least of proximal airways (Tucker et al., 2017), with this increase being maintained over 15-min after exercise cessation only in the normothermic trials. Finally, PEF is the parameter that showed the highest differences between normothermic and cold trials, slightly increasing for the former and decreasing for the latter conditions, especially in CH. Overall, these data confirm that intense exercise in the cold has an impact on post-exercise lung function, but the magnitude of this impact is still unclear and seems to be highly individual-dependant. Furthermore, environmental but most of all inhaled air temperature plays a pivotal role in triggering EIB response. In fact, also Kennedy and colleagues (Kennedy & Faulhaber, 2018) reported a 7%, 11% and 12% mean decrease in FEV1, FEF25-75% and FEF50%, respectively, after exercise at -20°C in a female cohort, as well as 3% and 4% decrease in FVC and FEV1 and a 10 to 12% decrease in FEF25-75%, FEF50% and PEF in highly trained male athletes after a cold air trial at -15°C (Kennedy et al., 2019b). Also, they reported a similar ≈7% post-exercise decrease in FEV1 at -20°C between men and women (Kennedy et al., 2020): however, in this study an increase in FEF 25-75% and FEF50% at 3 minutes post-exercise at 0°C but not at -20°C was detected, suggesting that exercise induced bronchodilation still happens for ambient temperatures ≈0 °C. Similarly, Sandsund et al. (Sandsund et al., 1997) demonstrated that exercise at a -15°C ambient temperature but inhaling air at -2°C had an overall positive effect on postexercise lung function in non-asthmatic athletes.

It should be noted that our study design may have affected the results in two important but opposite ways; firstly, post-exercise spirometry was performed at a constant ambient temperature of +20°C, this paradoxically being shown to exacerbate drying of the airway (McFadden et al., 1985), a phenomenon known as the 'burden of rewarming' (Farley et al., 1988). On the other hand, the EBPT has been performed 10' after a maximal CPET, and it is known that the time since the last exercise is important when assessing EIB, because some subjects become refractory to another exercise stimulus for up to 4 hours (Parsons et al., 2013), blunting EIB responses (Bonini, 2018). Potential physiological mechanisms have been proposed as to why exercise would cause a refractory period of EIB, including

depletion of catecholamines, increased circulation of prostaglandin and consequent desensitization of bronchial smooth muscle receptors, or degranulation of mast cell mediators which can lead to the temporary depletion of inflammatory mediators in the bronchial tissue (Stickland et al., 2012). However, Rundell and colleagues (K.W. et al., 2003) demonstrated no period of refractoriness when a second bout of interval-type exercise is initiated within 20 minutes after the completion of a prior session in elite cross-country skiers who were not asthmatic but suffered from EIB. Since depletion of mast cells seems to be crucial in refractory phenomena, but EIB development in non-asthmatic subjects partially follows a different pathway than in the asthmatic ones (Bougault et al., 2009), it is possible that the former do not present a marked refractory period. Moreover, Beck et al. (Beck et al., 1994) did not find significant refractoriness among eight laboratory-tested subjects with demonstrated exercise-induced asthma performing 36 min of '6 min on 6 min off' interval exercise, suggesting that refractoriness does not occur after interval-type exercises (similar to the one proposed in this study) even in an asthmatic population. To summarize, no important further impairment of combined stressor exposure (i.e., CH) on EIB insurgence following high-intensity exercise of short durations has been found. However, future studies should verify whether different exercising modes, comprising prolonged medium to high-intensity exercise, may lead to additive and/or synergistic effects of cold and hypoxia on considered variables.

Respiratory muscle strength

Our results showed no significant changes in respiratory muscle strength between PRE and POST CPET evaluation either in N or in one of the other conditions. This outcome partially disagrees with the results of Oueslati and colleagues (Oueslati et al., 2018), who demonstrated a ≈13% significant decrease in both MIP and MEP values after maximal normoxic running and cycling incremental tests to exhaustion in trained individuals. It is possible that the intermittent nature of the incremental test in the presented study (4-min of work interspersed by 2-min recovery period) allowed the respiratory muscles to recover, potentially delaying the onset of fatigue (Kurti, 2011). Interestingly, the only notable decrease observed was in MEP of the C condition, which mirrored the findings reported by Oueslati and colleagues,

showing a comparable reduction of -12.28% from PRE values. This may indicate an increased mechanical load on the respiratory muscles (mostly the expiratory ones) while exercising in this condition. A possible explanation, partially related to lung function results discussed above, could be a change in the ventilatory strategy adopted during exercise in the cold, which may have caused the diaphragm and the other respiratory muscles to work at a suboptimal portion of the length-tension curve, determining a loss of efficiency and consequent increased respiratory muscle work (Mediano et al., 2017). Also, reduced muscle temperature in the cold has been linked to decreased maximal force generation (K. Hinde et al., 2018; Lloyd et al., 2016; Oksa et al., 2004), but the authors tend to exclude this hypothesis since participants were dressed up properly to not feel cold at the end of the exercise**.**

Interestingly, the lowest impact between PRE-to-POST exercise changes in MIP and MEP outcomes has been found in H. Previous studies reported increased respiratory muscle fatigue in hypoxia if compared to normoxia due to i) increased ventilatory response (i.e. hypoxic ventilatory response) and consequently increased work of breathing for the same normoxic absolute external WL (Babcock et al., 1995; Vergès et al., 2005) and ii) decreased O2 availability that might enhance respiratory muscle fatigue per se, even when ventilatory demands between normoxia and hypoxia are matched: in fact, this situation forces the diaphragm to use anaerobic energy-generating processes, thus increasing diaphragm lactate production for similar ventilation rates of normoxic exercise and consequently anticipating the onset of fatigue for this muscle (Vogiatzis et al., 2007). In this study, respiratory muscle strength as an indirect index of fatigue has been measured, this possibly being a reason for the failed detection of greater impairments in hypoxia. In fact, Gudjonsdottir and colleagues (Gudjonsdottir et al., 2001) reported impaired diaphragmatic force-generating capacity measured by transdiaphragmatic pressure changes after a 10-min incremental test to exhaustion at high altitude if compared to equivalent work rates at sea level, concluding that hypoxia alone was the predominant causative factor of these impairments. Furthermore, the intermittent incremental protocol adopted in the present study may have caused the duration of continuous exercising time spent above 85% of $VO2_{max}$ to have been

too short for consistent reductions in respiratory muscle strength to occur (Johnson et al., 1993).

Finally, based on previous studies, additive, if not synergistic, effects of cold and hypoxia would have been expected on post-exercise respiratory muscle strength in CH. In fact, Hinde and colleagues (K. Hinde et al., 2018) found an additive effect of the two environments (FiO2 11.8%, temperature -10°C) on MIP decrements after an approximately 2-hour walk at different speeds and slopes independently of carrying or not an 18.2 kg backpack, while maintaining an exercising intensity ≈40%$VO2_{max}$. However, in this present study, the aforementioned reasons related to study design, especially the short and intermittent exercising time, generated an antagonistic inter-stressor effect in CH, meaning that impairments in both MIP and MEP resulted higher in single stressor (i.e. C) compared to the combined stressors (i.e. CH) exposure.

Lung function, respiratory muscle strength and ventilatory data during exercise

Multiple correlation analysis revealed a positive correlation between exercising ventilatory responses and post-exercise LF, showing a trend towards lower $\dot{V}E$ and VT values during exercise (i.e. C and CH conditions) being associated to higher post-exercise air-way responses. A possible explanation of our results is related to the theory that ventilatory strategy adopted during exercise might have consequences on post-exercise bronchoconstriction (Mediano et al., 2017): specifically, Mediano et al. demonstrated high dynamic hyperinflation (DH) prevalence during exercise in patients with asthma and EIB. DH refers to the temporary increase in operating lung volumes above their resting values while exercising, due to an increase in end expiratory lung volume (Stickland et al., 2022) as a consequence of the obstruction of the peripheral airways that results in inadequate ventilation (Kominami et al., 2023). Accordingly, our hypothesis is that exercising in the cold may trigger partial bronchoconstriction of distal airways already during exercise, and several minutes after exercise cessation, proximal airway narrowing occurs, as represented by the decrease in FEV1. These changes in operating lung volumes would also corroborate the theory that a tendency

towards greater respiratory muscle strength impairments in the cold is due to the respiratory muscle fibres working at a suboptimal portion of the length-tension curve (see above). However, these are only hypotheses, as there have been no actual measurements of Inspiratory Capacity during exercise to derive end-expiratory lung volume and assess the occurrence of dynamic hyperinflation while exercising in both cold and/or hypoxic conditions. Finally, contrary to the authors' hypothesis, no additional detrimental effects on ventilatory responses during exercise in CH were observed, probably due to the fact that the proposed study design did not superimpose respiratory muscle fatigue to lung function impairments during combined stressors exposure. Future studies should focus on gaining a more comprehensive understanding of these phenomena.

LIMITATIONS

The results of the study have to be interpreted in light of these considerations: first of all, the fact that we tested normobaric rather than hypobaric hypoxia exposed the participants to greater respiratory load and flow limitations due to air density not being decreased as in real high altitude exposure (Cogo et al., 1997; Deboeck et al., 2005). Moreover, we did not test the baseline bronchial responsiveness, missing interesting information concerning the broncho-reactivity of our subjects, which could have helped in the interpretation of the results. Finally, exercise modality (especially duration and intermittent nature) may have caused the hypoxic effect on respiratory muscle strength to be blunted, thus not permitting the evaluation of the actual presence of a possible interactive effect of cold and hypoxia on this parameter.

CONCLUSION

Our findings reveal that lung function and respiratory muscle strength are negatively impacted by cold exposure. However, the effects of hypoxia, both as an independent stressor and in combination with cold, on the respiratory system require further investigation, specifically when considering prolonged exercise at intensities exceeding 85% of $VO2_{max}$. Furthermore, a relationship between ventilatory responses to exercise and LF impairments has been found, suggesting

that the development of EIB post-exercise cessation and the manifestation of ventilatory constraints during exercise may be, to some extent, related. In conclusion, these results seem to exclude additive or interactive effects of cold and hypoxia on considered parameters, but further studies are necessary to exclude different outcomes when considering prolonged, medium-to-high-intensity exercising tasks.

SUPPLEMENTAL MATERIAL

Table 1 Absolute PRE and POST incremental test MIP and MEP measurements and PRE and POST Exercise Broncho Provocation Test (EBPT) FVC, FEV1, FEV1/FVC, PEF, FEF25-75 and MEF 50 measurements in each environmental condition.

Characteristics		N				H				C				CH			
		pre		post		pre		post		pre		post		pre		post	
		mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
MEP	cmH2O	144.0 ±	12.6	137.0 ±	14.5	137.6 ±	10.5	135.4 ±	13.4	144.8 ±	11.7	124.2 ±	9.3	141.3 ±	13.3	133.6 ±	16.0
MIP	cmH2O	119.8 ±	26.1	113.9 ±	30.6	114.9 ±	25.6	117.1 ±	32.9	129.9 ±	33.9	117.8 ±	36.6	119.9 ±	27.7	112.9 ±	32.7
FVC	L	5.5 ±	0.1	5.1 ±	0.1	5.5 ±	0.1	5.1 ±	0.1	5.6 ±	0.1	5.2 ±	0.1	5.4 ±	0.1	5.0 ±	0.1
FEV1	L/min	4.3 ±	0.1	4.2 ±	0.1	4.3 ±	0.1	4.2 ±	0.1	4.4 ±	0.1	4.1 ±	0.1	4.2 ±	0.1	3.9 ±	0.1
FEV1/FVC	ratio	78.3 ±	1.3	79.7 ±	1.5	79.0 ±	1.3	79.9 ±	1.4	79.0 ±	1.5	76.4 ±	1.4	78.0 ±	1.7	76.9 ±	2.1
PEF	L/min	10.4 ±	0.4	9.8 ±	0.5	10.4 ±	0.4	9.9 ±	0.4	10.6 ±	0.3	9.7 ±	0.4	10.2 ±	0.3	9.0 ±	0.4
FEF25-75	L/min	3.9 ±	0.2	4.0 ±	0.3	4.0 ±	0.2	4.0 ±	0.2	4.1 ±	0.3	3.6 ±	0.2	3.8 ±	0.2	3.5 ±	0.3
MEF50	L/min	4.5 ±	0.3	4.5 ±	0.3	4.6 ±	0.3	4.5 ±	0.3	4.6 ±	0.3	4.1 ±	0.3	4.4 ±	0.3	3.8 ±	0.3

Data reported as mean ± SE. MIP, maximal inspiratory pressure, MEP, maximal expiratory pressure; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 s; PEF, peak expiratory flow; FEF 25-75, forced expiratory flow at 25%–75%, MEF 50, mid expiratory flow at 50%. N: 18°C, 20.9% FiO2; H; 18°C, 13.5% FiO2; C: -20°C, 20.9% FiO2; CH: -20°C, 13.5% FiO2.

Table 2 Absolute PRE and POST Exercise Broncho Provocation Test (EBPT) FEV1 measurements, as well as D% PRE-to-POST change in FEV1, for each tested subjects.

	N			H			C			CH		
	pre	**post**	**D%**	**pre**	**post**	**D%**	**pre**	**post**	**D%**	**pre**	**post**	**D%**
sbj 1	4.71	4.73	0.4	4.7	4.79	1.9	5.01	4.76	-5.0	4.72	4.57	-3.2
sbj2	4.77	4.71	-1.3	4.7	4.5	-4.3	4.86	4.38	**-9.9**	4.26	4.06	-4.7
sbj 3	3.94	3.69	-6.3	4.05	3.98	-1.7	3.88	3.64	-6.2	4.06	3.71	-8.6
sbj 4	3.91	3.92	0.3	4.18	3.97	-5.0	4.04	3.76	-6.9	4.2	3.6	**-14.3**
sbj 5	4.13	4.06	-1.7	4.5	4.29	-4.7	4.53	4.26	-6.0	4.35	4.01	-7.8
sbj 6	4.2	3.68	**-12.4**	4.06	3.76	-7.4	4.2	3.43	**-18.3**	4.25	3.7	**-12.9**
sbj 7	3.05	3.4	11.5	3.14	3.4	8.3	3.22	3.18	-1.2	3.14	3.25	3.5
sbj 8	4.26	3.82	**-10.3**	4.04	3.44	**-14.9**	4.22	3.74	**-11.4**	3.96	2.98	**-24.7**
sbj 9	3.92	3.92	0.0				4.21	3.72	**-11.6**	3.66	3.66	0.0
sbj 10	4.43	4.48	1.1	4.57	4.5	-1.5	4.42	4.52	2.3	4.24	4.11	-3.1
sbj 11	4.58	4.45	-2.8	4.22	4.19	-0.7	4.51	4.17	-7.5	4.44	4.18	-5.9
sbj 12	4.92	4.52	-8.1	4.91	4.79	-2.4	4.95	4.71	-4.8	4.92	4.66	-5.3
sbj 13	4.59	4.67	1.7	4.5	4.4	-2.2	4.77	4.68	-1.9			
sbj 14	4.91	4.39	**-10.6**	4.74	4.08	**-13.9**	4.73	3.89	**-17.8**	4.86	4.3	**-11.5**
mean	4.31	4.17	-2.8	4.33	4.16	-3.7	4.40	4.06	-7.6	4.24	3.91	-7.6
sd	0.51	0.44	6.3	0.46	0.45	6.1	0.49	0.51	5.8	0.48	0.49	7.2
EIB prevalence		21%			14%			36%			29%	

Number in bold font indicate Exercise Induced Bronchoconstriction (EIB) development. N: 18°C, 20.9% FiO2; H; 18°C, 13.5% FiO2; C: -20°C, 20.9% FiO2; CH: -20°C, 13.5% FiO2.

STUDY 3 – SEX DIFFERENCES IN CARDIAC AND RESPIRATORY RESPONSES DURING EXERCISE UNDER HYPOXIC CONDITIONS

ABSTRACT

Introduction: Sex differences in exercise physiology are underrepresented in the literature, particularly in specific contexts such as under conditions of reduced oxygen availability (Normobaric Hypoxia, NH). The aim of this study was to investigate the influence of sex on the cardiac and respiratory responses to exercise at sea level (SL) and NH.

Methods: 10 women (F; $VO2_{max}$:49±4.2mL/kg/min) and 10 height-matched men (M; $VO2_{max}$:58.3±5.9 mL/kg/min) underwent a $VO2_{max}$ test, at SL (20.9 FiO2) and at a simulated altitude of 4500m (11.8 FiO2). These were followed by two pulmonary function (PF) evaluation sessions, one at SL and one at NH, where cardio-respiratory responses, Inspiratory Capacity (IC), and Diffusion Capacity (DLCO) were assessed during moderate (MOD), heavy (HEAV) and severe (SEV) exercise. Subsequently, considered parameters' values corresponding to 80% of $VO2_{max}$ were calculated for each subject at SL and NH, respectively.

Results: $VO2_{max}$ (F:-30.6%, M:-30.4%), VE_{max} (F:-6.3%, M:-7%) and HR_{max} (F:-5.3%, M:-5.7%) were significantly reduced at NH (all $p<0.001$). Absolute VO2 at 80%$VO2_{max}$ was significantly lower in females ($p<0.001$), but similar reductions between SL and NH values were observed for F (-30.7%) and M (-30.4%). SpO2 was significantly reduced at NH (≈ -29%), with no general effect of 'SEX', whereas [La] tended to be higher in females only at NH (FiO2*SEX interaction, $p=0.07$). HR and cardiac output (CO) were reduced at NH ($p<0.027$ and 0.023, respectively), but they did not show any effect of SEX or FiO2*SEX interaction. Ventilation (VE) and operational lung volumes (i.e. Inspiratory Capacity (IC) and Inspiratory Reserve Volume (IRV)) were lower in females at 80%$VO2_{max}$ both at SL and NH (SEX effect, $p<0.001$), but differences in IC and IRV disappeared once values were normalized for Total Lung Capacity. No effect of NH exposure on ventilatory parameters at 80%$VO2_{max}$ was detected. DLCO resulted lower in women ($p=0.003$) even when normalized for Alveolar Volume (DLCO/VA), and was significantly reduced in both sexes at NH ($p<0.001$). However, the effectiveness of pulmonary vascular recruitment (i.e. DLCO/CO) was unaltered by reduced oxygen availability, even though it was always significantly lower in females ($p<0.001$).

Conclusions: Similar ventilatory, lung diffusive, and cardiac changes in response to exercise at NH were detected in men and women, with no greater deterioration in females' physiological adjustments to exercise at NH when healthy trained subjects are considered.

INTRODUCTION

In the last decades, traveling to High Altitude (HA) has become increasingly popular for both recreational and performance interests (Chatre B, Lanzinger G, Macaluso M, 2010). As a consequence, scientific literature has recently focused on a better understanding of acute (José A.L. Calbet et al., 2009; Clark et al., 2007; Mourot, 2018; R. S.Mazzeo, 2006) and chronic (José A.L. Calbet & Lundby, 2009; S. A. Gallagher & Hackett, 2004; Mallet et al., 2023) physiological responses to exercise at HA, aiming at mitigating risks (J. Burtscher et al., 2023; Mazur et al., 2020; Richalet et al., 2012) and optimizing performance both when at altitude (Callovini et al., 2023; Fornasiero et al., 2020; Girard et al., 2017), as well as when returning to sea level (SL) (Kettunen et al., 2023; Gregoire P. Millet et al., 2010; Grégoire P. Millet et al., 2019; Mujika et al., 2019; Sinex & Chapman, 2015). Being HA exposure characterised by reduced oxygen availability, most attention has been posed on the oxygen cascade (David R. Bassett & Howley, 2000), conventionally described as an in-series system of four transport processes: ventilation, alveolar-capillary diffusion, circulation, and muscle capillary–mitochondrial diffusion. Based on exercising modality, different putative roles of each system in determining $VO2_{max}$ reductions at HA (≈6.3% decrease per 1000 m increasing altitude (Wehrlin & Hallén, 2006) have been established, with O2 delivery (thus comprising ventilatory, alveolar-capillary diffusion, and circulation mechanisms) rather than muscle O2 diffusing capacity being considered as the primary involved mechanism when whole body exercise is considered (José A.L. Calbet & Lundby, 2009). Unsurprisingly, most of these studies have been conducted on male subjects (Cowley et al., 2021; Raberin et al., 2023), despite the sex ratio of women engaged in mountain activities is significant, ranging from 15% of Everest climbers, to 40% of Everest trekkers, and over 40% of Swiss Alpine Club members (Horakova, 2023). This perpetuates the assumption that the physiological responses of women are the same as those of men (Sims & Heather, 2018): however, evidence (Ansdell et al., 2020; Archiza et al., 2021; J. Burtscher et al., 2023; Dominelli et al., 2013; Dominelli, Render, et al., 2015; Goldberg et al., 2017; Raberin et al., 2023; Santisteban et al., 2022) are present concerning a possible effect of biological sex on several aspects of the oxygen cascade, especially those related to convective

properties. Firstly, acute hypoxic ventilatory response (HVR) has been reported to be blunted in women (Goldberg et al., 2017), with this relative hypoventilation being explained as an interfering effect of female sex hormones with peripheral chemoreceptors governing the mechanism of ventilation (José A.L. Calbet & Lundby, 2009). Apart from reduced chemosensitivity, also mechanical constraints due to anatomical reasons may cause different ventilatory responses between men and women. In fact, females have smaller lungs than men, even when matched for standing height (Dominelli et al., 2013; A. William Sheel et al., 2016); furthermore, for similar lung sizes, females present smaller cross-sectional areas of the trachea and large-conducting airways, a phenomenon that is known as respiratory dysanapsis (Dominelli et al., 2012; A. William Sheel et al., 2016). Consequently, they could be more likely to reach their maximum capacity to generate expired flow during exercise, developing Expiratory Flow Limitations (EFL) (Dominelli et al., 2019) and thus being unable to properly increase ventilation in order to meet oxygen demand, especially when at HA.

Furthermore, scarce information is present regarding sex-related differences in alveolar-capillary oxygen diffusion capacity and their consequences on O2 delivery at HA. At sea level, Bouwsema et al. (Bouwsema et al., 2017) demonstrated that height-matched men and women increased diffusion capacity with normoxic incremental exercise (Tedjasaputra et al., 2016), but men had significantly greater absolute values of the measurement. However differences were eliminated once values were normalized for alveolar lung volume (VA), suggesting that the greater diffusion during exercise in men can be explained by greater lung size. At HA, for similar SL oxygen requests, diffusion capacity is supposed to increase in order to buffer i) a restriction in oxygen diffusion due to a decrease in alveolar-venous blood oxygen partial pressure gradient (Bartesaghi et al., 2014; Beretta et al., 2017) and ii) a possible reduction in lung capillary volume (Vc) due to hypoxic induced pulmonary vasoconstriction (Raberin et al., 2023). Despite this, the efficiency of the above anti-edemagenic mechanisms varies among subjects (Beretta et al., 2017), and if a role of sex is present on these responses is quite unexplored. The only study that addressed this topic (Olfert et al., 2004) demonstrated that sex never reflected into differences in Alveolar to Arterial O2 difference (A-aO2) while

exercising at both SL and simulated HA (12.5% Fraction of Inspired Oxygen, FiO2) suggesting that pulmonary end-capillary O2 diffusion equilibrium was not different between the sexes.

Cardiac responses (i.e. CO, SV and HR) present well known differences between sexes, even though most of these differences are reduced or even nullified when parameters are normalized for body surface area (Ansdell et al., 2020). It is known that exercise at altitude (acute exposure) increases HR but not SV for the same exercise performed at sea level (Clark 2007), but sex related differences in cardiac responses to hypoxic exercise are scarce. Furthermore, lower haemoglobin mass for the same quantity of blood flow, and overall lower blood volume relative to body size (Santisteban et al., 2022) could place additional burden in Oxygen delivery to the working muscles in females at HA. Finally, the relationship between diffusion capacity and cardiovascular aspects, named pulmonary vascular recruitment effectiveness (i.e. DLCO/CO), has been proposed as one of the most important mechanisms responsible for a fall in arterial partial pressure of Oxygen (PaO2) at high exercising intensities (J. A. Dempsey et al., 1984; Olfert et al., 2004), supposedly playing a primary role also in determining $VO2_{max}$ reductions at HA, and thus deserving more attention when addressing sex differences in response to exercise in this condition.

In summary, the effect of sex on the convective properties of the oxygen cascade while exercising under conditions of reduced oxygen availability is widely unexplored and warrants further investigation. Thus, the aim of this study is to compare ventilatory, diffusive, and circulatory aspects while exercising at SL and under hypoxic conditions in trained men and women.

MATERIALS AND METHODS

Subjects

10 women and 10 men volunteered for this study. Males and females were matched for height and sex-specific performance level classification (De Pauw et al., 2013; Decroix et al., 2016). All subjects were nonsmokers, had normal pulmonary function, and were physically active. Subjects' characteristics and baseline

pulmonary function data are presented in Table 1. Women included in the study were eumenorrheic (meaning they had a menstrual cycle (MC) that occurs regularly and lasts between 21 and 35 days (Carmichael et al., 2021), with no oral contraceptive assumption.

Before data collection, all participants were properly informed about the experimental procedures and gave their written informed consent for the measurements.

Table 1 Subjects baseline characteristics

Characteristic		All men (10) mean	sd	All women (10) mean	sd	p
Age	years	26.6 ±	5.1	27.8 ±	4.4	0.582
Height	cm	173.3 ±	3.1	171.0 ±	3.7	0.144
Weight	kg	67.1 ±	6.5	60.0 ±	4.8	**0.012**
BMI	kg/m^2	22.4 ±	2.6	20.5 ±	1.3	0.056
BSA		1.8	0.1	1.7	0.1	**0.007**
DLCO	ml/min/mmHg	31.0 ±	4.1	23.3 ±	3.3	**0.000**
DLCO/VA	mL/min/mmHg/L	4.9 ±	0.8	4.4 ±	0.6	0.179
TLC	L	6.5 ±	0.8	5.4 ±	0.5	**0.001**
IC	L	3.4 ±	0.4	2.9 ±	0.4	**0.014**
IC	% predicted	97.2 ±	9.6	111.8 ±	13.4	**0.012**
VC	L	5.1 ±	0.7	4.1 ±	0.4	**0.001**
VC	% predicted	99.2 ±	10.3	96.0 ±	8.3	0.435
ERV	L	1.7 ±	0.6	1.1 ±	0.2	**0.010**
ERV	% predicted	107.5 ±	33.9	88.1 ±	16.6	0.121
IRV	L	2.5 ±	0.5	2.2 ±	0.3	**0.053**
FEV1	L/min	4.2 ±	0.6	3.6 ±	0.4	**0.016**
FEV1	% predicted	98.3 ±	12.8	100.1 ±	9.6	0.727
FVC	L	5.3 ±	0.7	4.3 ±	0.4	**0.001**
FVC	% predicted	102.1 ±	10.3	100.2 ±	7.6	0.645
FEV1/FVC	ratio	80.4 ±	5.1	84.4 ±	5.2	0.099
VO2peak	ml/min	3907.2 ±	517.0	2946.0 ±	226.9	**<0.001**
VO2peak	ml/kg/min	58.3 ±	5.9	49.3 ±	4.2	**0.001**

Data reported as mean ± SD. BMI, Body Mass Index; BSA, Body Surface Area, DLCO: Diffusion Capacity for Carbon Monoxide; VA: Alveolar Volume; TLC: Total Lung Capacity; IC: Inspiratory Capacity; VC: Vital Capacity; ERV: Expiratory Reserve Volume; IRV: Inspiratory Reserve Volume; FEV1, forced

expiratory volume in 1 s; FVC, forced vital capacity. Statistical significance was set at $p<0.05$.

Study Design

Overview

Each participant visited the laboratory on 4 different occasions within the same time of the day. Males completed the protocol within a maximum of three weeks, whereas for female subjects the first experimental sessions were performed between the 5th and the 10th day post-ovulation (i.e. mid-luteal phase (Carmichael et al., 2021)), and the remaining sessions completed during the mid-luteal phase of the following MC. This allowed at least 48 hours of recovery to pass between each session for all participants. To detect menstrual phase, women were recruited several months before being enrolled in the study, and they were asked to track their MC through diaries or phone apps (Wideman et al., 2013), as well as to collect urine samples (Wondfo ovulation sticks) to detect effective luteinizing hormone (LH) surge and, consequently, the presence of ovulation (Barron et al., 2018). If regular MC length or LH surge were not registered, they were discarded from the study. Midlutheal phase is characterized by elevated progesterone and estrogen levels and it was chosen in order to maximize potential hormonal differences between sexes (Bouwsema et al., 2017). However, some previous work suggest that overall respiratory responses (MacNutt et al., 2012; Smith et al., 2015) may be enhanced in females during the midluteal compared to the early follicular phase; thus, the generalization of our findings to all MC phases should be approached with caution. The experimental sessions were randomly performed in an environmental chamber at either 18°C and 20.9% Fraction of inspired Oxygen (FiO2, sea level condition, SL) or 18°C and 11.8% FiO2 (≈4500m asl simulated high altitude exposure, Normobaric Hypoxia, NH). The hypoxic environment was created through the manipulation of the FiO2 (i.e. normobaric hypoxia) by means of an oxygen dilution system based on the Vacuum Pressure Swing Adsorption principle (B-Cat, Tiel, The Netherlands). Each session started with a 30-minute resting period to ensure that first short-term physiological responses to the hypoxic environment occurred

(Duffin, 2007), but was repeated within all conditions in order to guarantee subject's blindness to the experimental condition (i.e. SL vs NH).

During the first testing session, subjects were screened for any cardiopulmonary disorders and/or medications and then completed a full pulmonary function test, including spirometry and resting DLCO as described below. Subsequently, participants entered the chamber and after the 30-min resting period they performed an incremental $VO2_{max}$ test on a treadmill, either at SL or NH. At least 48-h later, subjects returned to the laboratory and completed either the second GXT in the opposite condition or a pulmonary function evaluation session (PF) at same FiO2 exposure of the first visit. During PF, subjects performed inspiratory capacity (IC) and DLCO manoeuvres (see below) while exercising at three progressively incremental external WorkLoads (WL) corresponding to moderate (MOD), heavy (HEAV) and severe (SEV) exercising domains derived from ventilatory thresholds detection (VT1 and VT2) in respective FiO2 matched GXT. Specifically, chosen speeds corresponded to 80% of VO2 at VT1(i.e. moderate), the median VO2 between VT1 and VT2 (i.e. heavy) and VO2 at VT2 (i.e. severe).

The last two sessions consisted of either the GXT in the missing FiO2 condition followed by the FiO2-matched PF trial, or the two PF sessions in opposite FiO2 exposures if randomization analysis dictated the execution of the two GXT tests initially.

Throughout rest, exercise, and recovery phases of all experimental sessions, beat-to beat heart rate (HR) was continuously recorded using a Polar RS800CX HR monitor (Polar, Kempele, Finland) and pulse oxygen saturation (SpO2) through a finger pulse oximeter (Nonin Medical, Minneapolis, MN).

Furthermore, cardio-respiratory measures were collected continuously with breath-by-breath method using automated open-circuit gas analysis systems (Quark CPET, Cosmed Srl, Rome, Italy), and Cardiac Output (CO) evaluated by impedance cardiography (PhysioFlow, Manatec Biomedical, Ebersviller, France). The individual RPE was divided into breath RPE and leg RPE and assessed using the CR100 Scale (Borg & Borg, 2002) at the end of each exercise intensity for the GXT, whereas only overall RPE on the same scale was asked for the three exercising bouts of PF session. Finally, to measure blood lactate, a blood sample

was collected from the finger at the end of each exercising intensity of GXT and PF sessions (Biosen C-line, EKF Diagnostics GmbH, Barleben, Germany).

Graded Exercise Test

The Graded Exercise Test (GXT) was conducted on a motorized treadmill (Technogym, Italy) at a fixed 25% slope. Before the test, each athlete performed a 5 min warm-up at a constant speed of 1.6 km/h. Initial speed of the GXT at SL was set to 2 km/h and was increased by 0.6 km/h every three minutes until volitional exhaustion. Differently, the GXT at NH started at 1.6 km/h with following increments of 0.4 km/h every three minutes. The two protocols were carried out in order to reach similar exhaustion times based on expected average $VO2_{max}$ decrease at ≈4500m asl (Wehrlin & Hallén, 2006). This avoided a too rapid work rate increment in NH, which is often associated with marked hyperventilation, an inability to determine ventilatory thresholds and premature termination of exercise due to lactate acidosis (Iannetta et al., 2019; Mezzani, 2017). $VO2_{max}$ validation was obtained according to the main criterion of a VO2 plateau occurrence and to secondary criteria (i.e., HR_{max}≥95% age-predicted HR_{max}; RER>1.10, maximal blood lactate concentration >8 mM) (Poole & Jones, 2017). $VO2_{max}$ was defined as the highest values of a 20-s average (Robergs et al., 2010), whilst ventilatory thresholds (i.e. VT1 and VT2) were defined by experienced exercise physiologists according to well established methods (Wasserman et al., 2005). Maximal workload (WL_{max}) achieved at athlete's exhaustion during both incremental tests was determined according to the following equation: WL_{max}(km/h)= speed last stage completed (km/h)+ [t(s)/step duration(s) * speed increment(km/h)], where t is the time of the uncompleted stage (Kuipers F T J; Keizer, H A; Geurten, P; van Kranenburg, G, 1985).

Cardio-respiratory data throughout the tests were processed and analysed with MATLAB 7.0 (The MathWorks, Inc., Natick, MA, USA), using a customized code that averaged data over the last 30 seconds of each exercising intensity.

Pulmonary Function Session

Resting Lung function and Lung volumes

After being exposed to the specific environmental condition (SL or NH) and immediately following the 30-minute resting period, subjects underwent a repetition of the comprehensive pulmonary function test they had become familiar with during the first experimental session.

Resting spirometry and lung volumes evaluation was performed using an ergospirometer (Quark PFT, COSMED, Rome, Italy) following current guidelines (Wanger et al., 2005). Each subject completed a minimum of three Forced Vital Capacity (FVC) and Slow Vital Capacity (SVC) manoeuvres, in order to assess resting lung function (FVC, FEV1, FEV1/FVC) and lung volumes (IC, Vital Capacity (VC), Expiratory Reserve Volume (ERV) and Inspiratory Reserve Volume (IRV)) within specific environmental exposure. Furthermore, subjects were given specific training on how to perform IC manoeuvres during exercise.

Resting DLCO

The high affinity of carbon monoxide (CO) with hemoglobin (Hb) is used to estimate diffusion capacity of the lung (DLCO, ml min−1 mmHg−1) (Macintyre et al., 2022). Resting haemoglobin corrected lung diffusing capacity for carbon monoxide (DLCO) (Quark PFT, COSMED, Rome, Italy) was determined with the single-breath breath-hold technique. Hemoglobin concentration ([Hb]) was measured at the beginning of each session (HemoCue 201, HemoCue, Angelholm, Sweden), and DLCO was adjusted for [Hb] with the following equation (Marrades et al., 1997):

$$DLCOcorr = DLCO \times \frac{10.22+[Hb]}{1.7x[Hb]}$$

Since our study took place in normobaric hypoxia, DLCO breath holds was carried out at two different FiO2 values at SL (21% tank O2 content) and NH (12% tank O2 content), being missing O2 content in the latter tank replaced through balance Nitrogen (N2). Methane (0.3%) was used in each gas mixture to measure alveolar volume (VA) and determine adequate gas equilibration.

The data acquisition software automatically corrected DLCO value once inspired FiO2 concentration was included in the program. Before data collection, subjects were coached in the proper breath-hold manoeuvre, and at least one simulated trial without CO rebreathing was conducted. After several normal breaths, subjects were instructed to exhale to residual volume, and then inhale to total lung capacity (TLC) within maximum 3 seconds, subsequently performing a breath hold for up to 8 seconds from the beginning of the maneuver, avoiding Valsalva or Müllerian maneuvers. During the exhalation, the methane tracing was monitored to ensure that the slope was horizontal, indicating that the test gas was well equilibrated in the lungs. Moreover, combining methane tracing and lung volumes during the manoeuvre (i.e. dilution technique), TLC was calculated by the software as follows:

$$\text{TLC} = V_{inhaled} \text{ x } (\frac{C_{initial}}{C_{final}} - 1)$$

where $V_{inhaled}$ is the volume of the inhaled gas mixture, $C_{initial}$ is the known concentration of methane in the inhaled gas mixture (0.3%) and C_{final} is the concentration of methane in the exhaled gas after breath holding.

Exercising Trials

Subjects started with a 5-minutes warm up at 1.6 km/h, and then performed in ascending order the three selected exercising intensities (i.e.. moderate, heavy and severe) for SL and NH, respectively. For moderate and heavy trials, subjects exercised continuously for 5 minutes during which cardiorespiratory data were recorded continuously, whereas for the severe intensity domain only three minutes of continuously monitored exercise were requested. Steady state achievement (at least for the firsts intensities) was defined by both visual inspection of ventilatory and gas exchange data, as well as by a consistent HR (<3 bpm change over 1 min). A paper worksheet on excel was used to average cardio-respiratory data over the last 30 sec of each exercise intensity, which were subsequently used for analysis.

Right after cardio-respiratory measurements recording, without any resting period and still with the mask on, subjects were requested to perform an IC manoeuvre,

which was prompted before its execution with the words: ‘at the end of a normal breath out, take a maximal breath all the way in’ (Dominelli, Molgat-Seon, et al., 2015). Strong encouragement was provided immediately prior to and during each manoeuvre during exercise. Our data acquisition software allowed visualization of real-time measurement so if a manoeuvre appeared inadequate, the subject was asked to perform another. The in-house data analysis program already provided measures of inspiratory (IRV) and expiratory (ERV) reserve volumes, whereas End Expiratory (EELV) was mathematically determined by subtracting inspiratory capacity volume from FVC (EELV = FVC−IC) (Dominelli, Molgat-Seon, et al., 2015; Moore et al., 2018). Besides operational lung volumes, Expiratory flow limitation (EFL) was evaluated at each workload by determining the percentage at which the VT curve derived from IC overlapped with the maximal flow-volume curve (VFL/VT) obtained from baseline spirometry of the same testing day (Moore et al., 2018). When VFL/VT was >0.5, EFL was assumed to happen.

Once a correct IC was registered, mask was taken off and DLCO manoeuvre for each intensity domain was evaluated while exercising. For moderate and heavy intensities, subjects performed the DLCO trial without any resting period, whereas for severe intensity a discontinuous protocol was used such that there was 30 sec passive recovery between IC and DLCO to limit fatigue. As the subject re-started to walk/run, DLCO manoeuvre was performed once heart rate reached the value consistent with that obtained during previous steady state evaluation (≈1.5 min) (Bouwsema et al., 2017). Instructed personnel evaluated the trial by both visual inspection of real-time measurements as well as immediate evaluation of DLCO outcomes, and as for IC, if a manoeuvre appeared inadequate, the subject was asked to perform another after at least 2 min from the previous one. [Hb] was measured during each exercising trial, and DLCO was subsequently corrected for the respective intensity [Hb] registered value as described above. We did not correct for HbCO because subjects were nonsmokers (Sansores et al., 1995) and sufficient time between DLCO breathhold tests occurred to sufficiently clear CO from the lungs (Ogilvie et al., 1957), especially considering that exercise promotes clearance of CO from the lungs and blood (Zavorsky, 2013).

Data analysis

For the purposes of this study, our objective was to compare ventilatory, diffusive, and cardiac aspects between men and women at a consistent percentage of $VO2_{max}$ under normoxic and hypoxic conditions, respectively. Accordingly, utilizing data collected at similar intensity domains, albeit not necessarily at the same $\%VO2_{max}$ in the respective conditions, we delineated a model that best elucidated the data trend using a third-degree polynomial. Subsequently, we extrapolated one value for all measured variables corresponding to 80% of the $VO2_{max}$ at sea level (SL) and simulated altitude (NH), respectively, for each subject. The selection of 80% of $VO2_{max}$ was deliberate, as it represented a point within the range of the three measured points for each subject, obviating the necessity to speculate on the curve's behaviour beyond the measured range.

Statistical analysis

Data are presented as means±SDs. Data were tested for normal distribution with Shapiro–Wilk test. Baseline characteristics between men and women were compared using paired sample T-test. Incremental data , as well as ventilatory, diffusive and cardiac responses at $80\%VO2_{max}$ calculated from data collected during the PF session were compared using a two-way analysis of variance (ANOVA) for repeated measures (RM), with ‘‘FiO2’’ (SL and HA) as within and ‘‘SEX’’ (M and F) as between subjects’ factors. When statistical significance was identified, a Sidak post hoc test was used to further delineate differences between FiO2 or SEX . Statistical analysis was completed using a Statistical software (SPSS, Inc., Chicago, IL). The level of statistical significance was set at $p < 0.05$.

RESULTS

GXT Test

The outcomes for maximal incremental test in normoxia and hypoxia are presented in Table 2. General effects of 'FiO2 and SEX' were found on $Speed_{max}$, $VO2_{max}$, VO2 at 80% of $VO2_{max}$ in respective condition (80%$VO2_{max}$) and VE_{max}, which were negatively impacted by both hypoxic exposure and female sex, with no 'FiO2*SEX' interaction. HR_{max} and $SpO2_{max}$ only showed a general effect of 'FiO2', decreasing in both sexes in at NH. Maximal Lactate, RPE_{breath} and RPE_{leg} showed no differences neither between SL and NH, nor between M and F.

Table 2 Results of the GXT test

		SL_MAN	NH_MAN	SL_WOMEN	NH_WOMEN	FiO2	SEX	FiO2*SEX
		mean ± sd	mean ± sd	mean ± sd	mean ± sd			
Speed	(km/h)	5.9 ± 0.7	4.2 ± 0.6	5.0 ± 0.4	3.5 ± 0.4	**<0.001**	**0.00**	0.18
VO2max	(ml/kg/min)	58.28 ± 5.86	40.56 ± 6.45	49.32 ± 4.19	34.21 ± 3.92	**<0.001**	**0.00**	0.13
80%VO2max	(ml/kg/min)	46.62 ± 4.69	32.44 ± 5.16	39.46 ± 3.35	27.31 ± 3.07	**<0.001**	**0.00**	0.15
HR	(bpm)	191 ± 11	180 ± 12	187 ± 8	177 ± 7	**<0.001**	0.34	0.60
VE	(L/min)	167.6 ± 15.1	155.9 ± 20.3	115.4 ± 14.0	108.0 ± 16.2	**<0.001**	**<0.001**	0.34
La	mMol	11.84 ± 2.93	11.42 ± 1.84	9.86 ± 2.33	10.72 ± 2.57	0.60	0.20	0.14
SpO2	%	93 ± 3	69 ± 5	94 ± 4	69 ± 4	**<0.001**	0.88	0.54
RPEbreath		90 ± 7	90 ± 9	89 ± 17	96 ± 14	0.16	0.67	0.15
RPEleg		95 ± 7	97 ± 10	90 ± 19	91 ± 6	0.64	0.20	0.96

Data reported as mean ± SD. HR, Heart Rate; Ve, Ventilation; La, Lactate concentration; SpO2, pulse Oxygen Saturation; RPE, Rate of Perceived Exertion. 80%VO2max represents the calculated value at 80% of maximal Oxygen consumption in respective sex and condition. SL, Sea Level (18°C, 20.9% FiO2); NH, Normobaric Hypoxia (18°C, 11.8% FiO2). FiO2, Fraction of inspired Oxygen. Bold characters represent statistical significance ($p<0.05$).

Pulmonary Function Session

Overall calculated data at 80%$VO2_{max}$ at SL and NH are presented in Table 3.

[La], SpO2 and RPE

[La] (Fig. 1A) and RPE at 80%$VO2_{max}$ showed no general effect of 'FiO2' or 'SEX', but a trend towards lower [La] and RPE at NH if compared to SL for male, and the opposite trend for women (i.e. higher [La] and RPE at NH if compared to SL values) has been detected ('FiO2*SEX' interaction, p=0.070 and 0.151 for [La] and RPE, respectively). SpO2 did not show any effect of 'SEX', but as expected was significantly lower at NH (general effect of FiO2, p<0.001), with no 'FiO2*SEX' interaction (Fig 1B).

Heart Rate (HR), Stroke Volume (SV) and Cardiac Output (CO)

HR showed a general effect of 'FiO2' (p=0.027), being lower at NH if compared to SL (Fig.2A); no general effect of 'SEX' nor 'FiO2*SEX' interaction were seen on this parameter. SV (Fig.2B) was similar for both sexes at SL and NH, either if it was considered in absolute terms or normalized for body surface area (SV index, SVi). Both CO in absolute terms (Fig.2C), and normalized for body surface area (Cardiac Index, Ci) showed a general effect of 'FiO2' (p=0.023 and 0.044, respectively), being slightly lower at NH if compared to SL. However, no general effect of 'SEX', neither a 'FiO2*SEX' interaction were found on these parameters.

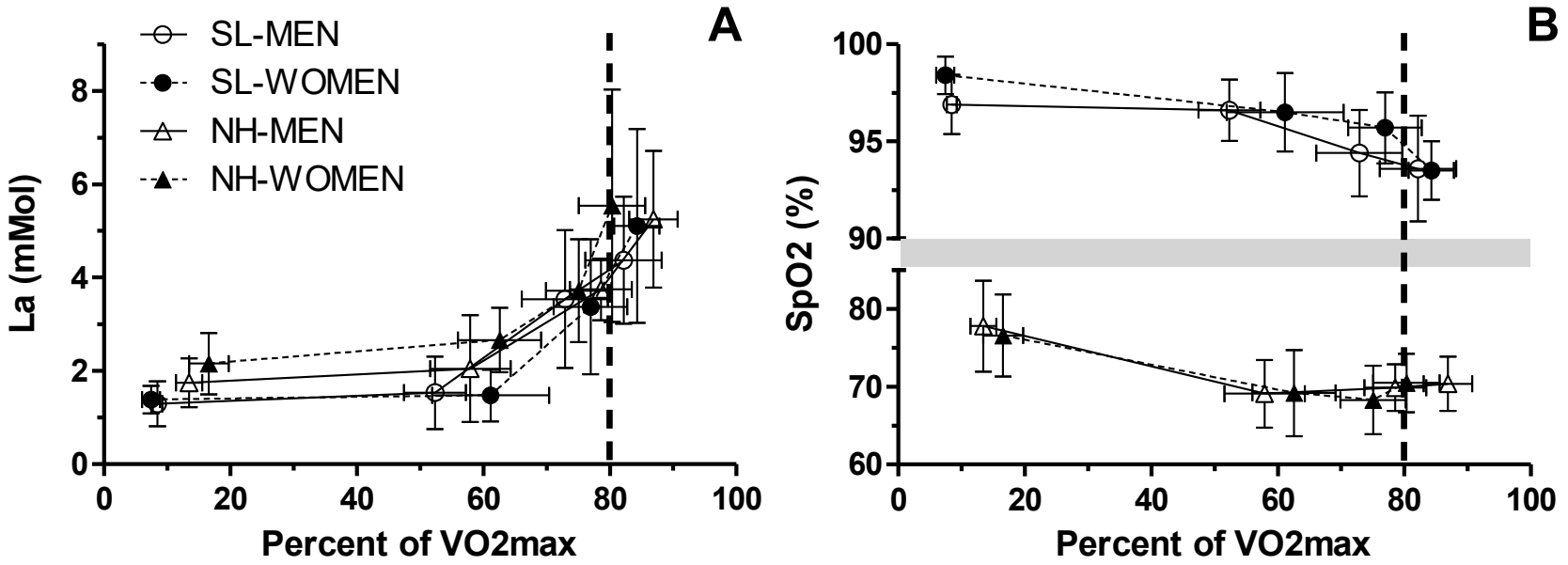

Figure 1 Lactate (La) and pulse Oxygen Saturation (SpO2) responses to exercise in all males (n 10) and females (n 10) (means ±sd) in relation to % of VO2max at Sea Level (SL) and Normobaric Hypoxia (NH), respectively. Data were collected at rest and moderate, heavy and severe intensities. Empty symbols represent men, full symbols represent women. Circles refers to SL, triangles to NH values. Dotted line represents the point calculated for each subject at 80%VO2max in respective condition.

Ventilatory aspects and Operational Lung Volumes

VE (Fig.2D) showed a general effect of 'SEX' (p=0.002), being lower in female subjects even after normalization for body surface area (VE/BSA, see Table 2). However, no general effect of 'FiO2' nor 'FiO2*SEX' interaction were detected. Tidal Volume (Vt) was significantly lower in females independently of environmental condition (general effect of 'SEX', p=0.001), but it also showed a general effect of 'FiO2' (p=0.010), being lower at NH if compared to SL in all subjects. Respiratory Frequency (Rf) was similar in both sexes and conditions. Inspiratory Reserve Volume (IRV) (Fig.2E) and Inspiratory Capacity (IC) (Fig2F) were unaffected by environmental condition, and they showed a general effect of 'SEX' only when not normalized for Total Lung Capacity (TLC) (see Table 2). Similarly, End Expiratory Lung volume (EELV) expressed as % of TLC did not show any general effect of 'FiO2' nor 'SEX'. Finally, no 'FiO2*SEX' interaction was found for these parameters.

Diffusion Capacity and Pulmonary Vascular recruitment

DLCO showed a general effect of 'FiO2' (p<0.001) and 'SEX'+ (p<0.001), with no 'FiO2*SEX' interaction, meaning that it was always lower in female subjects, but also that it was reduced in both sexes in NH. Even when normalizing this parameter for subjects' Alveolar Volume (DLCO/VA), still a general effect of 'FiO2' (p<0.001) and 'SEX' (p=0.003) were detected (Fig.3A). Effectiveness of pulmonary Vascular recruitment, represented by DLCO/CO ratio, was identical between environmental conditions at 80%$VO2_{max}$, but it was always lower in women if compared to men (general effect of 'SEX', p=0.005, Fig. 3B).

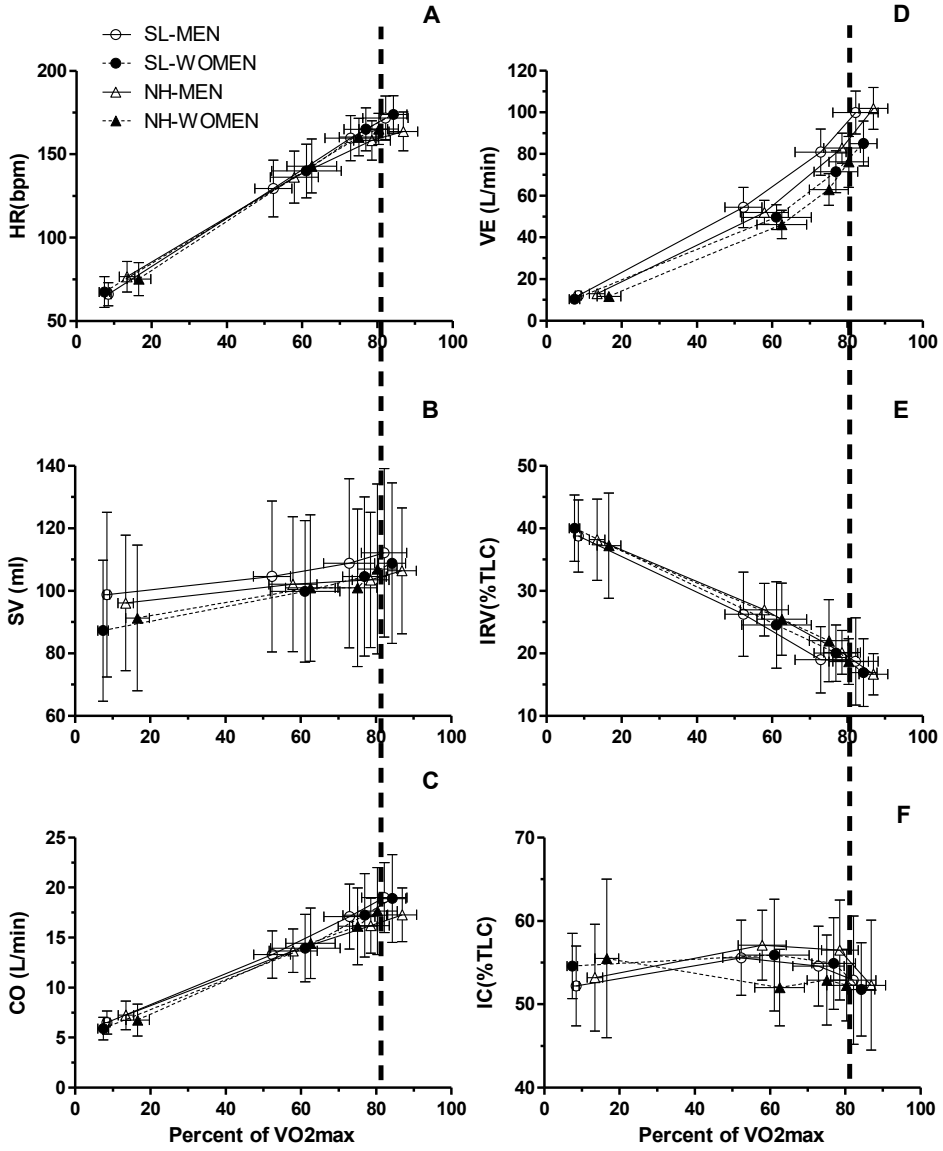

Figure 2 Heart Rate (HR), Stroke Volume (SV), Cardiac Output (CO), Ventilation (VE), Inspiratory Reserve Volume (IRV) expressed as percentage of Total Lung Capacity (TLC) and Inspiratory Capacity (IC) expressed as percentage of TLC responses to exercise in all males (n 10) and females (n 10) (means ±sd) in relation to % of $VO2_{max}$ at Sea Level (SL) and Normobaric Hypoxia (NH), respectively. Data were collected at rest and moderate, heavy and severe intensities. Empty symbols represent men, full symbols represent women. Circles refers to SL, triangles to NH values. Dotted line represents the point calculated for each subject at 80%$VO2_{max}$ in respective condition.

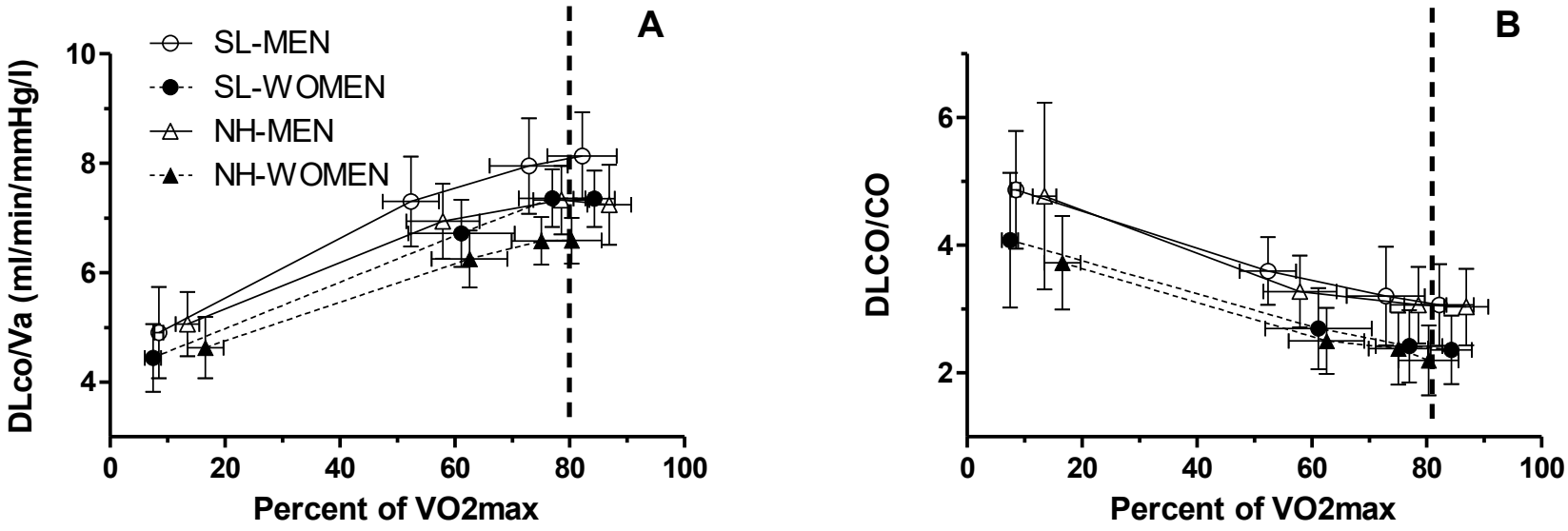

Figure 3 Diffusing capacity (DLCO) corrected for alveolar volume (Va) and DLCO expressed in relation to Cardiac Output (CO) as an index of pulmonary vascular recruitment in responses to exercise in all males (n 10) and females (n 10) (means ±sd) in relation to % of VO2max at Sea Level (SL) and Normobaric Hypoxia (NH), respectively. Data were collected at rest and moderate, heavy and severe intensities. Empty symbols represent men, full symbols represent women. Circles refers to SL, triangles to NH values. Dotted line represents the point calculated for each subject at 80%VO2max in respective condition.

Table 3 Data calculated at 80%$VO2_{max}$ in men (M) and women (W) at Sea Level (SL) and simulated altitude (NH), respectively.

	SL_M			NH_M			SL_W			NH_W			FiO2	SEX	FiO2* SEX
	mean	±	sd	mean	±	sd	mean	±	sd	mean	±	sd			
La (mMol)	4.3	±	1.5	3.8	±	0.7	3.8	±	1.8	5.2	±	3.0	0.393	0.562	0.070
SpO2 (%)	93.8	±	2.5	69.9	±	3.2	94.9	±	1.2	69.9	±	4.8	**<0.001**	0.641	0.567
RPE	56.1	±	19.3	45.4	±	16.8	48.9	±	17.1	56.4	±	20.2	0.800	0.730	0.151
HR (bpm)	170	±	14	160	±	11	167	±	12	165	±	8.02	**0.027**	0.798	0.129
SV (ml)	111	±	28	104	±	21	108	±	26	105	±	26	0.152	0.889	0.470
Svi (ml/m^2)	61.8	±	14.2	58.1	±	11.3	63.9	±	15.7	62.5	±	16.0	0.175	0.600	0.531
CO (l/min)	18.6	±	3.9	16.5	±	2.8	17.8	±	3.8	17.2	±	3.9	**0.023**	0.974	0.163
Ci (l/min/m^2)	10.3	±	1.9	9.2	±	1.6	10.4	±	2.3	10.3	±	2.3	**0.044**	0.507	0.140
VE (l/min)	95.8	±	11.1	86.7	±	15.2	77.3	±	11.2	74.0	±	14.3	0.133	**0.002**	0.468
VE/BSA (l/min/m^2)	53.3	±	4.7	48.6	±	9.9	45.8	±	5.9	43.8	±	8.1	0.15	**0.024**	0.543
Rf (bpm)	39.2	±	3.2	40.3	±	6.1	42.5	±	4.9	42.1	±	5.8	0.781	0.195	0.576
Vt (l/min)	2.5	±	0.3	2.2	±	0.3	1.8	±	0.4	1.8	±	0.3	**0.010**	**0.001**	0.148
IC (l)	3.8	±	0.4	3.8	±	0.4	3.1	±	0.4	2.9	±	0.5	0.603	**<0.001**	0.137
IC (%TLC)	54.3	±	6.1	55.0	±	7.9	54.0	±	6.1	50.6	±	6.3	0.435	0.344	0.239
IRV (l)	1.4	±	0.4	1.3	±	0.2	1.1	±	0.3	1.0	±	0.3	0.131	**0.009**	0.444
IRV (%TLC)	20.2	±	6.5	19.3	±	4.0	19.8	±	4.4	16.3	±	4.8	0.103	0.381	0.329
EELV (%TLC)	21.6	±	4.4	20.9	±	2.8	21.2	±	3.6	22.6	±	8.6	0.765	0.752	0.455
DLCO (ml/min/mmHg)	55.6	±	8.2	50.8	±	10.9	40.2	±	4.0	36.7	±	3.4	**<0.001**	**<0.001**	0.538
DLCO/VA (ml/min/mmHg/l)	8.1	±	0.8	7.7	±	0.8	7.2	±	0.5	6.5	±	0.4	**<0.001**	**0.003**	0.515
DLCO/CO	3.1	±	0.7	3.1	±	0.6	2.4	±	0.5	2.3	±	0.4	0.525	**0.005**	0.271

Data reported as mean ± SD. La, Lactate concentration; SpO2, pulse O_2 saturation; RPE, Rate of Perceived Exertion; HR, Heart Rate; SV, Stroke Volume; SVi, Stroke Volume Index; CO, Cardiac Output; Ci, Cardiac Index; Ve, Ventilation; BSA, Body Surface Area; Rf, Respiratory Frequency; Vt, Tidal Volume; IC, Inspiratory

Capacity; TLC, Total Lung Capacity; IRV, Inspiratory Reserve Volume; EELV, End Expiratory Lung Volume; DLCO, Diffusing capacity for Carbon Monoxide; VA, Alveolar Volume. SL, Sea Level (18°C, 20.9% FiO2); NH, Normobaric Hypoxia (18°C, 11.8% FiO2). FiO2, Fraction of inspired Oxygen. Bold characters represent statistical significance ($p<0.05$).

DISCUSSION

The aim of this study was to investigate the effects of sex on respiratory responses and operational lung volumes, diffusion capacity and cardiovascular adjustments to exercise under conditions of reduced oxygen availability (NH). In this study, we compared the above-mentioned responses at a similar percentage of $VO2_{max}$ in the respective environmental condition (i.e. SL or HA), thus the results have to be viewed in light of the fact that a lower absolute oxygen consumption in NH in both sexes compared to SL, as well as in females compared to males are expected (see Table 1). The main findings revealed that for similar exercising intensities at SL and HA, the difference in the ventilatory, diffusive and cardiac responses to exercise in NH remains constant between men and women, suggesting that no greater limitations in female subjects' physiological responses of the considered physiological systems while exercising in NH are present.

Cardiovascular adjustments

Previous studies demonstrated that during high-intensity exercise, women reach lower maximal stroke volumes compared to men, resulting in a lower maximal cardiac output as maximal heart rate is similar across sexes (Santisteban et al., 2022). This latter information is confirmed by our data, showing similar HR_{max} between sexes at SL, as well as a similar reduction in this parameter in NH (- 11 and -10 bpm for M and F, respectively), in agreement with, e.g., the 1.9 bpm per 1,000m altitude gain found in young subjects by Wehrlin et al. (Wehrlin & Hallén, 2006) and with the 9 bpm decrease of HRmax observed at 4,300 m (Lundby & van Hall, 2001; Mourot, 2018). Unfortunately, it was not possible to measure CO and SV at maximal exercising intensities, given the fact that the chosen exercising

modality (i.e. walking at 25% slope) did not allow to correctly measure cardiac variables when the subjects started to run due to excessive vibrations. However, assuming that maximal CO would have been reduced in women (Higginbotham et al., 1984), but HR, SV and CO were similar between sexes at 80% of respective $VO2_{max}$ even when normalized for body surface area (see Table 3), this suggests that women did need a greater proportion of their maximal CO to sustain a similar % of $VO2_{max}$ than their male counterparts, underlying a less efficient work of the cardiovascular system at submaximal exercising intensities. This would also explain the fact that exercise-induced arterial hypoxemia (EIAH) has been shown to develop at lower relative intensities in females if compared to men (Archiza et al., 2021; Santisteban et al., 2022): in fact, if CO reaches its upper limit at a lower % $VO2_{max}$ in females, pulmonary transit time for adequate red blood cell oxygenation may fall below critical levels earlier (≈0.3 s), thus causing faster development of EIAH in women due to gas exchange impairment secondary to diffusion limitation (Bouwsema et al., 2017; Smith et al., 2015). However, cardiovascular responses at similar $\%VO2_{max}$ in NH do not show greater differences between males and females, suggesting no amplified loss of efficiency in cardiovascular system function in the latter when oxygen availability is reduced.

Ventilatory responses

The ventilatory response to exercise has been proposed as one of the most sex-sensitive factors that may modify reactions to hypoxia between men and women (Raberin et al., 2023)**.** Once acutely exposed to HA, hyperventilation occurs due to the so-called Hypoxic Ventilatory Response (HVR) (Ainslie et al., 2013; J. A.L. Calbet et al., 2003; José A.L. Calbet & Lundby, 2009; C. A. Gallagher et al., 2014; Peacock, 1998), stimulated by peripheral chemoreceptor's detection of a drop in alveolar and consequently arterial partial pressure of oxygen (PAO2 and PaO2, respectively) (José A.L. Calbet & Lundby, 2009; Lundby et al., 2006). For this reason, ventilatory response to hypoxemia has been measured at rest in a large cohort of males and females as the change in VE (liter/minute) per change in percentage oxygen saturation (ΔVe /ΔSpO2) while performing a specific poikilocapnic test, rebreathing CO2 filtered exhaled air (Goldberg et al., 2017). The

change in ventilation for a given variation in SpO2 over time has been found to be lower in females if compared to males, thus the authors concluded blunted HVR in women. However, the impact of sex on HVR is not fully understood, with contradictory studies reporting either higher (with a range of 39±110% (Aitken et al., 1986)), lower , or similar (MacNutt et al., 2012) HVR between men and women. Specifically, in a large cohort of unselected subjects divided into 1793 men and 1474 women acutely exposed at 3466m asl, the latter presented slightly higher resting SpO2 levels of male counterparts, suggesting a possible role of sex-related respiratory differences immediately after reaching high altitude, with women presenting higher chemoreceptor sensitivity to reduced PaO2 (C. Vignati, 2021). Furthermore, MacNutt and colleagues (MacNutt et al., 2012) found that the percent change in exercising minute ventilation from normoxic to hypoxic condition was not different between males and females, and both sexes reached similar absolute SpO2 values in hypoxia while exercising at 40% of respective maximum normoxic power output. Our results confirm these findings, showing quantitatively similar ventilatory changes to hypoxic exercise between males and females, as well as similar absolute SpO2 values at respective 80%$VO2_{max}$ exercising intensity at HA. This is supported also by the fact that in this study women did not show higher prevalence of mechanical constraints such as expiratory flow limitations (EFL) (Dominelli et al., 2019) neither at SL nor in NH (see supplemental material 1). In fact, EFL has been proposed as one of the putative mechanisms connected to hypoventilation, since it is represented by a situation in which ventilatory demands during exercise reach ventilatory capacity, causing the inability to generate higher expired flow despite increased expiratory effort. Reduced lung volumes and/or airway calibre (A. William Sheel et al., 2016) in women even when matched for standing height to men have been proposed as possible causes for higher EFL prevalence, even though (as for ventilatory responses) contrasting results on this topic are present (Archiza et al., 2021). EFL is believed to result also in an upward shift toward higher operating lung volumes (i.e., reduced inspiratory capacity (IC) and inspiratory reserve volume (IRV)), with consequent increase in EELV and development of dynamic lung hyperinflation (Moore et al., 2018). Despite higher absolute IC and IRV values in males at both SL and NH in this study, sex

differences disappeared once these parameters were normalized for TLC, suggesting that operational lung volumes were similar between sexes and adequate to satisfy respective oxygen requests. Furthermore, no changes in IC, IRV or EELV due to reduced oxygen availability were detected neither in M or F, indicating similar operational lung volume responses at SL and NH in both men and women when exercising at a consistent percentage of respective specific condition $VO2_{max}$ (Tab 3).

Diffusion Capacity and Pulmonary Vascular recruitment

Sex differences in diffusion capacity are widely under-represented in literature, especially when whole-body exercise at high altitude is considered. Our results partially disagree with those shown in a previous work by Bouwsema and colleagues (Bouwsema et al., 2017), which found that at SL, highly fit females ($VO2_{max}$: 52.3 ml/kg/min) presented lower DLCO values than highly fit males ($VO2_{max}$: 62.1 ml/kg/min) when exercising at similar percentages of respective $VO2_{max}$, but these differences disappeared once DLCO was normalized for Alveolar Volume (i.e DLCO/VA). The authors concluded that the greater DLCO during exercise in men is explained by greater lung size, but once this aspect is considered (i.e. normalizing for Va), the pulmonary capillary blood volume response to incremental exercise is adequate to meet individual oxygen demand during exercise in both sexes. However, our results showed that in a similar context (comparing trained men and women), females presented lower diffusion capacity than male counterparts even when this value was normalized for Alveolar Volume (See Table 3). This difference persists at HA, in which also a general decreasing effect of reduced oxygen availability on diffusion capacity for both men and women is present. This reduction in DLCO with acute hypoxic exposure is in line with previous research conducted by Agostoni and colleagues at Capanna Regina Margherita (4559 m) (Agostoni et al., 2013). In fact, hypoxic-induced pulmonary vasoconstriction causes an increase in pulmonary pressure, with consequent pulmonary capillaries microvascular filtration and subclinical increase in interstitial lung fluid (Cogo & Miserocchi, 2011). This is paralleled by a reduction in DLCO due to a reduction in diffusion membrane (Dm) properties, with a partial

compensatory increase in pulmonary capillary volume (Agostoni et al., 2013). However, decreases in Dm are not associated with evidence for alveolar capillary barrier damage, being primarily related to the extravascular lung fluid accumulation that can probably be considered a ‘‘paraphysiological’’ condition that does not necessarily proceed to severe edema with alveolar flooding (Cogo & Miserocchi, 2011). Interestingly, DLCO impairments detected with acute exposure disappear after a three-week acclimatization period (Agostoni et al., 2011): on the contrary, DLCO and Dm increase with acclimatization if compared to Sea Level values, along with an increase in SpO2 and a decrease in Alveolar-to-Arterial O2 difference if compared to acute hypoxic exposure. Interestingly, A-aO2 difference decreases with no evident changes in ventilation to perfusion matching, implying a reduced resistance to gas transfer across the alveolar-capillary membrane with acclimatization (Agostoni et al., 2011).

Finally, as for ventilation, quantitatively similar changes in lung diffusive responses to hypoxic exercise between males and females have been found.

Apart from diffusion capacity per se, the actual presence of gas exchange impairments is evaluated through the relationship between pulmonary vascular recruitment and diffusion, represented by DLCO/CO ratio (Connie C W Hsia, 2002). Bouwsema and colleagues (Bouwsema et al., 2017) showed that above 70% of respective $VO2_{max}$, highly fit males and females present similar DLCO/CO ratio, differently from our findings which demonstrate significantly lower DLCO/CO at 80% $VO2_{max}$ in females. However, the decreasing trend (C C W Hsia et al., 1994) in this parameter from resting value to higher exercising intensities (i.e. SEV domain) appears to be identical between sexes (see Figure 3B). Furthermore, normobaric hypoxia did not affect DLCO/CO ratio neither in men or in women, showing similar values when matched $\%VO2_{max}$ exercising intensities at SL and NH are considered. Since we did not directly measure PaO2, it is not possible to conclude whether a lower DLCO/CO ratio in females is connected to a higher probability of reaching a functional threshold for gas exchange impairment as exercise intensity increases (Connie C.W. Hsia, 2002). However, the unique study that directly measured PaO2 in men and women (matched for age, height and VO2 max values) while exercising at similar intensity domains at SL and NH (FIO2:

12.5%) did not find any sex related difference in alveolar-arterial O2 difference in neither of the two conditions, estimating similar pulmonary end-capillary diffusion equilibrium between sexes both at SL and HA. Still we can conclude that the differences seen at SL between sexes remained constant at HA, thus no amplification effect of reduced oxygen availability on differences in diffusion properties was present.

LIMITATIONS

Performing IC and DLCO manoeuvre while walking on a treadmill is extremely challenging, thus it was impossible to obtain measurements at peak exercise without asking the subject to stop walking. However, since both DLCO and operational lung volumes are influenced by exercising modality (Tedjasaputra et al., 2016), we aimed at evaluating the most ecological locomotion mode that might occur at 4500m asl (i.e. walking uphill). For the same reason, cardiac data could not be registered at peak exercise.

Concerning DLCO manoeuvre, a 8-s breath-hold time was used in the present study as opposed to the standard 10- DLCO breath hold (Macintyre et al., 2022). However, previous work has demonstrated identical DLCO values from 6-s and 10-s breath holds in healthy subjects (Bouwsema et al., 2017) .

FUTURE PERSPECTIVE

The presented analysis investigated sex differences at equal $\%VO2_{max}$ at SL and HA, respectively. However, speed at SL and NH was adjusted considering similar intensity domains (i.e. MOD, HEAV and SEV) derived from GXT data analysis, which actually resulted in lower sustainable $\%VO2_{max}$ at SEV only in women once exposed to HA.

This is confirmed also by a tendency towards higher [La] at $80\%VO2_{max}$ in NH if compared to SL in females, which was instead not seen in males counterparts. This stands for a possible synergistic effect (Lloyd & Havenith, 2016) of sex (i.e. reduced convective properties in females) and reduced oxygen availability in determining greater reductions in endurance exercising capacity for women at HA.

As a consequence, future perspective aim at combining presented result with reduction in sustainable fraction of $VO2_{max}$ in the severe domain in women at HA, in order to better elucidate the role of the systems involved in the oxygen cascade in determining this phenomena .

CONCLUSIONS

This study examined the effect of sex on ventilatory aspects (i.e. Ventilation and Lung Volumes), Oxygen Diffusive properties (i.e. DLCO and DLCO/CO) and Cardiac responses (i.e. HR, SV and CO) while exercising at SL and HA. Women demonstrated consistently lower values of all parameters due to both reduced absolute oxygen consumption at considered exercising intensities, as well as anatomical differences related to respiratory and cardiovascular systems. However, differences observed at SL were not amplified at HA, suggesting that physiological responses to exercise are similar between trained men and women when oxygen availability is reduced. These results provide new insights within sex differences in the oxygen cascade responses to exercise at HA, but future studies are warranted in order to better elucidate the relationship between these responses and reduction in sustainable fraction of $VO2_{max}$ in NH in females.

SUPPLEMENTAL MATERIAL

Table 1 Prevalence of Expiratoy Flow Limitation (EFL) at Sea Level (SL) and Normobaric Hypoxia (NH) in men and women exercising at similar intensity domains in the 2 experimental conditions (i.e. moderate, heavy and severe).

	MEN		WOMEN		MEN		WOMEN		MEN		WOMEN	
	SL	HA	SL	HA	SL	HA	SL	HA	SL	HA	SL	HA
	MODERATE				HEAVY				SEVERE			
sbj 1	n	n	n	n	n	n	n	1	0.55	0.62	n	0.6
sbj2	n	n	n	n	0.46	0.47	n	n	0.44	0.59	n	n
sbj 3	n	n	n	n	n	n	n	n	n	n	n	n
sbj 4	n	n	n	n	n	n	n	n	n	n	n	0.64
sbj 5	n	n	n	n	n	n	0.74	n	n	0.5	0.55	n
sbj 6	n	n	n	n	n	n	n	0.46	n	n	n	0.66
sbj 7	n	n	n	n	n	0.89	n	n	0.89	0.81	n	n
sbj 8	n	n	n	n	n	n	n	n	n	n	n	n
sbj 9	n	n	n	n	n	n	n	n	n	n	n	n
sbj 10	n	n	n	n	n	n	n	n	0.65	n	n	n
EFL prevalence	0%	0%	0%	0%	10%	20%	10%	10%	40%	40%	10%	30%

The value represent the percentage at which the VT curve derived from IC overlapped with the maximal flow-volume curve (VFL/VT) obtained from baseline spirometry of the same testing day. When VFL/VT was >0.5, EFL was assumed to happen.

OVERVIEW OF THE MAIN FINDINGS (Fig. 15)

Study 1: The combination of cold (-20°C) and hypoxia (≈3500 m) exerted additive rather than synergistic effects on exercise performance, cardiac (i.e. HR at maximal and LT intensities) and ventilatory (i.e. Ve_{max}) responses, highlighting the need for careful consideration of independent and combined stressor impact on considered variables for optimal exercise intensity prescription and training load monitoring in athletes training/competing in hypoxic and/or cold environments.

Study 2: Respiratory Muscle Fatigue and Lung Function are adversely impacted by cold exposure, whereas the effects of hypoxia, both as an independent stressor and in combination with cold, on the respiratory system seem to be negligible when considering high-intensity exercise of short duration. Furthermore, a relationship between ventilatory responses to exercise and LF impairments has been found. Again, these results highlight the importance of considering combined stressor exposure in literature studies, as multiple stressors may operate on several physiological mechanisms in an interactive way, implying both increased health risks for practitioners as well as amplified reductions in exercise performance.

Study 3: Sex differences observed at SL in ventilatory aspects (i.e. Ventilation and Lung Volumes), Lung Diffusive properties (i.e. DLCO and DLCO/CO) and Cardiac responses (i.e. HR, SV and CO) while exercising were not amplified at HA, suggesting that physiological adjustments to exercise in hypoxic conditions are similar between trained men and woman.

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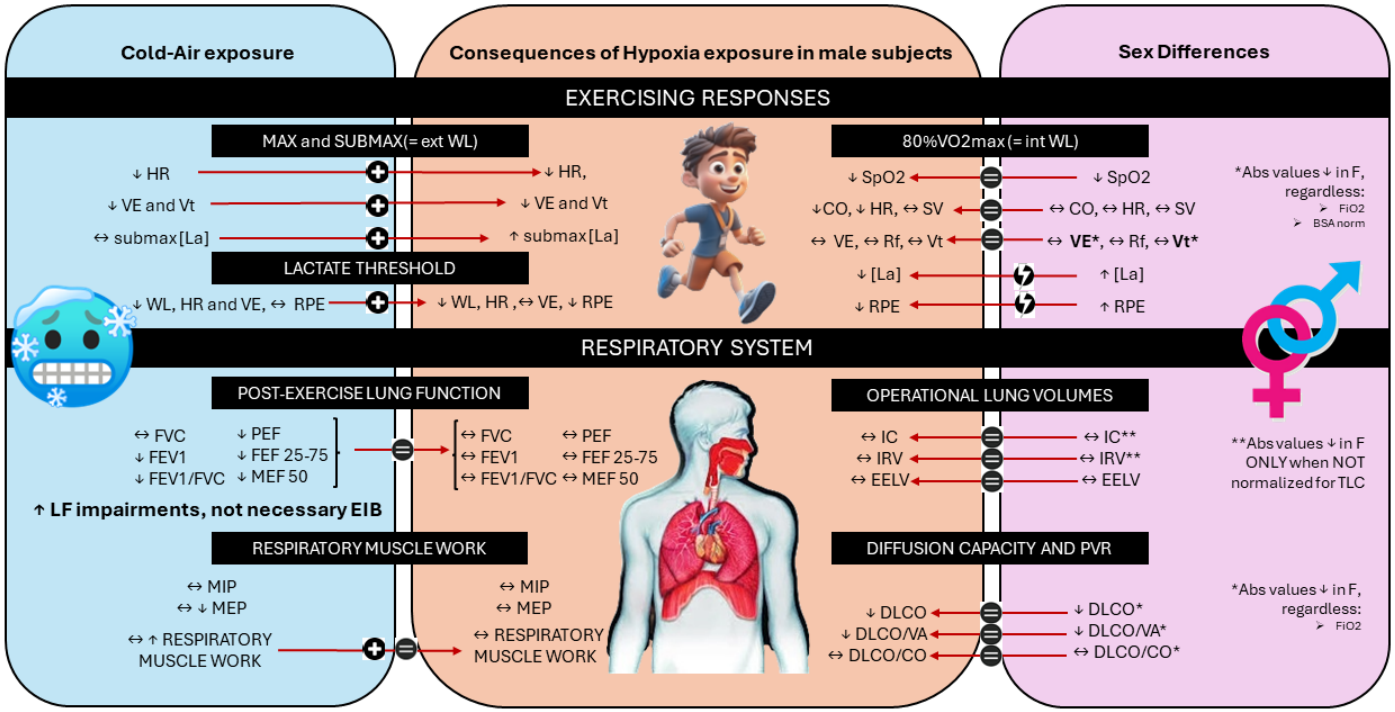

Figure 15 Overall individual responses to hypoxia in males (orange box), the cold in males (light blue box) and hypoxia in females (pink box) on exercising parameters and the respiratory system. Little black arrows indicate an increased (↑), decreased (↓) or equal (=) response if compared to normothermic exercise at sea level. Symbols indicate an attenuation (−), augmentation (+), similar (=) and opposite ( ) influence of cold-air exposure or female sex on hypoxic responses. HR:Heart Rate; CO:Cardiac Output; SV:Stroke Volume; VE:Ventilation; Vt:Tidal Volume; Rf: Respiratory Frequency; [La]:Lactate concentration; WL:WorkLoad; SpO2:Oxygen Pulse Saturation; RPE:Rate of Perceived Exertion; FVC: Forced Vital Capacity; FEV1: forced expiratory volume in 1 s; PEF, peak expiratory flow; FEF 25-75, forced expiratory flow at 25%–75%, MEF 50, mid expiratory flow at 50%; LF: Lung Function; MIP: Maximal Inspiratory Pressure; MEP: Maximal Expiratory Pressure; IC:Inspiratory Capacity; IRV:Inspiratory Reserve Volume; EELV:End Expiratory Lung Volume; DLCO:Diffusion Capacity for Carbon Monoxide; VA:Alveolar Volume; PVR:Pulmonary Vascular Recruitment; F:Females; FiO2: Fraction of Inspired Oxygen; BSA:Body Surface Area; TLC:Total Lung Capacity.

PRACTICAL APPLICATIONS OF THIS DOCTORAL PROJECT

As proposed at the beginning of this doctoral Thesis, the number of people venturing into the mountains for recreational purposes has been increasing in the last decades. Thus, a better understanding of physiological responses while exercising in this harsh environment is necessary to reduce health-related risks. Also, the growing interest in high-altitude sports (i.e. ski-mountaineering) drives attention not only to reducing risks but also to finding strategies to better cope with multiple stressors and reduce performance deterioration: however, a good strategy to avoid performance decrements at altitude requires great knowledge of the mechanisms responsible for these impairments. Applying the same principle, also recommendations to maximize partially known health benefits associated with altitude training or hypoxic conditioning must take into account the possible concomitant presence of rigid temperatures when not in laboratory-controlled situations (i.e., real High Altitude exposure).

Finally, but probably of greatest relevance from a practical point of view, the influence of sex on physiological adaptations to reduced oxygen availability requires much more attention in scientific literature. Global campaigns of famous brands promote the visibility of women in sports, and a rise of female participants not only in the agonistic field but also in leisure activities related to sports practice is evident. However, what may be limiting or advantageous in one gender may not be so in the other and it is necessary to overcome the assumption that women just respond as men to any physiological stimulus. This would help in designing targeted strategies to improve performance, reduce risks, and maximize health benefits related to altitude exposure.

OVERALL LIMITATIONS

First of all, normobaric hypoxia may present several differences if compared to hypobaric hypoxia, especially with regard to performance changes (Grégoire P. Millet & Debevec, 2020) and respiratory load and flow limitations considerations, due to air density not being decreased in NH as in real high altitude exposure (Cogo et al., 1997; Deboeck et al., 2005). Moreover, all three projects focused on cardio-

respiratory outcomes (convective components of $VO2_{max}$), but no assessment of peripheral microcirculation and oxygen utilization at muscle level (diffusive components of $VO2_{max)}$ was performed. This would have been of relevance to determine i) the mechanism of interaction between cold and hypoxia at the peripheral level and ii) sex differences within the diffusive components of the Oxygen cascade when oxygen availability is reduced. Furthermore, cerebral oxygenation assessment would have been useful to determine differences in coping with reduced oxygen availability at the level of the central nervous system in all considered situations (combined presence of cold and female sex). Finally, although invasive, direct measurement of PaO2 is advisable when considering situations that may strongly affect this parameter, in order to differentiate desaturation resulting from a drop in arterial O2 pressure from the one caused by changes in hemoglobin affinity for O2.

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Enhancing comprehension of physiological responses to exercise at high altitude entails the consideration of complex study designs in order closely simulate real-world conditions (Tipton, 2012), just as the inclusion of as many diverse populations as possible for the development of tailored interventions to ensure the safest high-altitude exposure to all subjects. With this Doctoral Thesis, I tried to focus on these aspects by investigating the effects of combined cold-hypoxic exposure on exercising physiological responses (Lloyd & Havenith, 2016) and by adding information on sex differences in the Oxygen Cascade responses to reduced Oxygen Availability (Sims & Heather, 2018). These approaches are temporally challenging as they require increased experimental sessions and complex data analysis, but they are necessary for an effective understanding of human body's reaction to real-world challenges at HA, especially considering the growing interest of the population in engaging in activities in this type of environments. Also, new technologies may be helpful to overcome several challenges related to both data collection (i.e when considering really cold temperatures that do not allow instruments to work properly) and menstrual cycle phase detection in women. In reference to this latter point, striking a balance between proper menstrual cycle

control to avoid uncertain results and, at the same time, a thorough understanding of what is necessary and what is feasible in certain contexts could be helpful for all researchers in promoting the inclusion of women in scientific research studies. Considering all these aspects, future perspectives in this research field, both in general and on a personal level, aim for a more targeted and realistic approach. There is a need for a shift towards increased use of research studies that closely resemble real-life situations, incorporating both physical (such as cold and real High Altitude exposure) and mental stressors. Furthermore, active inclusion of women in research projects is crucial, aiming at bridging the knowledge gap regarding the potential influence of sex on exercising responses at high altitude.

ACKNOWLEDGMENTS

Un proverbio inglese recita ‘Nobody queues for a flat rollercoaster’, che si traduce con ‘Nessuno fa la fila per delle montagne russe piatte’. Questo è stato il motto del mio percorso di Dottorato, composto di grandi soddisfazioni ma anche di qualche difficoltà che però ha sicuramente contribuito alla mia crescita sia accademica che (soprattutto) personale.

Vorrei innanzitutto ringraziare il Prof. Schena, per aver sempre creduto in me ed avermi dato la possibilità di svolgere questo Dottorato, lasciandomi la libertà di sviluppare tutte le mie idee. Inoltre, un sentito ringraziamento va anche a Barbara e Lorenzo, sempre presenti e disponibili, che mi hanno aiutato nella progettazione e nello sviluppo di tutti i progetti presentati.

Un ringraziamento particolare al ‘Forna’, che mi ha affiancato dalla definizione dell’idea fino alla discussione dei risultati in ogni progetto, insegnandomi ad essere una migliore ricercatrice e supportandomi nelle difficoltà. Spero di poter continuare questa collaborazione, promettendo di essere un po' meno ‘in ansia’. Ringrazio anche Aldo per tutte le discussioni interessanti e gli spunti di ricerca suggeriti, e per avermi aiutato a sviluppare un pensiero critico utile a migliorarmi sia come ricercatrice che come persona. Infine, rivolgo un immenso grazie a tutti i miei colleghi del ‘CeRiSM’, che si sono sempre dimostrati disponibili ad insegnarmi qualcosa di nuovo, e che hanno vissuto con me tutte le fasi di questo dottorato, condividendo i successi ma anche supportandomi nelle difficoltà. Grazie anche ai colleghi di Verona, che mi hanno sempre accolto nel loro gruppo senza mai farmi sentire ‘quella di Rovereto’.

Un immenso grazie va poi al mio fidanzato Frenk, che dalla terza superiore mi affianca nella mia crescita personale e professionale, e con il quale spero di condividere tutti i futuri successi, e alle mie amiche Alessandra, Elisa e Vittoria (in ordine alfabetico, per non fare torti), su cui so di poter sempre contare.

Infine, non solo ringrazio, ma dedico questa tesi alla mia mamma, Anita, e al mio papà, Roberto: è grazie a voi che sono arrivata fino a qui, perché avete creduto in me, ma soprattutto perché mi avete insegnato a credere in me. Da ‘grande’ spero di andare fiera di me quanto lo sono di voi.

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