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| **UNIVERSITY OF CHICHESTER**  An accredited institution of the University of Southampton  Department of Sport and Exercise Sciences |
| **The Use of Normobaric Hypoxia as a Method for Enhancing Weight Loss**  by  **Carla Amanda Gallagher** |
| Thesis for the degree of Doctor of Philosophy  August 2014 |

**UNIVERSITY OF CHICHESTER**

An accredited institution of the University of Southampton

ABSTRACT

DEPARTMENT OF SPORT AND EXERCISE SCIENCES

Exercise Physiology

Thesis for the degree of Doctor of Philosophy

**THE USE OF NORMOBARIC HYPOXIA AS A METHOD FOR WEIGHT LOSS**

Carla Amanda Gallagher

Obesity is now a worldwide epidemic which has driven a compelling demand for innovative weight loss programmes. Pioneering investigations have reported that oxygen (O2) variations in humans may produce changes in body composition. In support, reductions in body mass are consistently reported in lowlanders staying at high-altitude. Therefore, it is hypothesised that the hypoxic stimulus associated with this environment, with or without the addition of exercise, could be used as a non-pharmacological therapy for obesity. Using both in vitro and in vivo techniques, in this thesis, this method for weight loss is explored.

Using physiological hypoxia (5% O2), study 1 demonstrated that 24 hours of normobaric hypoxic exposure did not significantly affect myoblast proliferation as determined by cell counts and cell viability. In addition, mRNA expression levels of genes associated with myoblast cell proliferation (myoD, myogenin and myf5) remained unchanged. Study 2 demonstrated that myotubes exposed to 24 hours of hypoxia (5% O2) significantly increased the mRNA expression of MURF-1, MAFbx, and myostatin compared to the normoxic control exposure (21% O2). IGF-1 mRNA was significantly reduced following 24 hours of hypoxia. However, when the length of exposure was reduced to 90 minutes, a significant increase in MURF-1 and MAFbx, but no change was observed for IGF-1 mRNA expression. These results demonstrate that while acute normobaric hypoxia appears to have no detrimental effect on myoblasts, the effect of hypoxia on myotubes can be detrimental as demonstrated by an increased expression of skeletal muscle atrophy genes; however the volume of this response appears to be exposure dependent. Therefore, when conducting human studies, length of hypoxic exposure should be a primary concern.

Study 3 demonstrated that body mass and body composition are unchanged following a 4-week intermittent hypoxic exposure programme compared with a normoxic control period. Blood pressure, resting metabolic rate, respiratory exchange ratio, fasting blood glucose, blood lipid profile parameters and aerobic capacity, also remained unchanged. Leptin and adiponectin, two key appetite hormones were not significantly altered with intermittent hypoxic exposure.

The results of study 4 suggest that the use of age-predicted equations for the prescription of exercise intensity in hypoxia are inadequate. There was no significant reduction in peak heart rate in hypoxia (1000, 2000, 3000, 4000 m) when compared with normoxia, however, all 11 age-predicted equations over-estimated peak heart rate in at least 3 conditions or more. On the contrary, study 4 demonstrated that the ventilatory threshold is not reduced in hypoxia when compared to normoxia, which suggests it is a good model for the prescription of exercise intensity in normobaric hypoxia.

The conclusions drawn from this thesis are; (1) cell proliferation is maintained under hypoxic (5% O2) conditions, (2) myotubes exposed to 90 minutes or 24 hours of hypoxia (5% O2) show increased protein degradation, as demonstrated by an increase in skeletal muscle atrophy genes MURF-1 and MAFbx, which following 24 hours can be explained by an increase in myostatin and decrease in IGF-1 however the same principle does not appear to explain the response at 90 minutes, (4) intermittent hypoxic exposures do not result in significant weight loss, or improve risk markers associated with excess body mass, which may suggest that exercise is a key component in intermittent hypoxic training programmes not hypoxic exposure *per se,* (5) leptin and adiponectin are not altered with IHE, and finally, (6) peak heart rate is not an accurate measure for exercise intensity prescription in hypoxia, but use of ventilatory threshold is promising.

Overall, the use of hypoxia as a non-pharmacological therapy for obesity still warrants further exploration. It has been demonstrated in this thesis that 4 weeks of intermittent hypoxic exposures (90 min·d-1, 3d·wk-1) does not reduce body mass or other associated metabolic health risks. Therefore, it appears that intermittent hypoxic exposures alone without the combination of exercise is not sufficient enough to induce weight loss suggesting that exercise and hypoxia should be combined within weight loss interventions designed to produce significant losses in body mass. In the design of an intermittent hypoxic training programme, the ventilatory threshold has been shown to be an appropriate method to determine exercise intensity in normobaric hypoxia, however equations used to predict peak heart rate should be avoided.

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DECLARATION OF AUTHORSHIP

I, Carla Amanda Gallagher declare that the thesis entitled

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and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

* this work was done wholly or mainly while in candidature for a research degree at this University;
* where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
* where I have consulted the published work of others, this is always clearly attributed;
* where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
* I have acknowledged all main sources of help;
* where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
* parts of this work have been published, as stated overleaf:

Signed: ………………………………………………………………………..

Date:…………………………………………………………………………….

Publications and Presentations

Publications

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Presentations

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So a new chapter begins...thank you all for joining me on this one…time to climb a new mountain!

Definitions

|  |  |
| --- | --- |
| Adiponectin | A 244-amino acid protein expressed and secreted exclusively from white adipose tissue |
| AMP-activated protein kinase | Stored in white adipose tissue, AMPK is involved in appetite regulation, glucose uptake and fatty acid uptake |
| Anorexigenic | Reducing appetite |
| Basal Metabolic Rate | Daily energy expenditure at rest |
| Body Mass Index | An individual’s body mass divided by the square of the individual’s height |
| Cholecystokinin | An endogenous peptide found in the gastrointestinal tract and brain |
| Coronary Heart Disease | A narrowing or blockage of the coronary arteries and vessels that provide oxygen to the heart |
| Diet-induced Energy Expenditure | Increase in energy expenditure above basal fasting level divided by the energy content of the food ingested |
| Dyslipidemia | An abnormal amount of [lipids](http://en.wikipedia.org/wiki/Lipid) (e.g. cholesterol and/or fat) presented in the [blood](http://en.wikipedia.org/wiki/Blood) |
| Energy Digestibility | Proportion of the energy in a food which is digested |
| Ghrelin | A 28-amino acid protein mainly produced in endocrine cells of the human gastric mucosa, involved in the regulation of appetite |
| HIF | A heterodimeric complex consisting of the O2 regulated HIF-1α subunit and the O2-independent HIF-1β subunit |
| HIF-1α | A 826-amino acid protein with a basic helix-loop-helix domain and a PAS domain, is a transcription factor regulated by oxygen |
| HIF-1β | An encoded protein that forms the beta subunit of the hypoxia inducible factor complex. Regulated by oxygen availability |
| Hypertension | High blood pressure, a [medical condition](http://en.wikipedia.org/wiki/Disease) in which the [blood pressure](http://en.wikipedia.org/wiki/Blood_pressure) in the [arteries](http://en.wikipedia.org/wiki/Artery) is elevated – a reading of 140/90 mmHg is considered to be hypertensive |
| Hypobaric | Reduced barometric pressure |
| Hypoxemia | Abnormally low levels of oxygen in the arterial blood |
| Hypoxia Responsive Element | DNA sequences in a gene’s promoter region that mediate expression during hypoxia |
| Insulin-like Growth Factor 1 | A protein encoded by the IGF protein, plays an important role in the anabolic effects of protein in skeletal muscle |
| Intestinal Permeability | A condition relating to damaged or disturbed bowel lining |
| Leptin | A 146-amino acid protein secreted predominantly in white adipose tissue which regulates appetite |
| Lipofuscin | A finely granular yellow-brown [pigment](http://en.wikipedia.org/wiki/Pigment) [granules](http://en.wikipedia.org/wiki/Granule_(cell_biology)) composed of [lipid](http://en.wikipedia.org/wiki/Lipid)-containing residues of [lysosomal](http://en.wikipedia.org/wiki/Lysosomal) digestion |
| Muscle Atrophy F-box | E3 ubiquitin ligase involved in skeletal muscle atrophy |
| Muscle Ring Finger-1 | E3 ubiquitin ligase involved in skeletal muscle atrophy |
| Myoblast | A primitive muscle cell with the potential to develop into a muscle fibre |
| myoD | A myogenic transcription factor involved in skeletal muscle repair and differentiation |
| Myogenic Factor 5 | A myogenic factor involved in regulating muscle differentiation |
| Myogenic Regulatory Factor 4 | A gene involved in regulating myogenesis and muscle regeneration |
| Myogenin | A differentiation marker involved in the co-ordination of skeletal muscle development and repair |
| Myogenesis | The formation of muscle tissue, in particular during embryonic development - muscle fibres form the fusion of myoblasts into myotubes |
| Myogenic | Originating in myocytes or muscle tissue |
| Myostatin | A gene known to inhibit skeletal muscle differentiation and growth |
| Myotube | A developing skeletal muscle fibre with a centrally located nucleus |
| Normobaric | A normal barometric pressure equivalent to sea-level |
| Normoxia | A normal level of oxygen (20.93%) |
| Orexigenic | Increasing appetite |
| PGC-1α | A transcriptional coactivator which is a key regulator of energy metabolism, may be involved in the development of obesity |
| Peroxisome proliferator-activated receptor | Group of transcription factors that regulate the expression of genes. They play an essential role in carbohydrate, lipid and protein metabolism |
| Protein Degradation | The breakdown of proteins by proteases |
| Protein Synthesis | The accumulation of proteins which can occur when protein accumulation is greater than protein degradation. |
| Respiratory Exchange Ratio | The proportion of carbon dioxide volume generated to the oxygen volume consumption by using expired air in the calculation |
| Respiratory Quotient | The ration of the volume of carbon dioxide evolved to that of oxygen consumed by an organism, tissue, or cell in a given time |
| Serotonin | A hormone found in many tissues, including the brain, blood serum and gastric mucous membranes. Involved in appetite regulation |
| Transactivation | An increased rate of gene expression triggered either by biological processes or by artificial means |
| Triglycerides | An ester derived from glycerol and three fatty acids |
| Type II Diabetes | A metabolic disorder associated with insulin resistance - a condition in which a person’s body tissues have a lowered response to insulin |
| Ubiquitin | A protein which attaches to other proteins, transporting them to the proteasome for degradation |
| Ubiquitination | The inactivation of a protein by the attachment of a ubiquitin |
| Von Hippel-Lindau | A ubiquitin protein ligase that regulates the half-life of HIF-1α |

Abbreviations

|  |  |
| --- | --- |
| Akt | Protein kinase B |
| AMPK | AMP-activated protein kinase |
| ANOVA | Analysis of variance |
| bHLH | Basic helix-loop-helix |
| BMI | Body mass index |
| BMR | Basal metabolic rate |
| CaO2 | Arterial oxygen content |
| CCK | Cholecystokinin |
| cDNA | Complementary deoxyribonucleic acid |
| CO2 | Carbon dioxide |
| CT | Cycle threshold |
| CVO2 | Venous oxygen content |
| DBP | Diastolic blood pressure |
| DMEM | Dulbecco modified eagles medium |
| DNA | Deoxyribonucleic acid |
| FBS | Fetal bovine serum |
| FIO2 | Fraction of inspired oxygen |
| FOXO | Forkhead box protein |
| GLUT4 | Glucose transporter 4 |
| GM | Growth media |
| GXT | Graded exercise test |
| HDL | High-density lipoprotein |
| HIF | Hypoxia inducible factor |
| HIF-1α | Hypoxia inducible factor 1 alpha subunit |
| HIF-1β | Hypoxia inducible factor 1 beta subunit |
| HR | Heart rate |
| HRE | Hypoxia responsive element |
| HRpeak | Peak heart rate |
| HRVT | Heart rate at the ventilatory threshold |
| HSC | Hypoxic stimulus charge |
| HSE | Health Survey for England |
| H+ | Hydrogen ion |
| IGF-1 | Insulin-like Growth Factor 1 |
| IHE | Intermittent hypoxic exposure |
| IHT | Intermittent hypoxic training |
| LDL | Low-density lipoprotein |
| LLTH | Live low – Train high |
| MAFbx | Muscle atrophy F-box |
| MRF | Myogenic regulatory factor |
| MRF4 | Myogenic regulatory factor 4 |
| mRNA | Messenger ribonucleic acid |
| MURF-1 | Muscle ring finger 1 |
| Myf5 | Myogenic factor 5 |
| MyoD | Myogenic differentiation 1 |
| NICE | National institute for health care and excellence |
| NHS | National Health Service |
| OGTT | Oral glucose tolerance test |
| O2 | Oxygen |
| PBS | Phosphate buffered saline |
| PGC-1α | Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha |
| PHRpeak | Age-predicted heart rate peak |
| PI3k | Phosphatidylinositide 3-kinase |
| PO2 | Partial pressure of oxygen |
| PPAR | Peroxisome proliferator-activated receptor |
| PCR | Polymerase chain reaction |
| qPCR | Quantitative polymerase chain reaction |
| RCP | Respiratory compensation point |
| REE | Resting energy expenditure |
| RER | Respiratory exchange ratio |
| RH | Relative humidity |
| RNA | Ribonucleic acid |
| RPE | Rate of perceived exertion |
| RPM | Revolutions per minute |
| RPII-β | Ribonucleic Acid Polymerase II |
| RT-PCR | Real-time polymerase chain reaction |
| 1SD | One standard deviation |
| SBP | Systolic blood pressure |
| SL | Sea-level |
| SPO2 | Arterial oxygen saturation |
| SV | Stroke volume |
| Tamb | Ambient temperature |
| TBS | Tris-buffered saline |
| Ʋ | Air velocity |
| CO2 | Carbon dioxide production |
| E | Minute ventilation |
| Epeak | Peak minute ventilation |
| VHL | Von Hippel-Lindau protein |
| O2 | Oxygen uptake |
| O2peak | Rate of peak oxygen uptake |
| O2VT | Oxygen uptake at the ventilatory threshold |
| VT | Ventilatory threshold |
| WHR | Waist to hip ratio |
| peak | Peak power output |
| WVT | Power at the ventilatory threshold |

# General Introduction

## Obesity

Obesity is classified as a condition associated with excessive adipose tissue or fat accumulation beyond a threshold for which is considered a normative or reference value ([Kuczmarski, 2007](#_ENREF_153)). Over the last decade, there has been a dramatic rise in the incidence of obesity worldwide among men, women and children ([Borer, 2008](#_ENREF_29); [Flegal et al., 2002](#_ENREF_87)). Currently, it is reported that one in four adults living in England are classified as obese under the definition of a Body Mass Index (BMI) ≥ 30 kg·m-2 ([NICE, 2006](#_ENREF_204)). In 2011, the Health Survey for England (HSE) reported that 65% of men and 58% of women aged 16 or over were overweight or obese, and in 2010 and 2011, obesity in women was at its highest level since 1993. Consequently, obesity is becoming an increasing concern representing a major health burden as a growing epidemic emerges, and with predicted figures expanding rapidly the situation appears unlikely to improve in the immediate future.

Prominent changes in lifestyle over the last decade have played an important role in the contribution to the rapid rise in obesity. Cross-sectional data have found associations between the total amount of time spent sitting down and BMI ([Martinez-Gonzalez et al., 1999](#_ENREF_181)), while ‘a low participation in sports activities’, and a ‘lack of interest in taking exercise’ are also statistically significant predictors of obesity ([Martinez-Gonzalez, et al., 1999](#_ENREF_181); [Martinez, 2000](#_ENREF_183)). To further highlight this association, in 2008, only 6% men and 4% of women were conforming to the recommended physical activity guidelines of at least 30 minutes a day of moderate or greater intensity activity on five or more days of the week ([N.H.S, 2008](#_ENREF_200)). In addition to the decline in physical activity participation, individual food choices are becoming more energy dense and portion sizes are on the rise ([Nielsen & Popkin, 2003](#_ENREF_205)). Together, increases in food consumption in recent years coupled with declining physical activity rates has caused individuals to acquire positive energy balances on a more regular basis, which has led to the increasing prevalence of obesity worldwide. However, there are other factors such as genetic background, dietary macronutrient composition, distribution of energy expenditure or an individual’s substrate oxidation rate which can influence the energy balance equation in the human body ([Marti et al., 2008](#_ENREF_178)) and these factors should not be ignored. Thus, obesity is seen as a chronic multi-factorial condition resulting from the interaction between genotype, environment, physical activity patterns and diet ([Marti, et al., 2008](#_ENREF_178); [McCarthy, 2010](#_ENREF_187); [Urdampilleta et al., 2012](#_ENREF_276)).

Obesity is a major risk factor for a number of chronic diseases such as type II diabetes, hypertension, dyslipidemias (abnormal blood lipid levels), cardiovascular disease, pulmonary afflictions, osteoarthritis, and many more ([Anderson & Konz, 2001a](#_ENREF_7); [Brown et al., 2000](#_ENREF_34); [Carter et al., 2000](#_ENREF_42); [Kopelman, 2000](#_ENREF_152)). Moreover, the risk of coronary artery disease and stroke has been reported to increase by 2.4 fold in individuals who are clinically obese ([Aylott et al., 2008](#_ENREF_15)). Consequently, these comorbidities of obesity conspire to increase overall mortality, which, large human studies report, rise steadily as a function of increasing body mass ([Calle et al., 1999](#_ENREF_40); [Cummings & Schwartz, 2002](#_ENREF_62); [Mokdad et al., 2003](#_ENREF_194)). However, both obesity and its associated increase with disease risk factors are preventable, and research has demonstrated that lifestyle changes that reduce body mass such as physical activity are linked to reduced health risks ([Mokdad, et al., 2003](#_ENREF_194)).

A reduction in body mass is achieved when a negative energy balance is established; this can be readily achieved through a change in dietary or exercise habits, with a combination of these two modalities producing a greater loss in body mass ([Miller et al., 1997](#_ENREF_191)). Despite the compelling demand for innovative weight loss programmes and a substantial increase in research findings appointing several methods (e.g. dietetic and nutritional therapies, physical activity programmes, pharmacological therapies and bariatric surgery), effective management programmes for obesity remain elusive ([Lippl et al., 2010](#_ENREF_165)). However, original investigations have reported that reduced oxygen (O2) availability as a result of lowered barometric pressure in humans may produce changes in body composition ([Armellini et al., 1997](#_ENREF_10); [Boyer & Blume, 1984](#_ENREF_32); [Lippl, et al., 2010](#_ENREF_165)). In support of the investigations into O2 reductions, decreases in body mass are consistently reported in lowlanders staying at altitude ([Boyer & Blume, 1984](#_ENREF_32); [de Glisezinski et al., 1999](#_ENREF_67); [Fulco et al., 1985](#_ENREF_96); [Lippl, et al., 2010](#_ENREF_165); [Pugh, 1962](#_ENREF_218); [Rose et al., 1988](#_ENREF_238)) suggesting that the hypoxic stimulus associated with this environment, with or without the addition of exercise, could be an interesting approach to enhancing the effectiveness of weight loss programmes. The aim of the research described in this thesis was to assess the effectiveness of hypoxia as a non-pharmacological therapy for weight loss using both in vitro and in vivo techniques.

## Hypoxia

Hypoxia may be induced by exposure to high-altitude or in pathological conditions such as chronic obstructive pulmonary disease and chronic heart failure ([Etheridge et al., 2011](#_ENREF_80)). In metabolic terms, hypoxia is defined as a state in which the rate of O2 utilisation by the cells is inadequate to supply all of the body’s energy requirements. It provides an important challenge for the organism which compromises many bodily functions, including cardio-respiratory, endocrine, metabolic, nutritional and thermal homeostasis ([Magalhães & Ascensão, 2008](#_ENREF_174)). Simulation of high-altitude in healthy humans under artificial conditions of hypoxia (i.e. breathing O2 at reduced concentrations) allows us to understand the molecular and systemic mechanisms involved in O2 sensing and the ensuing adaptive responses (Etheridge et al., 2011).

Hypoxic exposure can be acute or chronic, with each leading to different human physiological responses, therefore before exploring further it is important to define these two concepts individually. The term “acute hypoxia” will be used in this thesis to identify a period of several hours (≤ 24 hours), whereas “chronic hypoxia” will be used to define a period of days (≥ 24 hours). Moreover, it is also important to establish that hypoxia can be either normobaric or hypobaric, normobaric hypoxia refers to those experiments carried out in environmental chambers where the level of O2 concentration is altered but the barometric pressure remains the same. Hypobaric hypoxia will be used to describe experiments which have been carried out at high-altitude or in a specialised hypobaric hypoxic chamber.

Exposure to a hypoxic environment triggers a complex machinery of responses, involving all cells through the activation of genes presenting a hypoxia responsive element [HRE ([Favret & Richalet, 2007](#_ENREF_83))]. The major genes representing HREs are known as hypoxia-inducible factors (HIF); the most understood being hypoxia-inducible factor-1α (HIF-1α) and hypoxia-inducible factor-1β (HIF-1β). Under physiological normoxia (21% O2), the HIF-1α protein becomes hydroxylated at two proline residues (-402 and -564) located in its O2-dependent degradation domain ([Jaakkola et al., 2001](#_ENREF_135)) and is targeted by the von Hippel-Lindau (VHL) protein for ubiquitination and proteasome-mediated degradation ([Ohh et al., 2000](#_ENREF_208)). In contrast, under physiological hypoxia [≤ 5% ([Guzy & Schumacker, 2006](#_ENREF_118))], HIF-1α does not undergo proteasome degradation, instead the stabilised HIF-1α translocates into the nucleus where it dimerises with the O2-independent HIF-1β protein and initiates transcription by binding to HREs in the promoter region ([Gustafsson et al., 2005](#_ENREF_117); [Lin et al., 2008](#_ENREF_163)). This action of HIF then activates the transcription of genes that allow the cell to adapt and survive in hypoxia [Figure 1.1 ([Semenza et al., 2006](#_ENREF_252))]. Further to this cellular response, a number of physiological responses are activated; at rest and during sub-maximal exercise, heart rate, cardiac output, and minute ventilation are elevated. This series of reactions consequently allows the physiological systems of the human to efficiently survive the decrease in the partial pressure of inspired O2 and the resulting drop in arterial O2 saturation ([Mazzeo, 2008](#_ENREF_185)).

Figure 1.. Hypoxia-inducible factor-1 pathway in normoxia and hypoxia ([LaManna et al., 2004](#_ENREF_154)).

**HIF-1α**

HO

**VHL**

HO

HO

**Nucleus**

**HIF-1α**

**HIF-1β**

**Translocation**

**HIF-1α**

**HIF-1α**

**HO**

Ubiquitin

**+**

**VHL**

**HIF-1α**

**HO**

**HIF-1α**

**HO**

**Fe+2**

**+ Fe+2**

**+ O2**

**O2**

**+**

**HO**

**Hypoxia**

**HIF-1β**

**HIF-1β**

**HIF-1β**

**Gene transcription**

**Normoxia**

## Methods of Achieving Hypoxia at Sea-Level

There are several methods by which normobaric hypoxia can be achieved; these include O2 filtration, nitrogen flushes, and chemical treatments. Moreover, there are different environments in which low O2 can be created which include hypoxic chambers, hypoxic tents or application through a respiratory mask system attached to a hypoxic generator.

Hypoxic chambers are designed using either of the following two methods; O2 filtration and nitrogen displacement. Oxygen filtrations systems such as those used within the studies presented in this thesis (chapters 4-7) are designed using a molecular sieve system enclosed of microscopic pores which only allow small or mobile molecules to permeate through [i.e. O2, carbon dioxide (CO2), water vapour]. Thus, the principle molecule unable to permeate through is nitrogen. Essentially, compressed air is passed down fibres in which a restriction is applied creating pressure, the greater the restriction applied, the higher the pressure leading to a lower number of permeated O2 molecules. When using this method the O2 level in the output stream can be changed from 20% to 1%.

Hypoxic tents and respiratory mask systems work on the same principle; both substitute normal oxygen concentrations for lower oxygen concentrations using a gas mixture while maintaining the same barometric pressure as is found at sea-level (SL). The gas mixture can contain as low as 12% O2, with the remainder of the mixture being nitrogen which displaces the O2 and excess CO2 to create a hypoxic environment. This method can also be used for smaller hypoxic chambers such as those used in cell culture and which have been used within studies presented in this thesis (chapters 2 and 3).

Hypobaric chambers are an alternative method of simulating a high-altitude environment, by a reduction in barometric pressure, which is achieved by regulating air flow within the chamber. Although this method possibly provides a better simulation of a high-altitude environment than normobaric hypoxic methods, they are a more expensive alternative and so very few are located within the UK.

## Hypoxia and Weight Loss

As previously stated, exposure to hypoxia consistently results in significant losses in body mass ([Armellini, et al., 1997](#_ENREF_10); [Boyer & Blume, 1984](#_ENREF_32); [Lippl, et al., 2010](#_ENREF_165); [Pugh, 1962](#_ENREF_218); [Rose, et al., 1988](#_ENREF_238)), which can approach 9% during sojourns of at least 7 days in duration ([Fusch et al., 1996](#_ENREF_97)). The first series of classic studies at high-altitude in 1960 and 1961 involved lowland native scientists performing tests over 8-months of exposure at a base camp of 5800 m, this Himalayan project (also known as the “Silver Hut Expedition”) was the earliest expedition to report a loss in body mass ([Pugh, 1962](#_ENREF_218)). Initially it was reported that participants lost 0.5-1.4 kg per week at an altitude of 5790 m, and that some of this loss was regained on descent (3960-4570 m). More importantly however, at the conclusion of the expedition body mass losses had increased further ranging from 6.4 to 9.0 kg. The 1981 American Medical Research Expedition to Everest (AMREE) confirmed this characteristic response to hypoxia, reporting that some participants experienced up to a 5 kg loss in body mass ([Boyer & Blume, 1984](#_ENREF_32)). During this expedition the effects of hypoxia on changes in body fat percentage and limb circumferences were also documented and both were reported to decrease as a result of high-altitude exposure. Additionally, the authors reported that body mass losses were directly proportional to initial body mass, suggesting that at altitude those with a higher body mass and body fat percentage will experience greater losses in body mass ([Boyer & Blume, 1984](#_ENREF_32); [Ge et al., 2010](#_ENREF_101); [Kayser, 1994](#_ENREF_142)). This relationship between starting body mass and total body mass loss is a promising finding for the present programme of research as it indicates that hypoxia with or without exercise could produce greater losses in body mass.

Studies investigating losses in body mass during altitude sojourns report that the degree of weight loss varies depending on the height/altitude achieved (i.e. level of hypoxia) and the length of stay (i.e. exposure), which could be regarded as the hypoxic dose. In the majority of studies reporting a reduction in body mass at altitude, most of the loss was attributed to a decrease in fat mass ([Boyer & Blume, 1984](#_ENREF_32); [Butterfield et al., 1992](#_ENREF_38); [Kayser et al., 1992](#_ENREF_143); [Westerterp et al., 1992a](#_ENREF_290)). However, the proportion of mass lost from various tissue stores appears to change over the duration of an expedition. In one classic high-altitude study, during the approach march to Everest base camp, fat represented 70% of the total 1.9 kg mass loss. However, after arrival above 5400 m, where the mass lost was over twice that at base camp (4 kg), fat accounted for only 27% ([Boyer & Blume, 1984](#_ENREF_32)). This shift away from fat loss suggests that a major metabolic shift toward muscle and protein catabolism occurs following prolonged periods at extreme altitude. This finding has been further supported by the observation that prolonged hypoxia leads to significant reductions in muscle volume of the order of 10% to 15%, with a concomitant decrease in muscle fibre size of 20% to 25% ([Green et al., 1989](#_ENREF_113); [MacDougall et al., 1991](#_ENREF_171)). It appears that muscle catabolism therefore plays a central role in weight loss at extreme altitudes, and this may have important implications for research examining the use of hypoxia to aid weight loss, since obese individuals may have a relatively small percentage of fat-free mass relative to fat mass. Consequently, any loss in fat-free mass should be minimised where possible. Exploring the optimum combination of hypoxia and rest or exercise is therefore paramount in the design of any hypoxic intervention in order to design evidence-based research.

The exact mechanisms underlying the responses to hypoxia-induced body mass losses remain unclear and appear to be multifaceted; reduced food intake as a result of appetite suppression ([Tschop & Morrison, 2001](#_ENREF_274)), increased energy expenditure ([Armellini, et al., 1997](#_ENREF_10)), increased metabolic rate ([Lippl, et al., 2010](#_ENREF_165)), fluid imbalance ([Okazaki et al., 1984](#_ENREF_210)), malabsorption of nutrients ([Boyer & Blume, 1984](#_ENREF_32)) and reduced protein synthesis ([de Theije et al., 2013](#_ENREF_68); [Rennie et al., 1983](#_ENREF_226)) are some of the mechanisms proposed as contributory factors. The noted mechanisms will be discussed within this thesis, some of which will be explored experimentally within the following thesis chapters.

### Hypoxia, Energy Intake and Appetite Suppression

Voluntary energy intake in response to a hypoxic environment is decreased, and it is common for individuals to not meet daily energy requirements, with reductions in calorie consumption of up to 40% reported ([Consolazio et al., 1968](#_ENREF_60); [Rose, et al., 1988](#_ENREF_238); [Vats et al., 2007](#_ENREF_278)). In a study examining energy intake in subjects exposed to 21 days at 6542 m, it was reported that energy intake was about 76% of the measured energy expenditure observed at SL and this coincided with losses in body mass of up to 7 kg over a 3-week period. It was suggested that losses in body mass occurred due to an inability to attain an equal energy balance, despite limited physical activity and ad libitum access to palatable food ([Westerterp et al., 1994](#_ENREF_291)). In support of these findings, Westerterp-Platenga and colleagues (1999) conducted a study exposing 8 men to a simulated ascent of Mount Everest in a hypobaric hypoxic chamber and found a significant reduction in food intake compared to that in normoxia ([Westerterp-Plantenga et al., 1999](#_ENREF_288)). Coinciding with the reduction in food intake the authors reported reduced meal size due to an earlier onset in satiety, symptoms of acute mountain sickness and reduced appetite. A change in meal pattern was also observed which developed from a ‘gorging’ to a ‘nibbling’ style, i.e. an increase in meal frequency; this observation was related to the change in appetite profile. Furthermore, Vats and co-workers (2007) reported a significant decrease in food intake at altitude similar to that of other research, with the decrease in energy intake again coinciding with decreases in body mass ([Vats, et al., 2007](#_ENREF_278)). In summary, food intake is reduced with exposure to a hypoxic environment. In response to these consistent findings research has since identified that particular appetite hormones (e.g. leptin, ghrelin, adiponectin) could be responsible for the reduction in appetite and premature satiety when individuals are exposed to a hypoxic environment ([Shukla et al., 2005](#_ENREF_256); [Tschop & Morrison, 2001](#_ENREF_274); [Tschop et al., 2000](#_ENREF_275)). An overview of the response of the major appetite regulating hormones to hypoxia are displayed in Figure 1.2 and discussed in detail in the sections following it.

|  |
| --- |
| Hypoxia  ↑ Leptin  ↑ Cholecystokinin  ↑ AMPK  ↑ Serotonin  ↓ Ghrelin  ↓ Adiponectin  ↓ Food Intake |
| Figure .. Overview of the major appetite regulating hormones/enzymes and their response to hypoxic conditions. |

Leptin, an appetite suppressing (anorexigenic) hormone secreted primarily in white adipose tissue (WAT), is a mediator of long-term regulation of energy balance, involved in suppressing food intake and thereby inducing weight loss (Klok et al., 2007). It has been observed that circulating leptin levels increase due to hypoxia and this event has been one of the keys for understanding the appearance of some of the accompanying symptoms i.e. appetite suppression and weight loss ([Quintero et al., 2010](#_ENREF_223)). Several human studies have measured the response of leptin with altitude exposure. Tschop and co-workers (2000) in a prospective study of 20 healthy men reported elevated plasma leptin levels following a climb to 4559 m, this increase coincided with a loss in appetite, increased energy expenditure, and substantial weight loss ([Tschop, et al., 2000](#_ENREF_275)). A study by Shukla and colleagues (2005) also reported increased plasma levels of leptin and weight loss following 7 days of exposure to hypoxic conditions (4300 m) in a group of 30 lowlanders ([Shukla, et al., 2005](#_ENREF_256)). However, the findings in this area are equivocal, with other studies performed under hypobaric hypoxic conditions reporting no change ([Barnholt et al., 2006](#_ENREF_19); [Woolcott et al., 2002](#_ENREF_296)) or decreases in plasma leptin levels ([Vats, et al., 2007](#_ENREF_278)). Nevertheless, support for an increase in plasma leptin levels comes from studies performed under normobaric hypoxia ([Snyder et al., 2008a](#_ENREF_261)); possibly as this controlled environment eliminates stressors such as cold, limited food availability, physical exertion and rugged terrain common in high-altitude field studies ([Barnholt, et al., 2006](#_ENREF_19)) and which may account for the discrepancies between research findings.

Although leptin has received much attention and its response to hypoxia explored, ghrelin, an appetite stimulating (orexigenic) hormone secreted primarily by endocrine cells within the gastrointestinal tract, also involved in short- and long-term appetite control is understudied in humans. In a study described previously, Shukla and colleagues (2005) reported that a 48 hour exposure to 4300 m, significantly decreased plasma ghrelin levels; a finding often linked to appetite suppression ([Cummings & Shannon, 2003](#_ENREF_63)). Moreover, ghrelin levels were still decreased after 7 days of altitude exposure compared with SL measures. The decrease in ghrelin observed alongside an increase in leptin levels (Figure 1.2) are thought to be responsible for appetite suppression at high-altitude. A study conducted at SL examining these two hormones simultaneously demonstrated the suppressive effect of leptin on ghrelin induced feeding and inhibitory effect of ghrelin on leptin induced reduction of food intake. The results confirm that both leptin and ghrelin play a significant role in appetite regulation and may alter each other’s activity under a given situation ([Nakazato et al., 2001](#_ENREF_201)). Therefore, it is important to explore the role of leptin and ghrelin in response to acute and chronic hypoxia in more detail given the limited findings on their simultaneous action in the role of appetite suppression.

Another hormone secreted within WAT which is thought to play an important role in the regulation of food intake is adiponectin or as it is also known, adipocyte complement-related protein ([Dridi & Taouis, 2009](#_ENREF_73)). Adiponectin has also been implicated with the high-altitude effects on appetite and body mass. This effect of hypoxia has been examined in Sprague-Dawley rats; it was found that following 8 weeks of continuous hypoxia adiponectin was significantly reduced ([Chaiban et al., 2008](#_ENREF_45)). Contrary to these findings, a study in humans demonstrated that 21 days at 4300 m did not alter adiponectin levels ([Barnholt, et al., 2006](#_ENREF_19)). However, animal studies examining the role of intermittent hypoxia have reported that adiponectin levels are reduced ([Magalang et al., 2009](#_ENREF_173); [Zhang et al., 2010](#_ENREF_302)) and thus the examination of the response of adiponectin to hypoxia is still warranted. It also remains to be noted that research has also explored the response of other major appetite sensors [Figure 1.2 (e.g. Cholecystokinin (CCK), AMP-activated protein kinase (AMPK) and serotonin)] and their action in hypoxia should not be ignored ([Bailey et al., 2000b](#_ENREF_17); [Chaiban, et al., 2008](#_ENREF_45); [Sachanska, 1998](#_ENREF_242)).

Each of the appetite hormones listed above play a different role in the control of food intake, however, all respond in a similar manner due to anorexigenic properties, increasing in response to hypoxic exposure. Cholecystokinin is mainly stored in the I-cells of the small intestine responsible for the digestion of fat and protein as well as controlling processes within the gastrointestinal system, such as, gastric emptying ([Chaudhri et al., 2006](#_ENREF_48); [Stanley et al., 2005](#_ENREF_263)). Since CCK is an anorexigenic hormone, any increases in CCK will reduce appetite and subsequently food intake. A study conducted at 5100 m demonstrated that CCK is increased following 7 days of hypobaric hypoxic exposure which was associated with decreased caloric intake and a substantial decrease in body mass (4.5-6.5 kg) despite availability of palatable food ([Bailey, et al., 2000b](#_ENREF_17)). It can be suggested from these findings that the anorexic effects of CCK contribute to the reduced caloric intake observed at altitude and subsequent weight loss.

Serotonin is primarily secreted by the gastrointestinal tract which partially controls food intake, protein intake and carbohydrate selection ([Gonzales, 1980](#_ENREF_110); [Urdampilleta, et al., 2012](#_ENREF_276)). An increase in serotonin leads to appetite suppression, therefore representing an inverse relationship with food intake. It has been demonstrated in rats exposed to 4340 m for 84 days that increased serotonin levels led to decreased body mass gain of up to 15% compared with SL controls ([Gonzales, 1980](#_ENREF_110)). In humans, it has been demonstrated that if basal levels of serotonin are low, exposure to acute hypoxia (5500 m, 30 minutes) will cause levels to increase. However, in those individuals in which basal serotonin levels are high (> 3,413 mmol·L-1), it will decrease ([Sachanska, 1998](#_ENREF_242)).

Since evidence suggests that levels of both serotonin and CCK are increased with hypoxic exposure, it is expected that appetite would be subsequently reduced which might in-turn stimulate weight loss. Together, observations from studies examining appetite hormones in response to hypoxia allow consideration of the hypothesis that intermittent hypoxia induces fat loss which may occur via changes in appetite and could ameliorate cardiovascular health, which might be of interest for the management of obesity ([Urdampilleta, et al., 2012](#_ENREF_276)).

### Hypoxia, Energy Expenditure and Metabolic Rate

Daily energy expenditure can be divided into three components: basal metabolic rate (BMR), diet-induced energy expenditure and physical activity-induced energy expenditure. The BMR is normally the largest component of our daily energy expenditure, whereas diet- and physical activity-induced expenditure can vary daily ([Westerterp, 2001](#_ENREF_289)). The effect of high-altitude on BMR has received some attention in research studies, however their findings are rather inconclusive, with some reporting an increase ([Butterfield, et al., 1992](#_ENREF_38)) and others reporting no change ([Armellini, et al., 1997](#_ENREF_10); [Westerterp, et al., 1992a](#_ENREF_290)). These discrepancies could be attributed to a number of factors including altitude height, length of exposure and measuring techniques, however research findings suggest that length of exposure plays the most important role.

Hannon and Sudman (1973) reported a 28% increase in BMR in women following 36 hours of altitude exposure [Pikes Peak 4300 m ([Hannon & Sudman, 1973](#_ENREF_121))] which was also confirmed later on the same location in men ([Butterfield, et al., 1992](#_ENREF_38)). Other studies examining acute exposure to 3650 m and 3800 m have reported increases in BMR of 6% and 10%, respectively ([Kellogg et al., 1957](#_ENREF_145); [Stock et al., 1978](#_ENREF_266)). Additionally, a 7-day stay at altitude (2650 m) in obese individuals supported the above findings, by reporting an increase in BMR with metabolic rate remaining elevated for up to 7 days on return to SL ([Lippl, et al., 2010](#_ENREF_165)). The initial rise in BMR has been attributed to the high levels of adrenaline individuals incur in response to an increased sympathetic drive with hypoxic exposure ([Kayser, 1994](#_ENREF_142); [Kayser & Verges, 2013](#_ENREF_144)). In contrast to the metabolic response observed in acute hypoxia, chronic exposure appears to diminish the increase in BMR. However, it should be noted that BMR does not appear to return completely to baseline values, at least when energy balance is maintained ([Kayser, 1994](#_ENREF_142)). In one study, where no change was reported, subjects were adapted to altitude for over 4 weeks and measured BMR was not systematically different from values calculated using an equation based on subject characteristics and BMR measured at SL ([Westerterp, et al., 1992a](#_ENREF_290)). It was also reported that diet-induced energy expenditure was decreased at altitude which resulted in physical activity as the main determinant of the increase in BMR at high-altitude. Moreover, despite a significant reduction in body mass (≈ 3 kg), a study examining the effect of a 16-day trek above 4500 m also reported no change in metabolic rate ([Armellini, et al., 1997](#_ENREF_10)). Consequently, the available literature suggests that BMR may not account for weight loss during high-altitude exposure/sojourns. Alternatively, since BMR appears to be increased up on immediate exposure to hypoxia, repeated acute exposures (intermittent hypoxia) may allow for short-term increases in BMR and subsequently enhance weight loss.

Many studies have reported increased physical activity-induced energy expenditure at high-altitude ([Armellini, et al., 1997](#_ENREF_10); [Gill & Pugh, 1964](#_ENREF_104); [Grover, 1963](#_ENREF_116); [Kellogg, et al., 1957](#_ENREF_145); [Klausen et al., 1968](#_ENREF_149)). A study conducted by Westerterp and colleagues ([1992a](#_ENREF_290)) on Mount Everest examined energy expenditure in five healthy participants who were exposed to altitudes between 3500 m and 8872 m. The activity level was calculated from these participants using the doubly-labelled water technique[[1]](#footnote-1), and was found to be above 2.2, a value usually observed only in highly active individuals at SL engaged in endurance exercise ([Westerterp, et al., 1992a](#_ENREF_290); [Westerterp et al., 1992b](#_ENREF_293)). Further studies have also confirmed this finding reporting similar ([Pulfrey & Jones, 1996](#_ENREF_220)) or higher values to those of Westerterp and colleagues ([Reynolds et al., 1999](#_ENREF_227)). Although it appears that physical activity plays a major role in the increased energy expenditure and subsequent weight loss observed with exposure to hypoxia, BMR and alterations in energy intake should not be excluded as contributory factors. If acute or intermittent exposures to hypoxia were found to increase metabolic rate it could have important implications for overweight/obese individuals, donating to their daily caloric expenditure which may contribute towards a negative energy balance. In this thesis, the response of metabolic rate to a series of hypoxic exposures will be explored.

### Hypoxia, Fluid Balance and Nutrient Malabsorption

A substantial portion of the initial weight loss observed in hypoxic environments is comprised of body water. Vats et al., ([2007](#_ENREF_278)) found total body water decreases of about 3.5% during the first 3 days of high-altitude (3500 m) exposure. Other research studies have reported values of 3.3 ([Westerterp, et al., 1992a](#_ENREF_290)) and 3 litres per day ([Boyer & Blume, 1984](#_ENREF_32)), consequently much of the literature appears to be in agreement. This loss of fluid is known to be further enhanced when ambient temperatures and relative humidity (RH) are lower ([Westerterp et al., 1996](#_ENREF_294)) due to cold-induced diuresis ([Stocks et al., 2004](#_ENREF_267)), which are typical of a high-altitude environment or when rapid compared to a gradual ascent to high-altitude is performed ([Consolazio, et al., 1968](#_ENREF_60)) as there is an increased risk for developing altitude sickness ([Roach & Hackett, 2001](#_ENREF_232)). A study overcoming the effects of cold exposure examined fluid loss under identical environmental conditions (temperature 18-24°C, RH 30-60%) at two altitudes, 5000-7000 m and 7000-8848 m. Results demonstrated a total water loss decrease of 3.7 to 3.3 litres per day, suggesting that fluid loss occurs at altitude regardless of environmental conditions ([Westerterp et al., 2000](#_ENREF_292)). However, other studies have concluded that water balance is maintained at altitude either via increased intake or metabolic water formation[[2]](#footnote-2), and therefore does not account for weight loss ([Hamad & Travis, 2006](#_ENREF_119)). Moreover, this process of fluid loss is known to be a hallmark of maladaptation to altitude resulting in altitude sickness ([Kayser & Verges, 2013](#_ENREF_144)) and thus acute exposures to hypoxia with adequate fluid intake are unlikely to cause this response.

Malabsorptions of food and changes in intestinal permeability have both been proposed as possible causes for the metabolic changes associated with hypoxia. A field study completed by Boyer and Blume ([1984](#_ENREF_32)) supported this proposal by reporting a decrease in fat absorption in climbers at high-altitude. However, subsequent studies by others have suggested the opposite. Kayser et al., ([1992](#_ENREF_143)) reported energy digestibility above 96% during a one-month stay at 5000 m and Westerterp et al., ([1994](#_ENREF_291)) showed a similar figure (94%) for participants exposed in a hypobaric chamber to 7000 m, which were no different from values obtained in normoxia. Intestinal permeability was also found to be unchanged at 5730 m ([Dinmore et al., 1994](#_ENREF_71)).

Protein and carbohydrate absorption have also been speculated to play a role in metabolic changes at altitude, however most research suggests that this is not possible. Kayser and colleagues ([1992](#_ENREF_143)) reported that protein absorption was not significantly impaired at altitude (5000 m) compared with SL (96 versus 97%, respectively ([Kayser, et al., 1992](#_ENREF_143)). Chesner, Small and Dykes ([1987](#_ENREF_51)) also found no evidence for carbohydrate malabsorption in men exposed to hypoxia (> 3100 m). Current research therefore suggests that nutrient absorption and intestinal permeability at high-altitude is relatively well maintained and most probably not responsible for the weight loss observed at high-altitude.

### Hypoxia and Protein Synthesis

It has been reported that hypoxia leads to an inexorable loss in skeletal muscle mass in healthy humans ([Boyer & Blume, 1984](#_ENREF_32); [Fulco, et al., 1985](#_ENREF_96); [Hoppeler et al., 2008](#_ENREF_127); [Sergi et al., 2010](#_ENREF_253)). Although initially reductions in body mass can be attributed to a reduction in fat-mass, a significant proportion (up to 70%) of chronic altitude exposure related weight loss has been attributed to a reduction in fat-free mass ([Boyer & Blume, 1984](#_ENREF_32); [MacDougall, et al., 1991](#_ENREF_171); [Magalhães & Ascensão, 2008](#_ENREF_174); [Rose, et al., 1988](#_ENREF_238)). In support, a loss in skeletal muscle mass has been demonstrated by a reduction of muscle volume (10-15%) and cross-sectional area, including a decrease in muscle fibre size (20-25%) above 5000 m ([Green, et al., 1989](#_ENREF_113); [Hoppeler et al., 1990](#_ENREF_126); [Vogt et al., 2001](#_ENREF_279)). Additionally, muscle oxidative capacity characterised by muscle mitochondrial volume density is decreased by as much as 20% with a corresponding decrease of enzyme activities of the citric acid cycle, fatty acid oxidation and respiratory chain ([Howald et al., 1990](#_ENREF_130)). Further indication of muscle deterioration after long-term altitude exposure is provided by the observation of an increase in lipofuscin[[3]](#footnote-3), thought to be indicative of atrophy ([Allaire et al., 2002](#_ENREF_2)) in muscle fibres of mountaineers returning to SL ([Martinelli et al., 1990](#_ENREF_180)). Consequences of a reduction in skeletal muscle mass and physiology are multiple and include a decrease in strength and power output, increased fatigability, and an increase in insulin resistance[[4]](#footnote-4) ([Amirouche et al., 2009](#_ENREF_6); [Favier et al., 2010](#_ENREF_82)). For the obese individual, these consequences could have a negative cost on their health, for example, increased insulin resistance in individuals already predisposed to type II diabetes due to enlarged adipose tissue mass and limited muscle mass could be of further detriment to their health. Subsequently, this could cause other health problems such as hypertension, hyperlipidemia, and atherosclerosis, which are often associated with type II diabetes ([Kahn & Flier, 2000](#_ENREF_140)).

Since a significant proportion of weight loss with chronic hypoxic exposure has been attributed to a loss in skeletal muscle mass, cell tissue culture research, using several cell lines, has started to form a body of evidence which allows for a greater understanding of this characteristic response to low O2 environments. Satellite cells, the resident stem cells of skeletal muscle are considered to be self-renewing, and serve to generate a population of differentiation-competent myoblasts that will participate as needed in muscle growth, repair and regeneration ([Hawke & Garry, 2001](#_ENREF_123); [Siegel et al., 2011](#_ENREF_257)). Satellite cells are involved in a myogenic program of skeletal muscle cell development which consists of two temporally seperated processes; myoblast proliferation[[5]](#footnote-5) and differentiation[[6]](#footnote-6) (Figure 1.3), and the understanding of these processes is essential in understanding both muscle development and repair (Lawson & Purslow, 2000) in response to different treatments and/or environments.

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| --- |
| Satellite cell  Myotube  Muscle precursor cells  **Fusion**  **Quiescence**  **Differentiation**  **Proliferation**  Myf5  myoD  Myogenin  MRF4 |
| Figure .. Schematic of the myogenic program. |

In standard cell tissue culture conditions proliferating myoblasts express myogenic regulatory factors (MRF); myogenic differentiation factor 1 (myoD) and myogenic factor 5 (myf5). Upon activation of myoblast dfferentiation myoD and myf5 induce the withdrawal of myoblasts from the cell cycle together with the expression of myogenin. Myogenin and myogenic regulatory factor 4 (MRF4) possess functions which regulate myoblasts terminal differentiation further in to myotubes ([Li et al., 2007](#_ENREF_162); [Yun et al., 2005](#_ENREF_299); [Zammit et al., 2004](#_ENREF_301)). The response of myoblast proliferation to hypoxia has demonstrated that the hypoxic environment increases the proliferative capacity of satellite cells and allows for the formation of larger myotubes when compared with normoxic conditions (Chakravarthy et al., 2001; Csete et al., 2001). In contrast, the effect of hypoxia on myoblast differentiation demonstrates that low O2 environments inhibit myogenesis via a reduction in myoD messenger ribonucleic acid (mRNA) expression (Yun et al., 2005; Liu et al., 2012; Sato et al., 2011) and a delayed induction of myogenin (Yun et al., 2005).

Hypoxia may also reduce protein synthesis and increase protein degradation in fully differentiated myotubes. In a study by Rennie et al., (1983) they showed that in humans protein synthesis was decreased by 50% while protein breakdown was increased by 25% ([Rennie, et al., 1983](#_ENREF_226)). These findings suggest that hypoxia has a significant impact upon protein synthesis. Furthermore, tissue culture studies have confirmed this response demonstrating that acute hypoxia impacts those pathways involved within protein degradation and synthesis ([Caron et al., 2009](#_ENREF_41); [de Theije, et al., 2013](#_ENREF_68); [Favier, et al., 2010](#_ENREF_82)). Together, these findings may have implications for future research involving the use of a hypoxic stimulus as part of a weight loss programme as any reduction in skeletal muscle mass would not be beneficial for the overweight/obese individual. It is hoped that lean muscle mass is maintained and the contribution of fat mass in weight loss as a result of hypoxic exposure is the greatest mediator. In chapters 2 and 3, the response of proliferating myoblasts and differentiated myotubes to hypoxia (5% O2) will be examined.

## Altitude Sojourns

As previously mentioned, exposure to altitude or the associated hypoxic environment may be of therapeutic use for individuals who are obese; however the amount of evidence available at this time is limited. Twenty-two male patients with metabolic syndrome[[7]](#footnote-7) exposed for three weeks to 1700 m showed improvements in resting heart rate, blood pressure, insulin resistance and glucose tolerance (Schobersberger et al., 2000). In a more recent study, Li et al., (2010) reported on the effect of a 33-day stay at 4678 m in 20 SL residents and 35 moderate altitude residents (2200 m). After 33 days, body mass for the SL residents was reduced by 10.4% (67.1 ± 9.5 versus 60.0 ± 8.1 kg), whilst body mass for the altitude residents was reduced by a substantially smaller 2.2% (63.1 ± 5.5 versus 61.7 ± 6.4 kg). The reported degree of weight loss showed a strong positive correlation with baseline body weight in the SL residents. These results suggest that obese individuals may be more susceptible to weight loss at altitude. Moreover, losses in body mass were more pronounced in the SL natives compared with the moderate altitude residents. At a moderate altitude, Lippl et al., (2010) reported on the effects of a 1-week stay at 2650 m in obese subjects. Immediately after their stay, the body mass and diastolic blood pressure (DBP) of the participants were significantly decreased, whilst BMR and leptin levels were significantly increased. These results confirm that BMR and leptin play a key role in reducing body mass with altitude exposure. Chen et al., (2010) also demonstrated that a 25-day expedition at 2200-3800 m reduced body fat percentage, fat mass and waist to hip ratio. Post-expedition, the area under the curve for insulin and glucose following an oral glucose tolerance test (OGTT) were also reduced suggesting an improvement in insulin action. In two different ethnic groups (Indian and Kyrgyz), a significant reduction in body mass and muscle mass following 21 days at 3200 m has also been reported ([Vats et al., 2013](#_ENREF_277)). It must be noted that the lack of a control group in all of the above studies makes distinguishing between the effects of hypoxia and other factors, such as cold exposure and exercise, difficult. Nevertheless, together, these studies demonstrate that prolonged altitude exposure and the associated hypoxia reduces body mass and ameliorates metabolic health in overweight and obese subjects. However, more controlled research studies are needed to establish the efficacy of hypoxia as a non-pharmacological therapy for obesity.

## Intermittent Hypoxic Exposure

Instead of continuous hypoxic exposure such as that experienced during altitude sojourns, repeated intermittent exposure to hypoxia may be another option to manipulate body mass in overweight and obese populations ([Kayser & Verges, 2013](#_ENREF_144); [Urdampilleta, et al., 2012](#_ENREF_276)), especially since this method may also serve to reduce any losses in fat-free mass which may occur. Intermittent hypoxic exposure (IHE) is broadly defined as exposure to hypoxia interspersed with periods of normoxia ([Millet et al., 2010](#_ENREF_193)). The specific protocols used experimentally vary greatly in cycle length, number of hypoxic exposures per day, and the total number of exposure days. Nonetheless, regardless of the protocol, the compelling outcome is that these repeated episodes of hypoxia elicit persistent changes in a variety of physiological responses in humans and animals (Neubauer, 2001). The use of IHE is a method yet to be examined specifically in relation to promoting weight loss in humans; however findings from animal models provide promise that this method could be effective in doing so. Ling and colleagues ([2008](#_ENREF_164)) demonstrated that when female mice were exposed to eight 15-minute hypoxic exposures (3000 m) a day for a period of 40 days, those in the IHE group were the lightest at the end of the study, and had the lowest growth rate despite weighing the same as SL controls at baseline. Another research group also examined the effect of IHE (7500 m) on body weight in mice. Following 35 days of IHE or sham IHE (alternating 30 seconds of progressive hypoxia followed by 30 seconds of normoxia for 8 hours each day), body weight was significantly reduced in mice exposed to IHE ([Martinez et al., 2010](#_ENREF_182)). These findings have also been confirmed using other protocols in Wistar and Sprague-Dawley rats ([Chen et al., 2010](#_ENREF_49); [Germack et al., 2002](#_ENREF_102)). Although research findings are still limited in this area, results from studies carried out thus far along with earlier research demonstrating a loss of body mass with chronic exposure to moderate- to high-altitude ([Boyer & Blume, 1984](#_ENREF_32); [Lippl, et al., 2010](#_ENREF_165)), suggests that IHE may have an important role to play within weight loss research programmes.

Intermittent hypoxic exposure may also ameliorate metabolic conditions associated with being overweight and obese. Evidence from studies using a model of diet-induced obesity in mice demonstrated that compared to normoxia, IHE reduced blood sugar and blood cholesterol, whilst increases in serum leptin and insulin were observed ([Ling, et al., 2008](#_ENREF_164); [Qin et al., 2007](#_ENREF_221); [Qin et al., 2010](#_ENREF_222)). In human studies, IHE has been shown to elicit positive changes in blood pressure, sub-maximal work capacity, anaerobic threshold, peak rate of O2 consumption, metabolic rate and substrate utilisation ([Saeed et al., 2012](#_ENREF_243); [Shatilo et al., 2008](#_ENREF_254); [Workman & Basset, 2012](#_ENREF_297)). Thus, IHE as a result of weight loss could produce beneficial changes in metabolic health in human overweight and obese populations without the negative consequences of long-term exposure to altitude/hypoxia; however optimisation of an effective hypoxic dose must first be clarified.

### Mechanisms Underpinning the Positive Changes Observed with Intermittent Hypoxic Exposures

Most animal model studies examining the role of IHE in weight loss have yet to elucidate the mechanisms that may underpin the observed losses in body mass. As with acute and chronic altitude studies in humans, a reduction in appetite as a result of increased leptin levels has been proposed and supported in animal models ([Ling, et al., 2008](#_ENREF_164); [Qin, et al., 2007](#_ENREF_221)). Adiponectin a hormone that partially controls food intake can also be proposed as contributing to the mechanism for weight loss in hypoxia. Adiponectin, when reduced, leads to decreased appetite (Figure 1.2), thereby demonstrating a direct relationship with food intake ([Okamoto et al., 2006](#_ENREF_209)). Chaiban et al., ([2008](#_ENREF_45)) showed that in rats exposed to normobaric hypoxia (5700 m) for 8 weeks adiponectin levels were reduced, leading to decreased body mass. However, in humans, no change in adiponectin levels were reported following 3 weeks at 4300 m ([Barnholt, et al., 2006](#_ENREF_19)) which could be attributed to length of stay. Still, studies in this area remain scarce. However, cell culture studies do provide support for the role of adiponectin in the weight loss response to hypoxia by demonstrating reduced adiponectin levels in response to IHE ([Magalang, et al., 2009](#_ENREF_173); [Zhang, et al., 2010](#_ENREF_302)).

It has also been proposed that glucose transporter 4[[8]](#footnote-8) (GLUT4) levels might be involved in weight loss and subsequent improvements in insulin resistance. However, this proposal has not been supported thus far with Chen et al., ([2010](#_ENREF_49)) reporting that GLUT4 levels remain unchanged following 35 days of IHE. Thus, it would appear at present that the major controller of weight loss with IHE may be appetite suppression and the concomitant reduction in food intake. Other mechanisms, which may play a role include, increased metabolic rate and a switch in substrate utilisation towards lipid sources ([Workman & Basset, 2012](#_ENREF_297)). To gain a greater understanding of the role of IHE as a non-pharmacological therapy for obesity, in chapters 4 and 5, the effect of IHE on weight loss and the mechanisms which may underpin any observed reductions in body mass will be examined in sedentary individuals.

## Intermittent Hypoxic Training

The concept of altitude or hypoxic training for improving SL performance in athletic populations has been known for nearly 40 years, over this time, several strategies of such training regimes have been explored, such as live high-train high, live high-train low and live low-train high (LLTH) ([Czuba et al., 2011](#_ENREF_65)). All these methods have provided interesting findings, however LLTH is the most recently developed and practical model, as it allows individuals to live under normoxic conditions whilst training under natural hypobaric or normobaric hypoxic conditions. A method often employed under LLTH is intermittent hypoxic training (IHT). Similar to IHE, a key advantage of IHT, is that it allows individuals to benefit from the hypoxic stimulus without undergoing the detrimental effects of a prolonged hypoxic exposure ([Bassovitch, 2010](#_ENREF_20); [Millet, et al., 2010](#_ENREF_193)). One particular benefit of IHT is its ability to increase exercise capacity following training despite a reduced training workload compared with normoxia ([Haufe et al., 2008](#_ENREF_122); [Saeed, et al., 2012](#_ENREF_243)). In an overweight or obese population this potentially allows individuals to receive a maximal metabolic and cardiovascular benefit while reducing the risk of injury ([Haufe, et al., 2008](#_ENREF_122)). Moreover, as intensity of exercise has been found to be negatively related to adherence ([Ekkekakis & Lind, 2006](#_ENREF_76)), any exercise programme or intervention which allows intensity or duration to be reduced may be appealing to individuals who are reluctant to exercise.

In clinical settings, IHT has been associated with significant improvements in selected risk factors of obesity (e.g. triglycerides and blood pressure) and losses in body mass ([Bailey et al., 2000a](#_ENREF_16); [Haufe, et al., 2008](#_ENREF_122); [Wiesner et al., 2010](#_ENREF_295)). Therefore, it would appear that altitude training serves as a supplementary training stimulus for eliciting compensatory adaptations within the human body both physiologically and metabolically ([Cheung, 2010](#_ENREF_52)). Hypothetically using the LLTH concept during exercise training to enhance weight loss is an interesting proposal and research has started to collate a body of evidence in support, which includes respiratory, cardiovascular, cellular and metabolic and weight loss adaptations.

Respiratory adaptations to hypoxia include an increased ventilatory response to sub-maximal exercise, increased total exercise time, increased total haemoglobin levels and increasing lung diffusion capacity in patients suffering chronic obstructive pulmonary disease and asthma ([Urdampilleta, et al., 2012](#_ENREF_276); [Vogtel & Michels, 2010](#_ENREF_280)). In healthy individuals, hypoxia combined with moderate cycling exercise (20-30 min, 3·wk-1, 4 weeks) increased the rate of maximum voluntary ventilation, however no other changes in lung volumes or flow rates were observed ([Bailey, et al., 2000a](#_ENREF_16)). Additionally, Weisner and colleagues reported a positive effect of IHT on the respiratory quotient, which following four weeks of IHT during treadmill exercise, was reduced, suggesting a shift towards fat metabolism ([Wiesner, et al., 2010](#_ENREF_295)) and thus metabolic adaptation. Any reduction in the respiratory quotient would have obvious beneficial effects for individuals on a weight loss programme.

The cardiovascular system is conditioned to hypoxia over a greater time period than respiratory adaptations. Bailey et al., ([2000a](#_ENREF_16)) has shown that normobaric hypoxic exercise training may reduce the risk of cardiovascular disease. Cycling in normoxia and hypoxia decreased resting plasma concentrations of nonesterified fatty acids, total cholesterol (TC), and low-density lipoprotein (LDL). In addition, hypoxic training resulted in reduced levels of homocysteine[[9]](#footnote-9), an acidic model implicated in heart disease, which was increased following normoxic exercise training. Additionally, IHT may also normalise or reduce blood pressure via a hypotensive effect ([Wiesner, et al., 2010](#_ENREF_295)). Evidence thus far suggests that physical exercise in hypoxia decreases the risk of cardiovascular disease ([Bailey, et al., 2000a](#_ENREF_16); [Burtscher et al., 2004](#_ENREF_37); [Milano et al., 2002](#_ENREF_190); [Urdampilleta, et al., 2012](#_ENREF_276)).

Studies examining the concept of IHT on weight loss report promising findings. Following a four-week IHT programme, Bailey et al., ([2000a](#_ENREF_16)) reported that body mass was unaltered in healthy lean males, however fat-free mass increased within the hypoxic group, a finding which was not observed for the normoxic training group. Using a combination of exercise modalities (treadmill, stepper and cycle ergometer), Netzer et al., ([2008](#_ENREF_202)) also reported a significant reduction in body mass with IHT compared with normoxic training. In their study, body mass losses equalled 1.14 kg in the hypoxic training group, but were unchanged in the normoxic group. Following a protocol utilising treadmill exercise, a trend towards greater body mass losses in hypoxia, with those subjects using IHT losing a greater percentage of body fat than those training in normoxia (4.2 versus 2.4%) has also been reported ([Haufe, et al., 2008](#_ENREF_122)). A recent study conducted by another group also reported similar findings in swimmers following a 3-week IHT programme ([Chia et al., 2013](#_ENREF_53)). Collectively, studies reporting on weight loss as a consequence of IHT provide preliminary evidence to suggest that this method could be used as a non-pharmacological therapy for obesity. However, many of the studies lack comparison with a control population and therefore further well-controlled studies are needed to clarify the impact of IHT on body mass in humans.

As previously mentioned, obesity is a major underlying risk factor for a number of chronic diseases such as type II diabetes, hypertension, dyslipidemias, coronary heart disease, pulmonary afflictions, osteoarthritis, and more ([Anderson & Konz, 2001a](#_ENREF_7); [Brown, et al., 2000](#_ENREF_34); [Carter, et al., 2000](#_ENREF_42); [Kopelman, 2000](#_ENREF_152); [Tschop & Morrison, 2001](#_ENREF_274)). In studies examining weight loss as a result of IHT, markers associated with the aforementioned diseases such as insulin resistance, blood pressure and blood lipid profiles were also measured. One study reported a significant improvement in HOMA-Index[[10]](#footnote-10), fasting insulin and the area under the curve for insulin during an OGTT after 4-weeks IHT compared with training in normoxia ([Haufe, et al., 2008](#_ENREF_122)). Weisner et al., (2010) also reported a significant reduction in fasting insulin and HOMA index following IHT – additionally a significant reduction was also observed in those training under normoxic conditions. In summary, exercise training combined with hypoxia ameliorates insulin sensitivity contributing to the benefits of IHT as a non-pharmacological therapy for obesity.

### Mechanisms Underpinning the Positive Changes Observed with Intermittent Hypoxic Training

It is thought that the greater losses in body mass and health benefits observed with hypoxic exercise training may be due to increases sympathetic drive ([Lippl, et al., 2010](#_ENREF_165)), and other metabolic changes, which are yet to be elucidated ([Urdampilleta, et al., 2012](#_ENREF_276)). At a molecular level training under hypoxia is known to increase HIF-1 production ([Haufe, et al., 2008](#_ENREF_122); [Semenza, et al., 2006](#_ENREF_252)). Subsequent to activation of HIF-1, the transcription factor HIF-1α targets genes involved in O2 transport, glycolysis and glucose transport such as, peroxisome proliferator-activated receptor- γ co-activator-1α ([Maffei et al., 1995](#_ENREF_172)) via transcription of peroxisome proliferator-activated receptor coactivator 1α (PGC-1α) ([Gilde & Van Bilsen, 2003](#_ENREF_103)). Expression of peroxisome proliferator-activated receptor (PPAR), a family of lipid activated nuclear hormone receptors, includes mitochondrial biogenesis and is known to play a key role in the regulation of muscle fatty acid oxidation. Therefore, it has been proposed that when hypoxia and exercise training are combined they may have a synergistic effect on body composition and metabolism ([Haufe, et al., 2008](#_ENREF_122); [Wiesner, et al., 2010](#_ENREF_295)).

Hypoxia may also serve to reduce body weight via a decrease in serum glucose and blood cholesterol levels. Previous research findings have identified a relative increase in glucose oxidation rates after exercise training in hypoxia ([Hoppeler et al., 2003](#_ENREF_129)), and this response has been attributed to transactivation of HIF-1. Activation of the regulatory subunit HIF-1α leads to cellular adaptations that counteract the effects of reduced O2 supply to cells under hypoxic conditions. Further, hypoxic training is thought to increase the glycolytic enzymes, enhancing the number of mitochondria and glucose transporter GLUT4 levels as well as improving insulin sensitivity ([Haufe, et al., 2008](#_ENREF_122)).

A hormone involved in the regulation of body mass, leptin, has also been examined in response to body mass losses in hypoxia. It has been demonstrated in both humans and rodents that leptin levels increase in hypoxia leading to a reduction in appetite and subsequently weight loss ([Shukla, et al., 2005](#_ENREF_256); [Tschop, et al., 2000](#_ENREF_275)). However, the combination of exercise and hypoxia, and its effects on leptin and adiponectin levels are less clear. Haufe and colleagues reported a trend (P = 0.07) towards reduced leptin levels following IHT but these changes were also observed in the control group. Moreover, in their study no change in adiponectin was observed. To our knowledge, this is the only study to examine the effect of IHT on leptin and adiponectin and thus further research is required. Therefore, it is still to be identified whether IHE alone induces losses in body mass and ameliorates cardiovascular health, if so the results may be of interest for the management of obesity ([Urdampilleta, et al., 2012](#_ENREF_276)).

### Intermittent Hypoxic Training Programme Design

As previously discussed, IHT requires the individual to exercise under hypoxic conditions whilst remaining in normoxic conditions for the remainder of the time ([Urdampilleta, et al., 2012](#_ENREF_276)). During the hypoxic exercise bouts careful consideration of the training load, which can be thought of as a combination of frequency, intensity, and duration, are important since hypoxia reduces aerobic capacity ([Friedmann et al., 2005](#_ENREF_94)). The application of a training load results in a cascade of physiological responses that allow the individual to adapt to the training stimulus, which elevates fitness levels and leads to greater tolerance for training ([Bompa & Haff, 2009](#_ENREF_27)). As the individual adapts to the prescribed training load, it is important that it is increased progressively in order for further physiological adaptations to occur. Therefore, continuous measurements of fitness levels is important, especially for the determination, and if required the adjustment of exercise intensity.

Traditional methods of prescribing exercise intensity prescription include the use of peak oxygen uptake (O2peak) and peak heart rate (HRpeak), but since both these methods require individuals to perform graded exercise to exhaustion, researchers will often use predictive equations, particularly for those individuals in which exhaustive exercise may be contraindicated (i.e. the obese). However, since aerobic capacity is reduced in hypoxia, the use of these equations may be impractical or inaccurate, as any reduction in O2peak or HRpeak would allow the equations to overestimate exercise intensity. Such overestimations may subsequently lead to the setting of unachievable goals and decreased adherence to exercise training sessions ([Perri et al., 2002](#_ENREF_214)). Therefore, identifying an appropriate method to prescribe exercise intensity and monitor changes in fitness in hypoxia for individuals in whom exhaustive exercise may be contraindicated is important. In chapters 6 and 7 methods of achieving this are evaluated.

## Conclusion

Body mass losses occur at high-altitude and although a critical altitude is yet to be defined; it appears that body mass losses generally occur at altitudes above 5000 m. The cause of this loss in body mass is likely a combination of increased energy expenditure and decreased energy intake. The reduction in energy intake appears to be due to a reduction in appetite and increased satiety during high-altitude sojourns. Initially it was thought that the decrease in appetite was due to the symptoms associated with acute mountain sickness, however research has now identified leptin as a key hormone playing a role in the energy balance equation at altitude. Other hormones controlling food intake such as ghrelin and adiponectin may also play a role in the reduced appetite profile observed in response to hypoxia. Furthermore, the major metabolic shift towards muscle and protein catabolism at extreme altitudes ([Boyer & Blume, 1984](#_ENREF_32)) should not be ignored and provides an important implication for future weight loss research designs, as any reduction in lean muscle mass in obese individuals would have negative consequences.

Results of studies examining IHE and IHT suggest that hypoxic exposure is a promising approach for the management of obesity; importantly O2 availability may play a crucial role in the regulation of body weight and energy homeostasis ([Quintero, et al., 2010](#_ENREF_223)). The combination of exercise and hypoxia appears to ameliorate metabolic disturbances often associated with obesity such as hypercholesterolemia, hyperlipidemia, hypertension, type II diabetes and cardiovascular disease. Molecular mechanisms underlying this response point to changes in HIF-1α, PGC-1α and an increase in levels of glucose transporters (e.g. GLUT4) as a result of IHT. However, the results of studies examining the effect of IHE on weight loss are scarce and therefore further research is required. If successful, IHE may be an appealing approach for obese individuals, as it provides practicality and does not require individuals to perform physical exercise, thus potentially enhancing adherence to the programme. This thesis will examine the role of hypoxia at rest and its potential as a tool to aid weight loss using a combination of in vivo and in vitro techniques.

## Proposed Aims of the Thesis

Obesity managements can be placed into four categories; (1) dietetic and nutritional managements, (2) physical activity programmes, (3) pharmacological managements and (4) bariatric surgery. Findings within the literature pertaining to each of these categories suggest that an effective management still remains elusive. However, research investigations have reported that O2 variations may produce changes in body composition ([Boyer & Blume, 1984](#_ENREF_32); [Lippl, et al., 2010](#_ENREF_165)). It is also widely accepted that reductions in body mass occur with moderate- to high-altitude exposure ([Boyer & Blume, 1984](#_ENREF_32); [Lippl, et al., 2010](#_ENREF_165); [Rose, et al., 1988](#_ENREF_238)).

Evidence from animal models suggests that IHE stimulates weight loss; however this effect remains to be examined in humans. Intermittent hypoxic exposure, if proven to be effective, could be used as a preventative or therapeutic therapy in the fight against the obesity epidemic. However, since the literature also suggests a reduction in lean body mass with chronic altitude exposure, exploring whether acute hypoxia promotes satellite cell self-renewal while eradicating the debilitative effect of chronic hypoxia is important in order to determine whether lean muscle mass can be maintained with IHE.

Using in vitro cell culture techniques, the aims of the thesis were firstly to measure the effects of hypoxia on the proliferation of mouse myoblast satellite cells, and secondly to measure the effects of hypoxia on protein degradation in C2C12 myotubes. Based on results within the literature examining chronic hypoxic exposure, it was hypothesised that acute hypoxic exposure will increase the proliferation of satellite cells and increase the expression of genes associated with skeletal muscle degradation.

Using in vivo techniques, the whole body response to IHE at rest on weight loss and associated metabolic markers will be measured. It was thought that the findings will mimic those demonstrated within animal models, showing a reduction in appetite hormones and a subsequent reduction in body mass. The final aim of the thesis was to determine an effective method for prescribing exercise intensity in hypoxia for subsequent proposal of an IHT protocol.

# Effect of Low Oxygen Conditions on the Proliferation of C2C12 Myoblasts

## Introduction

Oxygen is an essential component in generating energy within mammalian cells and survival is classically viewed as being dependent upon the counterbalance of O2 supply and O2 demand ([Arthur et al., 2000](#_ENREF_14)). Oxygen deprivation, otherwise known as hypoxia, such as that experienced at altitude, provides an important challenge to the organism compromising body functioning including cardio-respiratory, endocrine, metabolic, nutritional and thermal homeostasis ([Di Carlo et al., 2004](#_ENREF_69); [Hoppeler & Vogt, 2001](#_ENREF_128); [Li, et al., 2007](#_ENREF_162); [Magalhães & Ascensão, 2008](#_ENREF_174)). Furthermore, depending on the severity, duration and rapidity of the onset of hypoxia, the decreased levels of O2 may severely compromise body metabolism, promoting reversible or irreversible loss of tissue and cell homeostasis leading to organic and functional decay ([Magalhães & Ascensão, 2008](#_ENREF_174)). Evidence for this occurrence has been demonstrated within the high-altitude research literature.

It is well known that altitude hypoxia leads to an inexorable loss in skeletal muscle mass in healthy human individuals ([Hoppeler, et al., 2008](#_ENREF_127); [Magalhães & Ascensão, 2008](#_ENREF_174)). Loss in skeletal muscle mass has been demonstrated by a reduction of muscle volume (10-15%) and cross sectional area, including a decrease in muscle fibre size (20-25%) above 3000 m ([Green, et al., 1989](#_ENREF_113); [Hoppeler, et al., 1990](#_ENREF_126); [Hoppeler & Vogt, 2001](#_ENREF_128)). Additionally, muscle oxidative capacity characterised by muscle mitochondrial volume density is decreased by as much as 20% with a corresponding decrease of enzyme activities of the citric acid cycle, fatty acid oxidation and respiratory chain ([Hoppeler, et al., 1990](#_ENREF_126); [Howald, et al., 1990](#_ENREF_130)). Further indication of muscle deterioration after long-term altitude exposure is provided by the observation of an increase in lipofuscin in muscle fibres in mountaineers after return to SL ([Martinelli, et al., 1990](#_ENREF_180)). The consequences of a reduction in skeletal muscle mass are multiple and include a decrease in strength and power output, increased fatigability, and an increase in insulin resistance ([Favier, et al., 2010](#_ENREF_82)). However, the exact mechanisms underlying this response to hypoxia at the cellular level remain to be elucidated. The findings from such research could have important implications as maintenance of fat-free mass while reducing fat mass within weight loss research programmes is pertinent.

Skeletal muscle growth and development is dependent upon two temporally separated processes; myoblast proliferation and differentiation. Skeletal muscle differentiation is characterised by myoblasts withdrawal from the cell cycle, induction of muscle-specific gene expression, and cells fusion into multinucleated myotubes ([Di Carlo, et al., 2004](#_ENREF_69); [Li, et al., 2007](#_ENREF_162); [Sato et al., 2011](#_ENREF_247)). The antagonism between proliferation and differentiation implies that signalling pathways driving proliferation must be suppressed to allow induction of differentiation ([Di Carlo, et al., 2004](#_ENREF_69); [Li, et al., 2007](#_ENREF_162)). The proliferation and differentiation of myoblasts is a highly coordinated process orchestrated by a family of MRFs; myoD, myf5, myogenin and MRF4 ([Yun, et al., 2005](#_ENREF_299)). These factors form heterodimers with ubiquitous basic helix-loop-helix (bHLH) proteins and, through their subsequent binding to specific sequences termed E-boxes, in the promoter-regulatory regions of muscle-restricted target genes, activate myogenic differentiation ([Andres & Walsh, 1996](#_ENREF_9); [Arnold & Winter, 1998](#_ENREF_13); [Di Carlo, et al., 2004](#_ENREF_69); [Moran et al., 2002](#_ENREF_196)).

Specifically, proliferating myoblasts expressing myoD and myf5, are committed to the muscle lineage and will continue to proliferate in the presence of mitogens[[11]](#footnote-11) under high-serum conditions in vitro ([Moran, et al., 2002](#_ENREF_196)). Upon serum deprivation, differentiation is activated, and myoD and myf5 expression is reduced which induces the withdrawal of myoblasts from the cell cycle together with the elevated expression of myogenin. Myogenin and MRF4 possess functions that regulate myoblasts terminal differentiation further into myotubes ([Li, et al., 2007](#_ENREF_162); [Yun, et al., 2005](#_ENREF_299); [Zammit, et al., 2004](#_ENREF_301)).

At the cellular level, decreased O2 tension or hypoxia alone may be sufficient to alter the proliferation, differentiation, myotube formation and gene expression of myoblasts ([Yun, et al., 2005](#_ENREF_299)). Whilst a number of studies examining the response of cells to low O2 conditions have been carried out over the last decade research evidence still remains sparse. Chakravarthy, Spangenberg and Booth (2001) were the first group of researchers to examine the effects of decreased O2 levels on the activation and proliferation of satellite cells. Satellite cells taken from 31-month old male rats were cultured under atmospheric conditions containing 21% O2 and 3% O2 for up to 35 days. Findings included a significant increase in proliferation and formation of larger myotubes after 7 days in cells cultured under 3% O2 compared with 21% O2, no further differences were found after the 7 day period. A down-regulation of the cell cycle inhibitor p27Kip1 protein was also documented confirming the results of previous work ([Gardner et al., 2001](#_ENREF_99)). These findings suggest that lower atmospheric levels of O2 provide a milieu that stimulates proliferation by allowing continued cell cycle progression ([Chakravarthy et al., 2001](#_ENREF_46)).

More recently Csete and colleagues ([2001](#_ENREF_61)) examined the effects of low O2 levels on the developmental potential and proliferative capacity of the adult skeletal muscle fibre progenitor population and cell lines in female mice. Under 6% O2 both satellite cell proliferation and survival of mature fibres were found to be enhanced compared with 21% O2. The low O2 condition also accelerated the up-regulation of multiple myoD family MRFs ([Csete, et al., 2001](#_ENREF_61)). The findings of Csete and colleagues suggest that regulatory pathways affected by O2 are important for satellite cell proliferation, execution of cell fate, and parent muscle survival in culture. Confirming these results, a study by Zhao et al., (2003) examining the effect of hypoxia on rat myoblasts reported that the number of myoblasts cultured in 3% and 10% O2 conditions increased by 1.5 and 2.5 times compared with cells cultured under 21% O2, respectively ([Zhao et al., 2003](#_ENREF_303)).

In myogenic cells, growth suppression induced by hypoxia has been demonstrated clearly by DiCarlo et al., showing G1 arrest [[12]](#footnote-12) of the cell cycle responding to hypoxia. This growth arrest is associated with p27 accumulation in C2C12 myoblasts. Moreover, hypoxia-dependent inhibition of differentiation was associated with myoD degradation by the ubiquitin-proteasome pathway. Thus, hypoxia by inducing myoD degradation blocked accumulation of early myogenic differentiation markers, myogenin, myf5, cyclin-dependent kinase inhibitor-1 and retinoblastoma protein resulting in cell cycle withdrawal ([Di Carlo, et al., 2004](#_ENREF_69)). In summary, DiCarlo and colleagues demonstrated that C2C12 myoblasts cultured in hypoxia showed neither myotube formation or myosin heavy chain protein accumulation, indicating that hypoxia inhibited myogenic differentiation of myoblasts.

Yun, Lin and Giacca (2005) investigated the effects of three different levels of hypoxia on C2C12 myogenesis; physiological hypoxia (2% O2), pathological hypoxia (0.5% O2), and extreme pathological hypoxia (0.01% O2). Their findings indicated that myogenesis was inhibited at ≤2% O2, with the strongest inhibition occurring at 0.01% O2. Inhibition of myogenesis also coincided with a transient inhibition of myoD mRNA expression and a significantly delayed induction of myogenin ([Csete, et al., 2001](#_ENREF_61)). However, Yun and colleagues also reported that when cells were transferred to 21% O2 after the cells induced myogenic differentiation at 2%, 0.5% and 0.01% O2 for 72 hours they recovered to continue differentiation from 2% and 0.5% O2, but only to some extent following 0.01% O2 exposure. DiCarlo et al., (2004) also observed that myoblasts differentiation was reversibly inhibited by hypoxia, as myoblasts retain their capacity to proliferate or differentiate when normal O2 concentrations were restored.

Sato et al., (2011) investigated the growth, differentiation and motility of myogenic C2C12 cells in response to hypoxia (2, 5 and 10% O2) using in vitro morphology, immunohistochemistry and immunoblotting. It was found that hypoxia affected the cells in different ways depending upon their stage in the growth phase. Specifically, hypoxia induced suppression of myoblast proliferation in the growth phase however this hypoxia induced suppression diminished in the differentiation phase. Expression of differentiation marker proteins (i.e. myosin heavy chain) were also inhibited accompanying myoD mRNA suppression, a result reported previously within other research findings ([Di Carlo, et al., 2004](#_ENREF_69); [Itoigawa et al., 2010](#_ENREF_134)). The authors concluded that hypoxia might shift cells in to the growth phase providing cells with the ability to translocate in to the appropriate area.

Liu, Wen, Bi, et al., (2012) studied the response of primary mice myoblasts to 48 hours of hypoxia (1%) versus normoxia. In this study it was demonstrated that hypoxic culture conditions favour the quiescence of satellite cell-derived primary myoblasts by upregulating paired box protein 7, a key regulator of satellite cell self-renewal, and downregulating myoD and myogenin. Overall this research confirms the results of others by demonstrating enhanced proliferation and inhibited differentiation. Furthermore, it was found that during myoblast division, the hypoxic condition promoted asymmetric self-renewal divisions while inhibiting asymmetric differentiation divisions without impacting the overall rate of proliferation ([Liu et al., 2012](#_ENREF_166)).

A recent study conducted by Koning et al., (2011) examined the effect of hypoxia (2% O2) on human satellite cell proliferation and differentiation during different passage phases ([Koning et al., 2011](#_ENREF_151)). In accordance with previous literature, the results demonstrated that during early passage phases (0-5) hypoxically cultured satellite cells proliferated twice as fast as those cultured in normoxia. However at high passage phases (6-15) these effects were eliminated and passage doubling time was similar in both normoxically and hypoxically cultured satellite cells. On the contrary, gene expression of myogenic transcription factors (myf5, myoD) was up-regulated by hypoxia during differentiation. Koning and colleagues concluded that during myogenesis in vitro satellite cell proliferation is enhanced. Moreover, although satellite cells differentiate in to myotubes, they maintain a pool of quiescent satellite with qualities that are unaffected by hypoxia. Similar findings were reported by Martin et al., (2009); their findings demonstrate that 5% O2 increases proliferative capacity of human myoblasts compared with 20% O2. However, unlike in rodent and bovine myoblasts, the increase in myoD and myogenin during differentiation was similar in 5 and 20% O2. Therefore, their data indicates that low O2 culture conditions maintain, but do not enhance the differentiation potential of human skeletal myoblasts ([Martin et al., 2009](#_ENREF_179)).

Table 2.. Summary of findings.

|  |  |  |
| --- | --- | --- |
| Authors | Methods | Findings |
| Chakravarthy, Spangenberg & Booth (2001) | Satellite cells (male rats) cultured under 21% and 3% O2 | * ↑ proliferation and formation of larger myotubes after 7 d in cells cultured under 3% O2 compared with 21% O2 * p27Kip1 downregulated |
| Csete et al. ([2001](#_ENREF_61)) | Female mice cell lines cultured under 21% O2 and 6% O2 | * Satellite cell proliferation enhanced * Survival of mature fibres increased with 6% O2 compared with 21% O2 * Upregulation of myoD family MRFs accelerated with 6% O2 |
| Zhao et al., (2003) | Rat myoblasts cultured under 3%, 10% and 21% O2. | * Number of myoblasts cultured in 3% and 10% O2 conditions ↑ by 1.5 and 2.5 times compared with cells cultured under 21% O2 |
| DiCarlo et al., | C2C12 myoblasts. | * G1 arrest [[13]](#footnote-13) of the cell cycle in response to hypoxia * growth arrest is associated with p27 accumulation * myoD degradation - blocked accumulation of early myogenic differentiation inhibitor-1 and retinoblastoma protein resulting in cell cycle withdrawal |
| Yun, Lin and Giacca (2005) | C2C12 cell line cultured under 2%, 0.5%, and 0.01% O2. | * Myogenesis was inhibited at ≤2% O2, with the strongest inhibition occurring at 0.01% O2. * Transient inhibition of myoD mRNA expression and a significantly delayed induction of myogenin * When cells were transferred to 21% O2 after the cells induced myogenic differentiation at 2%, 0.5% and 0.01% O2 for 72 hours they recovered to continue differentiation from 2% and 0.5% O2, but only to some extent following 0.01% O2 exposure |
| Sato et al., (2011) | C2C12 cell line cultured under 2, 5, 10 and 21% O2 | * Hypoxia affected the cells in different ways depending upon their stage in the growth phase. * Hypoxia induced suppression of myoblast proliferation in the growth phase however this hypoxia induced suppression diminished in the differentiation phase * Expression of differentiation marker proteins (i.e. myosin heavy chain) were also inhibited accompanying myoD mRNA suppression |
| Liu, Wen, Bi, et al., (2012) | Primary mice myoblasts cultured for 48 h under 1% and 21% O2 | * Hypoxic culture conditions favour the quiescence of satellite cell-derived primary myoblasts by upregulating paired box protein 7, and downregulating myoD and myogenin |
| Koning et al., (2011) | Human satellite cells cultured under 2% and 21% O2 | * During early passage phases (0-5) hypoxically cultured satellite cells proliferated twice as fast as those cultured in normoxia * At high passage phases (6-15) effects were eliminated and passage doubling time was similar in both normoxically and hypoxically cultured satellite cells * Gene expression of myf5 and myoD were up-regulated by hypoxia during differentiation |
| Martin et al., (2009) | Human myoblasts cultured under 20% and 5% O2. | * 5% O2 increases proliferative capacity of human myoblasts compared with 20% O2 * Increase in myoD and myogenin during differentiation was similar in 5 and 20% O2 |

Collectively, evidence examining the effects of hypoxia on cell proliferation suggests that low O2 conditions can promote proliferative capacity. Increased cell proliferation has also been associated with down-regulation of cell cycle inhibitors and up-regulation of myogenic regulatory factors. Conversely, the hypoxic effects of growth are still unclear in differentiating cells, i.e., cells cultivating in differentiation medium ([Sato, et al., 2011](#_ENREF_247)). Data available from studies examining the effect of hypoxia on differentiation would suggest that hypoxia has a debilitative effect, inhibiting cell differentiation. However, the work of Yun and colleagues provides positive support for intermittent exposure to acute hypoxia due to retention of cell capacity to proliferate or differentiate when normal O2 concentrations are restored ([Yun, et al., 2005](#_ENREF_299)). The aim of the present study was to examine the response of C2C12 myoblasts to 24 hours of hypoxia (5% O2) at different time points during proliferation. It is thought that the cells may respond differently depending upon which part of the growth phase they were in, previous work by others has demonstrated that 24 hours of hypoxia may alter atrophic genes at a given time phase, however it is still unknown whether different lengths of time spent in normoxia before hypoxic exposure yields the same results. It is hypothesised that hypoxia would increase proliferative capacity which would be demonstrated by an increase in cell number and mRNA expression of myogenic regulatory factors myoD, myogenin and myf5.

## Methods

### Cell Culture

All cell culture was carried out using a Class II Heraeus Biological Safety cabinet under sterile conditions. C2C12 murine myoblasts were provided by the Health Protection Agency (HPA cultures, Salisbury, UK). Cells were grown in growth medium, designated as GM, consisting of Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, Haverhill, UK) supplemented with 20% fetal bovine serum (FBS, PAA, Paisley, UK) and 1% PenStrep (Sigma-Aldrich, Haverhill, UK). Cells were incubated at 37°C in a humidified Heracell 240 incubator (Thermo Fisher Scientific, Rosklide, Denmark) under 5% CO2, which is the conventional 21% O2 condition (normoxia). To ensure reproducibility all experiments presented in this chapter were carried out at passage 7.

Once confluent, C2C12 cells were seeded on to pre-gelatinised (0.2%) 6-well plates (15 000 cells/well) in 2ml GM. GM was replaced every 48 hours. Each 6-well plate consisted of 3 wells for ribonucleic acid (RNA), 1 well for cell counting, 1 well for staining (this well contained 3 13mm glass coverslips) and 1 well for O2 probe measurement. Following cell seeding, C2C12 cells were incubated in a humidified incubator at 37°C under normoxic conditions (21% O2, 5% CO2, 74% N2). For cells exposed to physiological hypoxia (5% O2), C2C12 cells were placed in an adjustable hypoxia chamber with real-time partial pressure of oxygen (PO2) readout (MCO-5M, Sanyo, Japan) in which air was flushed out by 95% N2 and 5% CO2 until the chosen O2 concentration of 5% was attained. Actual concentration of 5% O2 in the chamber was based on a direct measurement inside the chamber using a microelectrode O2 probe (Microelectrodes Inc, USA). Cells were sampled for RNA and immunostaining every 24 hours up to a 96 hour time point (Figure 2.1).

**0-24 h**

**24-48 h**

**48-72 h**

**72-96 h**

Normoxic Incubation (FIO2 0.209)

Hypoxic Incubation (FIO2 0.05)

RNA Isolation/extraction

**H1**

**H2**

**H3**

**H4**

**N1**

**N2**

**N3**

**N4**

Figure .. Schematic diagram of study design (N; Normoxic exposure, H; Normoxic exposure with 24 h hypoxic exposure).

### Cell Counts

Cells were counted on a haemocytometer using the Trypan blue exclusion method (at Appendix 5). Firstly, the GM was removed from the well and washed thoroughly with phosphate buffered saline (PBS), 1 ml of Trypsin was then added to well and was subsequently incubated for five minutes at 37°C, which allows for detachment of cells from the well. Following detachment, the cells were re-suspended in 2 ml of GM which neutralises the effect of Trypsin. If cells were clumped together, the serum was carefully triturated using a pipette. Once the cells had been re-suspended in 2 ml of GM, 20 μl of cell suspension was added to 20 μl of trypan blue (Sigma-Aldrich, Haverhill, UK) solution and mixed by pipetting—10μl of this solution was then added to either end of the haemocytometer coverslip and allowed to fill the chambers. The number of cells in each of the four quadrants of a chamber was then counted under a light microscope (10 X magnification). Once counted each value was added together and divided by 4 to give an average of the four counted chambers. This value was then multiplied by 2 to account for the trypan blue dilution, and then further by 1 x 104. Finally, the value was multiplied by the amount of GM used to re-suspend the cells to give an estimation of the total number of cells present in the original solution.

### RNA Extraction and Quantification

Total RNA was isolated with Trizol reagent (Fisher Scientific, Loughborough, UK) as per the manufacturer’s instructions. After experimentation, GM was removed and a PBS wash was completed, 6-well plates were then scraped in the presence of 500 μl of Trizol reagent before being transferred to a 1.5 ml RNase free tube (Fisher Scientific, Loughborough, UK) and stored at -80°C.

Samples were defrosted and vortexed for 10 seconds prior to RNA extraction before 100 μl chloroform (Sigma-Aldrich, Haverhill, UK), was added to each sample tube and shaken thoroughly for 10 seconds by hand prior to incubation at room temperature for 5 minutes. Samples were then spun at 12 000 x g for 15 minutes using a micro-centrifuge (Fisher Scientific, Loughborough, UK) after which the upper aqueous phase containing RNA was transferred to a new 1.5 ml tube. RNA was precipitated from the remaining solution by the addition of 250 µl isopropyl alcohol (Fisher Scientific, Loughborough, UK). After incubation at room temperature for 10 minutes, the tubes were spun for 10 minutes at 12 000 x g resulting in the pelleting of RNA at the bottom of the tube. The supernatant was then carefully removed before washing the pellet in 500 µl of 75% molecular grade ethanol (Fisher Scientific, Loughborough, UK), by vortexing and centrifugation at 7500 x g for 5 minutes. The ethanol supernatant was then removed and the pellet was allowed to air dry for 10-15 minutes before being re-suspended in 50 µl of RNA storage solution (Sigma-Aldrich, Haverhill, UK).

Following Trizol extraction RNA yield (absorbance at 260 nm[[14]](#footnote-14)) and purity (260/280 ratio[[15]](#footnote-15)) were determined using a Nanodrop 3000 Spectrophotometer (Fisher Scientific, Loughborough, UK; Table 2.2). Using an L-2 pipet (Rainin Instruments, Bedfordshire, UK), 1 µl of undiluted RNA was placed directly onto the lower measurement pedestal of the Nanodrop 1000. The upper optical pedestal was lowered and the measurement was taken. After each reading, the sample was wiped from both the upper and lower pedestals. Each RNA sample was quantified in this manner.

Table 2.. RNA yield and purity of extracted RNA samples.

|  |  |  |  |
| --- | --- | --- | --- |
| Condition | Time (h) | 260 nm | 260/280 |
| Normoxia | 0 | 79.8 ± 30.7 | 1.97 |
| 24 | 199.7 ± 69.8 | 2.00 |
| 48 | 164.9 ± 21.4 | 2.01 |
| 72 | 227.9 ± 26.9 | 2.01 |
| 96 | 141.1 ± 17.3 | 2.06 |
| Hypoxia | 24 | 99.9 ± 26.8 | 1.96 |
| 48 | 148.1 ± 49.0 | 1.97 |
| 72 | 161.8 ± 22.9 | 1.96 |
| 96 | 201.6 ± 11.8 | 1.94 |

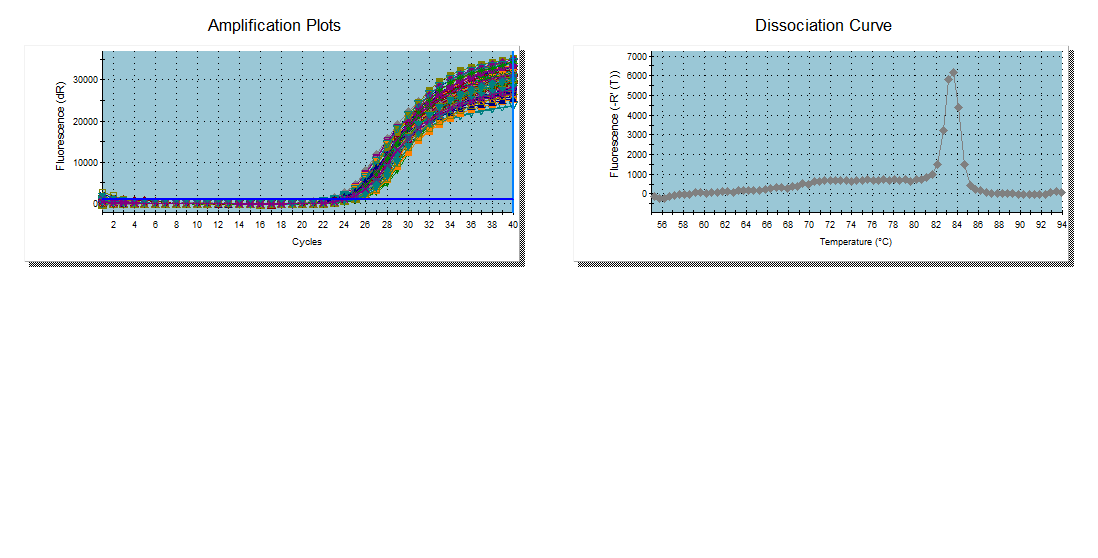
### Quantitative Real-time Polymerase Chain Reaction (qPCR)

Proliferation and the onset of differentiation of the 2D culture were determined by qPCR analysis of myoD, Myf-5 and Myogenin expression using the Stratagene Mx3005P PCR system (Applied Biosystems, Warrington, UK). Real-time PCR was performed with the QuantifastTM SYBR® Green PCR Kit (Qiagen, Manchester, UK). RNA Polymerase II-β (RPII-β) mRNA was used as an internal control. Primers used are presented in Table 2.3.

Table 2.. Primer sequences for myf5, myoD, myogenin and RPII-β.

|  |  |  |
| --- | --- | --- |
| **Target Gene** | **Primer Sequence** | **Ref.seq.number** |
| **Myf5** | F: CGTATTATGAACTCTCTC  R: CAAGACAGTATTTACAAC | NM\_008656 |
| **MyoD** | F: CCAACTGAGATTGTCTGTC  R: GGTGTTAGCCTTATGTGAAT | NM\_010866 |
| **Myogenin** | F: CCAACTGAGATTGTCTGTC  R: GGTGTTAGCCTTATGTGAAT | NM\_153798.2 |
| **RPII-β** | F: GGTCAGAAGGGAACTTGTGGTAT  R: GCATCATTAAATGGAGTAGCGTC | NM\_031189.2 |

The PCR reaction mixture (10 µl SYBR Green, 0.2 µl RT mix, 0.15 µl forward primer, 0.15 µl reverse primer) was created for each gene to be analysed in duplicate over a 96-well plate (Applied Biosystems, Warrington, UK). Of this reaction mix, 10.5 µl of solution was added to each well along with 9.5 µl of solution containing 70 ng of RNA dissolved in RNase-free water. Real-time PCR[[16]](#footnote-16) (RT-PCR) was performed as follows: 50°C, 10 min (cDNA[[17]](#footnote-17) synthesis), 95°C, 5 min (transcriptase inactivation), followed by: 95°C, 10 s (denaturation), and 60°C, 30 s (annealing/extension) for 40 cycles. Upon completion, dissociation curve analyses were performed to confirm primer specificity (i.e. to check for amplification of unwanted products or pseudogenes). Primer specificity was determined by a single peak as shown in Figure 2.2.

Figure 2.. Quantitative PCR dissociation curve analysis showing a single peak confirms the specificity of a primer set. Melt curve is shown from primers designed to amplify the mouse TNF-α gene.

### Quantitative PCR Data Analysis

A cycle threshold (CT) was defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe exceeded a fixed threshold above the baseline. A comparative CT method outlined by Applied Biosystems (<http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042115.pdf>), was used to quantify the amount of each target gene present. The mean CT values of the duplicate samples from each sample was determined and normalised to RPII-β, which acted as the endogenous housekeeping gene. Real-time PCR were analysed using the 2(-ΔΔCT) method for determining relative gene expression ratios, where the relative expression is calculated as 2(-ΔΔCT) and where CT represents the threshold cycle ([Livak & Schmittgen, 2001](#_ENREF_167); [Schmittgen & Livak, 2008](#_ENREF_249)). An example of the 2(-ΔΔCT) calculation is displayed in Table 2.4. In this example, there is a control and treatment group in which myf5 is the gene of interest and RPII-β is used as an internal control reference gene.

Table 2.. Quantitative PCR data analysis showing the calculation of relative gene expression using the 2(-ΔΔCT) method.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Myf5 CT | | RPII-β CT | | Calibrator Myf5 CT | Calibrator RPII-β CT | ΔCT1 | ΔCT2 | ΔΔCT | 2(-ΔΔCT) | Mean ± 1SD |
| Control | | 22.36 | | 22.72 | 22.63 | 22.56 | -0.36 | 0.07 | -0.43 | 1.35 |  |
| Control | | 22.55 | | 22.41 | 22.63 | 22.56 | 0.14 | 0.07 | 0.07 | 0.95 |  |
| Control | | 22.78 | | 22.53 | 22.63 | 22.56 | 0.25 | 0.07 | 0.18 | 0.88 | **1.06 ± 0.25** |
| Treatment | | 23.55 | | 22.62 | 22.63 | 22.56 | 0.93 | 0.07 | 0.86 | 0.55 |  |
| Treatment | | 24.24 | | 23.32 | 22.63 | 22.56 | 0.92 | 0.07 | 0.85 | 0.55 |  |
| Treatment | | 22.93 | | 22.88 | 22.63 | 22.56 | 0.05 | 0.07 | -0.02 | 1.01 | **0.70 ± 0.27** |

**Note:** ΔCT1 = Myf5 CT – RPII-β CT; ΔCT2 = calibrator Myf5 CT – Calibrator RPII-β CT; ΔΔCT = ΔCT1 – ΔCT2.

### Immunocytochemistry

Following experimentation the media from each well containing 13 mm coverslips was removed and the well was rinsed twice in 2 ml PBS. Cells were fixed in 1ml ice-cold methanol and acetone diluted in PBS for 10 minutes, followed by a further 5 minute incubation in 1ml ice-cold methanol and acetone alone. Once fixed, cells were rinsed twice in 500 µl Tris-buffered saline (TBS) before being blocked in 300 µl TBS containing 5% goat serum and 0.2% Triton X-100 for 30 minutes at room temperature. Cells were then rinsed again 3 times with TBS before being incubated with rabbit anti-desmin antibody (Abcam, Cambridge, UK) diluted 1 in 200 in TBS containing 2% goat serum and 0.2% Triton X-100 for two hours at room temperature. After another TBS rinse the samples were incubated with 300 µl goat anti-rabbit IgG TRITC (Abcam) diluted 1 in 200 in TBS) for one hour at room temperature in the dark. Following another rinse in TBS, a fluorescent minor-groove deoxyribonucleic acid (DNA) binding probe DAPI (4,6-diamidino-2-phenylindole; 1.0ng/ml; Sigma-Aldrich) was added to act as a nuclear stain and left for 10 minutes. After rinsed in TBS, coverslips were then mounted on coverslides using 10µl MOWIOL (MOWIOL 4-88, Sigma Aldrich). Coverslides were stored at 4ºC and subsequently imaged using a Leica DM2500 microscope (Leica software LAC V3.7, Leica Microsystems, Wetzler, Germany). All procedures took place at room temperature.

### Statistical Analyses

Data were analysed using SPSS software (release 20.0; SPSS; Chicago, IL). To test for differences between normoxia (21% O2) and physiological hypoxia (5% O2), a two way analysis of variance (ANOVA) with repeated measures on time was employed. Post hoc Bonferonni adjusted t-tests were used if ANOVA identified significant differences. All values are presented as mean ± 1 standard deviation (1SD) and for all statistical analyses an alpha of P ≤ 0.05 was considered significant unless otherwise stated.

## Results

### Cell Counts and Viability

Figure 2.3 shows representative growth curves of C2C12 cells both under normoxic and hypoxic conditions in experiments performed in duplicate. C2C12 cells grew exponentially with a population doubling time of less than 72 hours in both conditions. Total cell counts are presented in Figure 2.4, there were no significant differences between conditions however, a significant increase in cell counts over time was found (F(3,6) = 16.413, P = 0.03). Individual comparisons identified significant differences between 24 and 96 (t(3) = -7.561, P = 0.005) and 48 and 96 hours of culturing in both conditions (t(3) = -7.697, P = 0.005). Cell viability (%) within the total cell count was not significantly different at any of the time points between cells cultured under normoxic or hypoxic conditions (Figure 2.5).

Figure 2.. Growth curve of C2C12 cells under normoxic and hypoxic conditions.

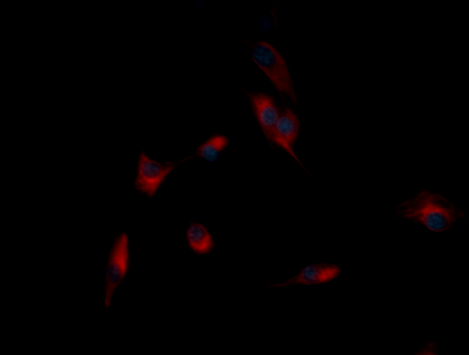
\*

\*

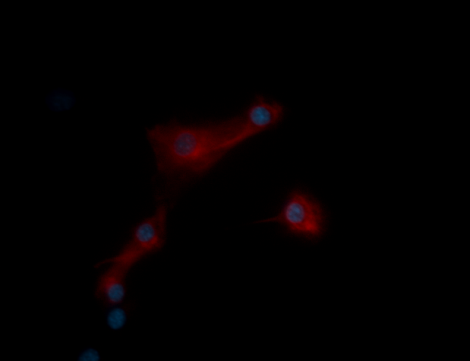
Figure 2.. Total cell count of adhered cells, taken every 24 hours over a 96 hour period in vitro, as determined by haemocytometer (n=6). \*Significantly lower than 96 hours, P ≤ 0.05.

Figure 2.. Percentage of viable cells adhered to coverslips taken every 24 hours over a 96 hour period in vitro, as determined by haemocytometer and Trypan blue exclusion (n=6).

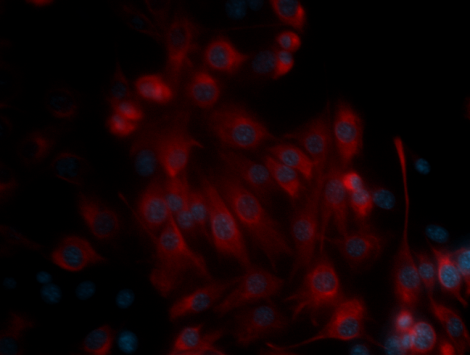
Examination of cell images suggest that cells exposed to both normoxia and hypoxia present similar morphology (Figure 2.6). All images demonstrate that the C2C12 myoblasts are relatively dormant in nature up till 72 hours, at 96 hours cells show some appearance of alignment.



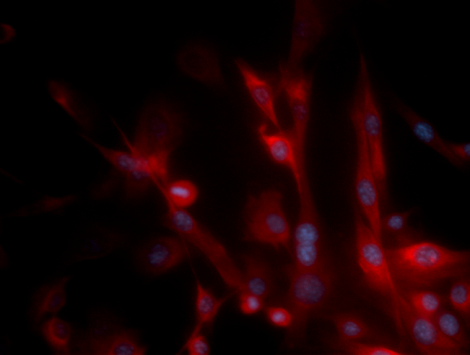
A24



B24



A96

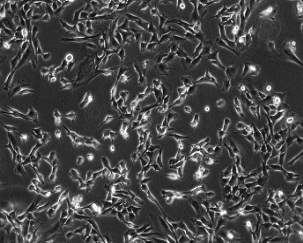
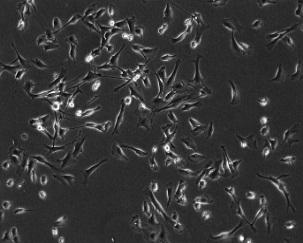
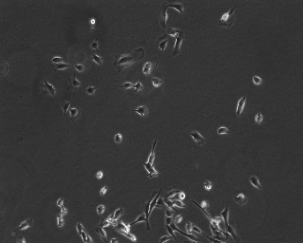
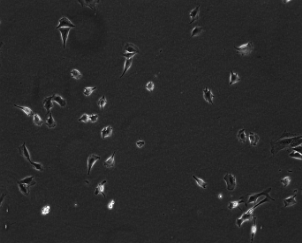
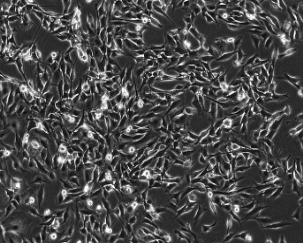
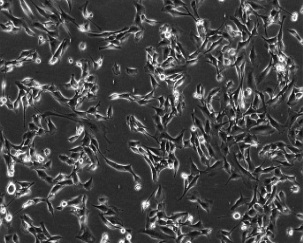
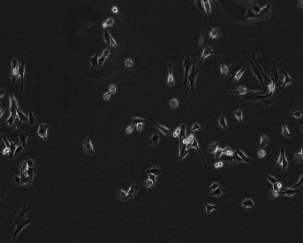
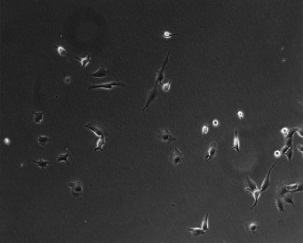


B96

Figure .. C2C12 cells cultured in GM for a total of 4 days. Cells were fixed every 24 hours. (A) Cells incubated under normoxic conditions (21% O2) (B) Cells incubated under normoxic conditions (21% O2) and exposed to acute hypoxia (5% O2) during the final 24 hours of culturing. Images were chosen at random from a selection of ten. Scale bar = 20 µm.

Figure 2.7. C2C12 cells cultured in GM immunostained for desmin (red) with a DAPI nuclear stain (blue). A24, cells incubated under normoxic conditions (FIO2 0.209) for 24 hours; A96, cells incubated under normoxic conditions (FIO2 0.209) for 96 hours; B24, cells incubated under hypoxic conditions (FIO2 0.05) for 24 hours; and B96, cells incubated under normoxic conditions (FIO2 0.209) for 72 hours with acute hypoxic exposure (FIO2 0.05) during the final 24 hours of culturing. Images selected randomly from a selection of ten. Scale bar = 20µm.

**24 hours 48 hours 72 hours 96 hours**



**A**

**B**

### Quantitative PCR Analysis

The mRNA expression of myf5, myoD and myogenin are presented in Figure 2.7 (a-c). No significant differences in relative myf5, myoD and myogenin mRNA expression levels between the two conditions were found. Compared with mRNA expression levels at 24 hours, myoD and myf5 mRNA expression levels were unchanged. Myogenin significantly changed over time (F(3,6) = 5.636, P = 0.03), however individual comparisons at all of the time points revealed no significant differences.

**B**

**A**

**C**

**Figure 2.7. Myf5 (A), myoD (B) and myogenin (c) mRNA expression levels from C2C12 cultures taken every 24 hours over a 96 hour period in vitro, as determined by qPCR. CT values were normalised to an internal housekeeping gene (RPII-β) and expressed relative to levels recorded following 24 hours of culture in normoxia (n = 6).**

## Discussion

The aim of the present study was to examine the effect of acute hypoxia on the proliferation rate, cell viability and MRF expression of C2C12 cells in vitro. The main findings demonstrate that there was no change in proliferative rate, cell viability or mRNA expression of myf5, myoD and myogenin with hypoxic conditions (FIO2 0.05) when compared with normoxic conditions (FIO2 0.209). These results demonstrate that C2C12 cells are able to maintain their proliferative capacity and myogenicity under conditions of low O2.

Cell survival is classically reported as being dependent upon the counterbalance of O2 supply and O2 demand ([Arthur, et al., 2000](#_ENREF_14)). Cell apoptosis in a number of cell types is well known, however the severity of hypoxia determines whether cells become apoptotic or adapt to hypoxia and survive ([Greijer & van der Wall, 2004](#_ENREF_114)). Due to this understanding the effect of chronic hypoxia (> 24 h) on cell viability has received some attention in previous studies, however findings are rather inconclusive, with some research reporting an increase ([Csete, et al., 2001](#_ENREF_61); [Erkkila et al., 1999](#_ENREF_79)), some a decrease ([Ramirez et al., 2011](#_ENREF_224)) and others reporting no change ([Santilli et al., 2010](#_ENREF_245)). On the contrary, little evidence is available concerning the effect of acute hypoxia (< 24 h). In the present study an acute bout of low O2 concentration (24 h) had no significant effect on total cell viability. Unlike the cascade of events which occur under severe hypoxia leading to cell apoptosis it appears that during acute hypoxia the cell is able to sense and adapt to the low O2 environment by activating a number of genes involved in metabolic adaptation, and stimulating cell proliferation allowing for increased oxygenation of the tissue ([Greijer & van der Wall, 2004](#_ENREF_114)). Therefore, both the length of exposure and O2 concentration appear to play a crucial role in the maintenance of cell viability. As previous studies have shown increased apoptosis following 48 hours of exposure and the present study shows no effect after 24 hours, length of exposure could be most important.

It is generally recognised that cells cultivated under low O2 conditions (0.02-0.10 FIO2) demonstrate increased proliferative capacity when compared with cells cultivated under standard O2 conditions ([Chakravarthy, et al., 2001](#_ENREF_46); [Csete, et al., 2001](#_ENREF_61)). However, some research has shown that hypoxia may reduce proliferative capacity and it is thought that exposure length may play a pivotal role in the effects hypoxia presents. One study reported that culturing of C2C12 cells for 122 h at 0.02, 0.05 and 0.10 FIO2 had no significant effect on growth rate for the first 48 h after seeding. After this time point proliferative capacity was significantly decreased by up to 60% at an FIO2 of 0.05, however the effect was diminished at 0.10 FIO2 ([Sato, et al., 2011](#_ENREF_247)). Results of the present study are in line with Sato and colleagues however no significant decline was observed following 24 h in normoxia plus 24 hours of exposure to an FIO2 of 0.05suggesting that proliferative capacity was maintained. The differing results may be due to methodology, Sato and colleagues used a single bout of exposure to hypoxia for 48 h whereas the present study exposed cells to 24 h of normoxia followed by 24 hours of hypoxia. Another possible explanation for the discrepancy between findings may be related to cell passage number. A study examining the effect low O2 conditions on proliferative rate in C2C12 cells demonstrated that an increase in proliferative capacity is only observed during low passages (0-5), at higher passages (6-8) there appears to be no change ([Koning, et al., 2011](#_ENREF_151)). The C2C12 cells used in the present research were exposed to acute hypoxia at passage 7 so this could be a possible explanation of why proliferative capacity was not enhanced under low O2 conditions. However, this interpretation must be taken with caution as Sato and colleagues did not report passage number.

Proliferating myoblasts express myoD and myf5, which are markers of commitment to the muscle lineage, and under high serum conditions in vitro C2C12 cells will continue to proliferate ([Moran, et al., 2002](#_ENREF_196)). Myogenin is then up-regulated as cells begin to undergo differentiation and fuse to form multinucleate myotubes ([Li, et al., 2007](#_ENREF_162); [Yun, et al., 2005](#_ENREF_299)). Therefore, a change in proliferative capacity with low O2 concentrations has been associated with both up- and down-regulation of genes associated with cell proliferation. Cultivation of murine satellite cells under low O2 (FIO2 0.06) expressed increased levels of myf5 and myoD ([Csete, et al., 2001](#_ENREF_61)). However, at more extreme levels of hypoxia (0.02-0.01 FIO2) a down-regulation of myf5, myoD and myogenin become apparent ([Di Carlo, et al., 2004](#_ENREF_69); [Gustafsson, et al., 2005](#_ENREF_117); [Liu, et al., 2012](#_ENREF_166); [Majmundar et al., 2012](#_ENREF_175); [Yun, et al., 2005](#_ENREF_299)). Results of the present study, i.e. no reduction in cell viability or increase in proliferation, are supported by mRNA expression findings. No significant differences in myogenin, myoD or myf5 were found between cells cultivated under normoxia and hypoxia at any time point.

In summary, it would appear that 24 hours of hypoxia (FIO2 0.05) at any time point during the proliferative phase is not sufficient enough to alter cell equilibrium in C2C12 myoblasts. In the present study we used a 0.05 FIO2 concentration to represent hypoxia. In skeletal muscle the partial saturation of O2 is reported to be between 1 and 10% ([Sato, et al., 2011](#_ENREF_247)). It is still uncertain whether the hypoxic condition used here represents the drop in cellular O2 pressure induced by a high-altitude environment at rest. Nevertheless from the results it would appear that an intramyocellular O2 pressure of 5% for 24 hours is safe for use within in vivo hypoxic exposure studies. Chapter 3 focuses on the effects of acute hypoxia on C2C12 myotubes in order to determine whether protein degradation is apparent within mature cells.

# Effect of Low Oxygen Conditions on C2C12 Myotube mRNA Expression

## Introduction

High-altitude and the associated hypoxia poses a biological challenge to the living organism and can have major consequences for survival compromising whole body metabolism, promoting reversible or irreversible loss of tissue and cell homeostasis which can lead to organic and functional decay ([Magalhães & Ascensão, 2008](#_ENREF_174)). This characteristic response to hypoxia has been demonstrated by a reduction in body mass at high-altitude ([Armellini, et al., 1997](#_ENREF_10); [Boyer & Blume, 1984](#_ENREF_32); [Lippl, et al., 2010](#_ENREF_165); [Rose, et al., 1988](#_ENREF_238)). Although below 5000 m the loss in body mass is largely attributable to a loss in fat mass ([Boyer & Blume, 1984](#_ENREF_32); [Butterfield, et al., 1992](#_ENREF_38); [Kayser, et al., 1992](#_ENREF_143); [Westerterp, et al., 1992a](#_ENREF_290)), research suggests that exposure to altitudes above 5000 m causes an inexorable loss in fat-free mass ([Boyer & Blume, 1984](#_ENREF_32); [Sergi, et al., 2010](#_ENREF_253)), which has been demonstrated by others to be caused by a significant reduction in muscle volume with a concomitant decrease in muscle fibre size ([Green, et al., 1989](#_ENREF_113); [MacDougall, et al., 1991](#_ENREF_171)) and a significantly lower ability to regenerate skeletal muscle ([Mancinelli et al., 2011](#_ENREF_176)). Taken together these observations suggest that muscle catabolism may play a central role in weight loss with exposure to high-altitude, which is an obvious negative effect.

The study of intermittent hypoxia with or without exercise training to enhance weight loss and improve metabolic health is becoming increasingly popular ([Bailey, et al., 2000a](#_ENREF_16); [Haufe, et al., 2008](#_ENREF_122); [Saeed, et al., 2012](#_ENREF_243); [Wiesner, et al., 2010](#_ENREF_295)) and has thus far provided promising results. However, it is still yet to be identified whether skeletal muscle atrophy occurs with acute/intermittent exposures to hypoxia and contributes to the reductions which have been observed within body mass ([Bailey, et al., 2000a](#_ENREF_16); [Haufe, et al., 2008](#_ENREF_122); [Netzer, et al., 2008](#_ENREF_202)). Any reduction in body mass as a result of a reduction in fat-free mass could have negative consequences for individuals in which fat-free mass is already limited (e.g. overweight/obese). Such consequences include decreased strength, increased fatigue and increased insulin resistance ([Amirouche, et al., 2009](#_ENREF_6); [Favier, et al., 2010](#_ENREF_82)) which can lead to other health problems such as hypertension, hyperlipidemia, and atherosclerosis, which have been associated with type II diabetes ([Kahn & Flier, 2000](#_ENREF_140)). Therefore, it is essential that the skeletal response to acute hypoxia is explored as it will enable researchers to identify whether the use of intermittent hypoxia as a non-pharmacological therapy of obesity is effective whilst remaining safe for the individual.

Skeletal muscle mass is maintained by the balance between protein synthesis and protein degradation, and any disruption in this equilibrium will promote either muscle hypertrophy or muscle atrophy ([Caron, et al., 2009](#_ENREF_41); [Favier, et al., 2010](#_ENREF_82); [Franch & Price, 2005](#_ENREF_91); [Kandarian & Jackman, 2006](#_ENREF_141)). Several signalling pathways are known to regulate skeletal muscle atrophy ([Russell, 2010](#_ENREF_241)) however, one pathway which has received considerable attention is the PI3k[[18]](#footnote-18)/Akt[[19]](#footnote-19)/foxO[[20]](#footnote-20) pathway ([Kandarian & Jackman, 2006](#_ENREF_141)). Deactivation of Akt phosphorylation leads to the progression of skeletal muscle atrophy ([Doucet et al., 2007](#_ENREF_72); [Kandarian & Jackman, 2006](#_ENREF_141)) through the activation of FoxO transcription factors which subsequently increase the expression of E3 ubiquitin ligating enzymes leading to synthesis of targeted proteins of the ubiquitin proteasome pathway ([Favier, et al., 2010](#_ENREF_82)). The addition of ubiquitin to a protein substrate has come to be recognised as a regulated signalling process in skeletal muscle atrophy ([Bodine et al., 2001](#_ENREF_26); [Foletta et al., 2011](#_ENREF_89); [Franch & Price, 2005](#_ENREF_91); [Glass, 2003](#_ENREF_105), [2005](#_ENREF_106); [Gomes et al., 2001](#_ENREF_109)). Two E3 ubiquitin-ligating enzymes most notably expressed in skeletal muscle are atrogenes muscle atrophy F-box (MAFbx) and muscle ring finger-1 (MURF-1) ([Franch & Price, 2005](#_ENREF_91); [Sandri et al., 2004](#_ENREF_244)). The role of both these E3 ligases is to attach activated ubiquitin to targeted substrates that will be degraded by the proteasome ([Caron, et al., 2009](#_ENREF_41)) which subsequently leads to skeletal muscle atrophy.

Whilst the research studies which have examined the role of low O2 on protein synthesis and/or degradation are sparse, some evidence is available. Arthur, Giles and Wakeford ([2000](#_ENREF_14)) examined the effect of severe hypoxia (0.01 FIO2) on protein synthesis. Following 150 minutes, a significant decline (43%) in the rate of protein synthesis in C2C12 cells was shown, however this result was not observed with moderate hypoxia (FIO2 0.05). Since hypoxia may contribute to the development of skeletal muscle atrophy Caron et al., ([2009](#_ENREF_41)) examined the response of L6 myoblasts to 24 and 48 h of hypoxic exposure (0.01 FIO2). Protein degradation and synthesis was evaluated in hypoxic myotubes and a significant reduction in total protein content was reported which was thought to be due to an increase in MAFbx mRNA expression following 24 hours of exposure. Theije et al., (2013) recently reported similar findings, reporting that hypoxia (0.04 FIO2) decreased the absolute amount of phosporylated Akt which subsequently led to an increase in MURF-1 and MAFbx mRNA expression via an increase in foxO1 (Theije et al., 2013). Together, these studies suggest that hypoxia reduces protein synthesis and increases protein degradation.

Myostatin, is a member of the transforming growth factor-β family of proteins ([Glass, 2010](#_ENREF_107)), it is a negative regulator of skeletal muscle mass ([Lee et al., 2004](#_ENREF_158)) and is also thought to control the PI3k/Akt/foxO pathway. Myostatin up-regulation inhibits the activation of Akt phosphorylation via a down-regulation in insulin-like growth factor-1 (IGF-1) in both myoblasts and myotubes ([Glass, 2010](#_ENREF_107); [Trendelenburg et al., 2009](#_ENREF_273)). The inhibition of Akt phosphorylation subsequently activates foxO transcription factors via phosphorylation which in turn increases the expression of atrogenes MURF-1 and MAFbx (Elkina et al., 2011), known to participate in protein degradation. Thus, myostatin alongside MURF-1 and MAFbx may play a role in the skeletal muscle response to hypoxia. Hayot et al., ([2011](#_ENREF_124)) examined the hypothesis that a potential mechanism for the effect of hypoxia on skeletal muscle may be through the regulation of myostatin. An up-regulation of myostatin expression in muscles of male Wistar rats exposed to five weeks of continuous hypoxia (0.12 FIO2) was reported which suggests that myostatin plays an essential role in the adaptation of skeletal muscle to hypoxic environments. In addition to exploring the role of myostatin the authors also recognised the role of MAFbx in response to skeletal muscle atrophy. Northern blot[[21]](#footnote-21) analysis showed an increase in MAFbx mRNA expression in hypoxic versus normoxic muscle. Thus, the findings of Hayot and colleagues confirm increased protein degradation with hypoxia.

Since research findings regarding the effects of acute hypoxia on skeletal muscle tissue are limited, the aim of the present study was to examine the effect of acute hypoxia (0.05 FIO2, 90 mins and 24 h) on myostatin, IGF-1, MURF-1, MAFbx and HIF-1α in C2C12 myotubes. It was hypothesised that 24 hours of hypoxic exposure would increase protein degradation and the activity of the PI3k/Akt/foxO pathway, but 90 minutes of exposure would have no effect on mRNA expression of genes associated with skeletal muscle atrophy.

## Methods

### Cell Culture

All cell culture was carried out using a Class II Heraeus Biological Safety cabinet under sterile conditions. C2C12 murine myoblasts were provided by the Health Protection Agency (HPA cultures, Salisbury, UK). Cells were grown in GM, consisting of DMEM (Sigma-Aldrich, Haverhill, UK) supplemented with 20% FBS (PAA Laboratories, Somerset, UK) and 1% PenStrep (Sigma-Aldrich, Haverhill, UK). Cells were incubated at 37°C in a humidified Heracell 240 incubator (Thermo Fisher Scientific, Rosklide, Denmark) under 5% CO2, which is the conventional 21% O2 condition (normoxia). To ensure reproducibility all experiments were carried out at passages 8-15.

Once confluent, C2C12 cells were seeded on to pre-gelatinised (0.2%) 6-well plates (150 000 cells/well) in 2ml GM, GM was replaced every 48 hours. Each 6-well plate consisted of 3 wells for RNA and 1 well immunostaining. Confluent myoblasts were placed in differentiation media, consisting of DMEM supplemented with 2% horse serum (HS; PAA Laboratories, Somerset, UK) and 1% PenStrep (Sigma-Aldrich, Haverhill, UK). After three days, differentiated myotubes were either incubated in a humidified incubator at 37°C under normoxic conditions (21% O2, 5% CO2, 74% N2) or exposed to physiological hypoxia (5% O2) for either 90 minutes or 24 hours. For cells exposed to physiological hypoxia C2C12 cells were placed in an adjustable hypoxia chamber with real-time PO2 readout (MCO-5M, Sanyo, Japan) in which air was flushed out by 95% N2 and 5% CO2 until the chosen O2 concentration of 5% was attained. Actual concentration of 5% O2 in the chamber was based on a direct measurement inside the chamber using a microelectrode O2 probe (Microelectrodes Inc, USA). Cells were sampled for RNA at 90 minutes or 24 hours.

### RNA Extraction and Quantification

Total RNA was isolated with Trizol reagent (Fisher Scientific, Loughborough, UK) as per the manufacturer’s instructions. After experimentation, GM was removed and a PBS wash was completed, 6-well plates were then scraped in the presence of 500 μl of Trizol reagent before being transferred to a 1.5 ml RNase free tube (Fisher Scientific, Loughborough, UK) and stored at -80°C.

Samples were defrosted and vortexed for 10 seconds prior to RNA extraction. Subsequently 100 μl chloroform (Sigma-Aldrich, Haverhill, UK) was added to each sample tube and shaken thoroughly for 10 seconds by hand before incubation at room temperature for 5 minutes. Samples were then spun at 12 000 x g for 15 minutes using a micro-centrifuge (Fisher Scientific, Loughborough, UK) after which the upper aqueous phase containing RNA was transferred to new 1.5 ml tubes. A 250 µl aliquot of isopropyl alcohol (Fisher Scientific, Loughborough, UK) was then added to each RNA sample before incubation at room temperature for 10 minutes. The tubes were spun for 10 minutes at 12 000 x g resulting in the pelleting of RNA at the bottom of the tube. The supernatant was then carefully removed before washing the pellet in 500 µl of 75% molecular grade ethanol (Fisher Scientific, Loughborough, UK), by vortexing and centrifugation at 7500 x g for 5 minutes. The ethanol supernatant was then removed and the pellet was allowed to air dry for 10-15 minutes before being re-suspended in 50 µl of RNA storage solution (Sigma-Aldrich, Haverhill, UK).

Following Trizol extraction RNA yield (absorbance at 260 nm) and purity (260/280 ratio) were determined using a Nanodrop 3000 Spectrophotometer (Table 3.1; Fisher Scientific, Loughborough, UK). Using an L-2 pipet (Rainin Instruments, Bedfordshire, UK), 1 µl of undiluted RNA was placed directly onto the lower measurement pedestal of the Nanodrop 1000. The upper optical pedestal was lowered and the measurement was taken. After each reading, the sample was wiped from both the upper and lower pedestals. Each RNA sample was quantified in this manner.

Table 3.. RNA yield and purity of extracted RNA samples.

|  |  |  |  |
| --- | --- | --- | --- |
|  |  | 260 nm absorbance | 260/280 |
| Normoxia | 24 h | 578 ± 28 | 2.0 ± 0.0 |
| 90 mins | 528 ± 105 | 1.9 ± 0.1 |
| Hypoxia | 24 h | 456 ± 52 | 2.0 ± 0.1 |
| 90 mins | 642 ± 90 | 1.9 ± 0.1 |

### Quantitative Real-time PCR

The mRNA expression of the 2D culture was determined by qPCR analysis of myostatin, MURF-1, MAFbx, IGF-1, and HIF-1α using the Stratagene Mx3005P PCR system (Agilent technolgies, Workingham, Berkshire, UK). Real-time PCR was performed with the QuantifastTM SYBR® Green PCR Kit (Qiagen, Manchester, UK). RNA Polymerase II-β mRNA was used as an internal control. Primers used are presented in Table 3.2.

Table 3.. Primer sequences for myostatin, MURF-1, MAFbx and RPII-β.

|  |  |  |
| --- | --- | --- |
| Target Gene | Primer Sequence | Ref. seq. number |
| Myostatin | F: CTATAAGACAACTTCTGCCAAG  R: AGAAAGTCAGACTCTGTAGG | NM\_010834.2 |
| MURF-1 | F: CCAAGGAGAATAGCCACCAG  R: CGCTCTTCTTCTCGTCCAG | NM\_001039048.2 |
| MAFbx | F: CTGAAAGTTCTTGAAGACCAG  R: GTGTGCATAAGGATGTGTAG | NM\_026346.3 |
| IGF-1 | F: GACATTGCTCTAACATCTCC  R: CAGGTAGAAGAGGTGTGAA | NM\_010512.4 |
| HIF-1α | F: CGATGACACAGAAACTGAAG  R: GAAGGTAAAGGAGACATTGC | NM\_010431.2 |
| RPII-β | F: GGTCAGAAGGGAACTTGTGGTAT  R: GCATCATTAAATGGAGTAGCGTC | NM\_031189.2 |

The PCR reaction mixture (10 µl SYBR Green, 0.2 µl RT mix, 0.15 µl forward primer, 0.15 µl reverse primer) was created for each gene to be analysed in triplicate over a 96-well plate (Applied Biosystems, Warrington, UK). Of this reaction mix, 10.5 µl of solution was added to each well along with 9.5 µl of solution containing 70 ng of RNA dissolved in RNase-free water. RT-PCR[[22]](#footnote-22) was performed as follows: 50°C, 10 min (cDNA synthesis), 95°C, 5 min (transcriptase inactivation), followed by: 95°C, 10 s (denaturation), and 60°C, 30 s (annealing/extension) for 40 cycles. Upon completion, dissociation curve analyses were performed to confirm primer specificity. Primer specificity was determined by a single peak as shown in Chapter 2 (Figure 2.2).

### Quantitative PCR Data Analysis

A CT was defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe exceeded a fixed threshold above the baseline. A comparative CT method outlined by Applied Biosystems, was used to quantify the amount of each target gene present. The mean CT values of the triplicate samples from each sample was determined and normalised to RPII-β, which acted as the endogenous housekeeping gene. Real-time PCR were analysed using the 2(-ΔΔCT) method for determining relative expression ratios, where the relative expression is calculated as 2(-ΔΔCT) and where CT represents the threshold cycle ([Livak & Schmittgen, 2001](#_ENREF_167); [Schmittgen & Livak, 2008](#_ENREF_249)). An example of the 2(-ΔΔCT) calculation is displayed in Chapter 2 (Table 2.4).

### Statistical Analyses

Data were analysed using SPSS software (release 20.0; SPSS; Chicago, IL). To test for differences between the normoxia (FIO2 0.209) and physiological hypoxia (FIO2 0.05), a paired t-test was employed. All values are presented as mean ± 1SD and for all statistical analyses an alpha of P ≤ 0.05 was considered significant unless otherwise stated.

## Results

### Quantitative PCR Analysis

Measurement of genes involved in skeletal muscle atrophy was assessed in myotubes by measuring mRNA expression of MURF-1 and MAFbx, two specific E3 ubiquitin ligases known to be up-regulated in multiple models of skeletal muscle atrophy. The mRNA expression of the two atrogenes was measured at 90 minutes and 24 hours in myotubes exposed to 0.05 FIO2. Both MURF-1 and MAFbx were significantly up-regulated after 90 minutes and 24 hours exposure to physiological hypoxia (Figure 3.1and Figure 3.2). In more detail, following 90 minutes exposure MURF-1 was increased 1.6-fold (t(9) = -5.029, P ≤ 0.01) and MAFbx was increased 1.5-fold (t(9) = -3.426, P ≤ 0.01). Following 24 hours hypoxic exposure, MURF-1 was increased 1.5-fold (t(10) = -4.100, P ≤ 0.05) and MAFbx was increased 2.5-fold (t(14) = -8.289, P ≤ 0.01) compared with normoxia (0.209 FIO2).

\*

\*

Figure 3.. MURF-1 mRNA expression levels from C2C12 myotube cultures at 90 mins and 24 h, as determined by qPCR. CT values were normalised to an internal housekeeping gene (RPII-β) and expressed relative to levels recorded at 0 hours (n = 10 for 90 mins and n = 15 for 24 h). \*Greater than normoxia, P ≤ 0.05.

\*

Figure 3.. MAFbx mRNA expression levels from C2C12 myotube cultures at 90 mins and 24 h, as determined by qPCR. CT values were normalised to an internal housekeeping gene (RPII-β) and expressed relative to levels recorded at 0 hours (n = 10 for 90 mins and n = 15 for 24 h). \*Greater than normoxia, P ≤ 0.01.

Since the IGF-1/PI3k/Akt and PI3k/Akt/foxO are thought to play a role in skeletal muscle atrophy, to determine which genes may also be involved in hypoxia-induced skeletal muscle atrophy along side MURF-1 and MAFbx, the mRNA expression of myostatin and IGF-1 were measured following 24 hours of exposure to hypoxia (FIO2 0.05), following 90 minutes exposure, only IGF-1 was measured. The expression of Myostatin increased significantly (t(13) = -3.401, P ≤ 0.01), whilst IGF-1 was significantly decreased (t(14) = 2.789, P ≤ 0.05) following 24 hours exposure. After a 90 minute exposure, IGF-1 was unchanged. Results are displayed in Figure 3.3 and Figure 3.4.

\*

Figure 3.. Myostatin mRNA expression levels from C2C12 myotube cultures at 24 h, as determined by qPCR. CT values were normalised to an internal housekeeping gene (RPII-β) and expressed relative to levels recorded at 0 hours (n = 15). \*Greater than normoxia, P ≤ 0.01.

Figure 3.. IGF-1 mRNA expression levels from C2C12 myotube cultures at 90 mins and 24 h, as determined by qPCR. CT values were normalised to an internal housekeeping gene (RPII-β) and expressed relative to levels recorded at 0 hours (n = 10 for 90 mins and n = 15 for 24 h). \*less than normoxia, P ≤ 0.05.

Also measured following 90 minutes and 24 hours of exposure to hypoxia (FIO2 < 0.05) was the mRNA expression of the oxygen sensing gene HIF-1α, no significant change was found between the normoxic and hypoxic condition.

## Discussion

The aim of the present study was to examine the effect of acute physiological hypoxia (FIO2 0.05, 24 h) on skeletal muscle atrophy through the measurement of myostatin, IGF-1, MURF-1, MAFbx and HIF-1α mRNA expression. The main findings include significant increases in MURF-1, MAFbx and myostatin with a significant decrease in IGF-1 following 24 hours exposure. Post 90 minute exposure, both MURF-1 and MAFbx were increased, but IGF-1 remained unchanged. No change was observed for HIF-1α following either 90 minutes or 24 hours hypoxic exposure.

It has been previously demonstrated that prolonged exposure to hypoxia leads to skeletal muscle atrophy ([Green, et al., 1989](#_ENREF_113); [MacDougall, et al., 1991](#_ENREF_171); [Mancinelli, et al., 2011](#_ENREF_176)); however the mechanisms underlying this response are still poorly understood. A change in either protein synthesis or degradation can have important implications for skeletal muscle mass ([Caron, et al., 2009](#_ENREF_41)) and therefore understanding hypoxia-induced atrophy is vital. Research exploring the role of intracellular signalling in several models of skeletal muscle atrophy (e.g. starvation, denervation, disease, immobilisation) have appointed several pathways responsible which include the IGF/PI3k/Akt pathway, and the PI3k/Akt/foxO pathway.

The role of the PI3k/Akt/foxO pathway in skeletal muscle atrophy/protein degradation has been extensively reviewed ([Bonaldo & Sandri, 2013](#_ENREF_28); [Glass, 2005](#_ENREF_106); [Kandarian & Jackman, 2006](#_ENREF_141)). In summary, this pathway acts via deactivation of Akt phosphorylation, which activates foxO transcription factors and in turn increases the expression of E3 ubiquitin ligating enzymes (e.g. MURF-1 and MAFbx) leading to synthesis of targeted proteins of the ubiquitin proteasome pathway and skeletal muscle atrophy ([Doucet, et al., 2007](#_ENREF_72); [Kandarian & Jackman, 2006](#_ENREF_141)). Therefore, it is thought that the role of the PI3k/Akt/foxO pathway may play a role in hypoxia-induced skeletal muscle atrophy through increased MURF-1 and MAFbx mRNA expression. In the present study, both 90 minutes and 24 hours of hypoxic exposure (FIO2 0.05) resulted in significant increases in MURF-1 and MAFbx mRNA expression. This observation is in line with previous reports showing increased MURF-1 and/or MAFbx following exposure to hypoxia (0.01-0.04 FIO2 ([Caron, et al., 2009](#_ENREF_41); [de Theije, et al., 2013](#_ENREF_68)) and suggests that skeletal muscle breakdown is increased with acute exposure to hypoxia. Moreover, the extent to which these atrogenes are upregulated appears to be exposure time dependent, for example, following 90 minutes exposure MAFbx was increased 1.5-fold, after 24 hours MAFbx was increased 2.5-fold.

An important connection has been made between the deactivation of the IGF-1/PI3k/Akt pathway and the expression of proteolytic genes ([Kandarian & Jackman, 2006](#_ENREF_141); [Sandri, et al., 2004](#_ENREF_244)), evidence of this connection comes from research demonstrating that increased expression of MURF-1 and MAFbx mRNA can be reversed by an increase in IGF-1 ([Stitt et al., 2004](#_ENREF_265)). Therefore, a down-regulation of IGF-1 results in an increased expression of atrogenes MURF-1 and MAFbx and subsequent atrophy. The present study demonstrated a 1.2-fold decrease in IGF-1 which appears to be concommitent with a 1.5-fold increase in MURF-1 and a 2.5-fold increase in MAFbx mRNA. However, this was only the case for myotubes exposed to 24 hours of hypoxia, following a 90 minute exposure, IGF-1 remained unchanged. These differences in the IGF-1 response may suggest that different mechanisms are involved in a time-dependent manner. However, as the interplay between genes was not measured, interpretations of the data are only speculative and should be taken with caution. It could be useful for future studies to run measurements on the conditioned media following hypoxic exposure in order to gain an understanding of which proteins might be present in order to elucidate to the mechanisms involved. Moreover, examining the interplay between selected genes may highlight the mechanisms involved in hypoxia-indcuced atrophy.

Evidence of cross-talk between myostatin, a negative regulator of skeletal muscle mass ([Bonaldo & Sandri, 2013](#_ENREF_28); [Lee, et al., 2004](#_ENREF_158)) and IGF-1 has been identified. Under normal cell culture conditions, IGF-1 signalling is dominant and blocks the myostatin pathway, however, an inhibition of IGF-1 is observed when myostatin is overexpressed ([Amirouche, et al., 2009](#_ENREF_6); [Elkina et al., 2011](#_ENREF_78); [Morissette et al., 2009](#_ENREF_198)). Thus, an increase in myostatin could lead to increased skeletal muscle atrophy via a reduction in IGF-1, which may also explain the present findings. The mechanism by which IGF-1 regulates myostatin signalling is thought to include the PI3k/Akt pathway ([Elkina, et al., 2011](#_ENREF_78); [Glass, 2010](#_ENREF_107)). Indeed, up-regulation of myostatin inhibits the activation of Akt phosphorylation which activates foxO transcription leading to increased mRNA expression of MURF-1 and MAFbx ([McFarlane et al., 2006](#_ENREF_188)). The results of the present study suggest that there may be a role for myostatin up-regulation in hypoxia-induced skeletal muscle atrophy, as a decrease in IGF-1 and increase in MURF-1 and MAFbx mRNA expression following 24 hours of hypoxic exposure was present. However, further evidence is needed to confirm such findings, it would play dividence to repeat the experiment while inhibiting myostatin. Therefore, if IGF-1 didn’t change as a result of myostatin inhibition, it would provide stronger evidence for the role of IGF-1 and myostatin in hypoxia-induced atrophy.

Changes in O2 concentration can be sensed and cause changes in the degree and pattern of gene expression for a wide variety of cells ([Arthur, et al., 2000](#_ENREF_14); [Wang & Semenza, 1996](#_ENREF_281)), as has been observed within the present study. An important gene involved in the regulatory response to a change in O2 concentration is HIF-1α ([Semenza, et al., 2006](#_ENREF_252)). Under physiological hypoxia [≤ 0.05 FIO2 ([Guzy & Schumacker, 2006](#_ENREF_118))], HIF-1α translocates in to the nucleus where is dimerises with the O2-independent HIF-1β protein and initiates transcription which subsequently activates the transcription of genes that allow the cell to adapt and survive under hypoxic conditions ([Gustafsson, et al., 2005](#_ENREF_117); [Lin, et al., 2008](#_ENREF_163); [Semenza, et al., 2006](#_ENREF_252)). Interestingly, the present data suggest that HIF-1α was not significantly altered by hypoxia when compared with the normoxic control condition, despite a large reduction in O2 concentration. It is thought that this result may be due to the rapid degradation (half-life) of HIF-1α in normoxia, which has been reported to be between 5 and 8 minutes ([Berra et al., 2001](#_ENREF_25); [Chun et al., 2002](#_ENREF_55)). Since the present study did not have access to a hypoxic chamber with glove box access, which allows for minimal exposure to normoxia during analysis, it is possible that during RNA extraction HIF-1α was rapidly degraded due to normoxic exposure during this process. Therefore, an increase in HIF-1α in the present study cannot be eliminated since the results suggest that the two conditions must have differed environmentally in order for significant differences in mRNA expression to be observed.

In summary, the present study demonstrates that hypoxia (FIO2 0.05, 90 mins and 24 h) significantly alters the mRNA expression of genes involved within the PI3k/Akt pathway. A significant up-regulation in MURF-1 and MAFbx following hypoxic exposure are in line with previous research ([Caron, et al., 2009](#_ENREF_41); [de Theije, et al., 2013](#_ENREF_68)). Moreover, the results indicate that IGF-1 and myostatin may play a key role in the regulation of the PI3k/Akt pathway in response to hypoxia-induced skeletal muscle atrophy, when exposure time equals 24 hours. Since the aim of this study was to understand whether the use of intermittent hypoxia as a non-pharmacological therapy for obesity is effective and safe (i.e. no loss in fat-free mass), it can be concluded that for limited protein degradation, hypoxic exposures should be kept to 90 minutes or less.

In Chapter 4 the effect of IHE on weight loss and associated metabolic risk markers will be examined. Since 24 hours of hypoxia did not significantly inhibit the growth of C2C12 myoblasts but did have a detrimental effect on C2C12 myotubes, each IHE was a maximum of 90 minutes. Moreover, exposures of this length were chosen to mimic a typical exercise session that would be used in a weight loss exercise programme session.

# Effect of Intermittent Normobaric Hypoxic Exposures at Rest on Body Mass and Metabolic Risk Markers in Sedentary Individuals

## Introduction

Intermittent hypoxic exposure or periodic exposure to hypoxia is broadly defined as exposure to hypoxia interspersed with periods of normoxia or less hypoxic conditions lasting from seconds to hours that is repeated over several days to weeks ([Millet, et al., 2010](#_ENREF_193); [Neubauer, 2001](#_ENREF_203); [Powell & Garcia, 2000](#_ENREF_217)). The specific protocols used experimentally vary greatly in cycle length, number of hypoxic exposures per day, and the total number of exposure days. Thus protocols vary from those that examine the effect of as few as 3-12 relatively short (2-10 minute) bouts of hypoxia interspersed with 2- to 20-minute episodes of normoxia on a single day ([Belik et al., 1990](#_ENREF_22); [Nieuwenhuijs et al., 2000](#_ENREF_206)) to those that examine longer daily exposures (1-12h) over periods ranging from 2 to 90 days ([Hamlin & Hellemans, 2007](#_ENREF_120); [Rodriguez et al., 1999](#_ENREF_236); [Rodriguez et al., 2000](#_ENREF_237)). Regardless of the protocol, the compelling outcome is that these repeated episodes of hypoxia elicit persistent changes in a variety of physiological responses in humans and animals ([Neubauer, 2001](#_ENREF_203)).

Often IHE is employed in combination with physical training (i.e. IHT) in athletic populations with the aim of improving SL performance ([Millet, et al., 2010](#_ENREF_193)), or improving high-altitude acclimatisation prior to sporting competition ([Powell & Garcia, 2000](#_ENREF_217)). In addition to its use within athletic populations research has also explored the use of IHT within clinically overweight and obese populations as a method of enhancing weight loss and reducing the metabolic health risks associated with weight gain ([Bailey, et al., 2000a](#_ENREF_16); [Haufe, et al., 2008](#_ENREF_122); [Netzer, et al., 2008](#_ENREF_202)). Netzer et al., (2008) reported a trend towards greater body mass losses with IHT compared with normoxic training. In their study body mass losses equalled 1.14 kg in the hypoxic group, but remained unchanged in the normoxic training group. Haufe et al., (2008) also reported similar findings; again reporting a trend towards greater body mass losses in hypoxia. In addition to losses in body mass within this study individuals using IHT also lost a greater percentage of body fat compared with those training in normoxia. Although in lean individuals, Bailey et al., (2000) also reported a significant increase in fat-free mass following training in hypoxia, a finding which was not observed with normoxic training. Cumulatively, although findings remain equivocal, the use of IHT within overweight and obese populations may be a promising method for enhancing weight loss. However, the effects of IHE independent of exercise on weight loss are unknown.

It is important to highlight that research is yet to examine the effects of IHE alone on weight loss and associated metabolic risk markers. Currently relatively little evidence is available within the literature to suggest that this concept could be beneficial and eliminate the need for exercise training within weight loss programmes. Yet there are a few studies to-date which provide promise including, chronic altitude studies and IHE studies both in human and animal research.

Weight loss with chronic exposure to hypoxia (> 5000 m) is well documented ([Boyer & Blume, 1984](#_ENREF_32); [Fusch, et al., 1996](#_ENREF_97); [Rose, et al., 1988](#_ENREF_238)) however often these studies are confounded by extraneous variables such as cold exposure, intense physical exertion and malnutrition. Moreover, much of the research published has been performed in lean physically active populations making application to overweight and obese populations problematic. Eliminating these variables Lippl et al., ([2010](#_ENREF_165)) determined the independent effect of a 7 day exposure to hypobaric hypoxia (2650 m) on body mass in obese individuals. In their study physical activity was controlled and food was provided ad libitum. Despite no significant increase in physical activity from baseline a significant reduction in body mass was observed alongside reductions in diastolic blood pressure, and blood lipid profile parameters. Similar results were also provided by the Austrian Moderate Altitude Study 2000 ([Schobersberger et al., 2003](#_ENREF_250)). The Austrian study showed that a three week exposure to 1700 m caused significant reductions in heart rate, blood pressure, and glucose concentrations in obese men. Together these studies demonstrate that chronic exposure to high-altitude can have beneficial effects for weight loss and associated health risks. Nonetheless this long-term method is impractical and unrealistic for weight loss programmes to widely adopt. Therefore, research using IHE provides an opportunity for hypoxic exposure to be utilised in such programmes, whilst being time and cost effective, and avoid altitude related risks such as acute mountain sickness.

Rodríguez et al., ([2000](#_ENREF_237)) examined the effect of IHE on haematological and physiological responses in humans. In this study eight lean physically active men were exposed to 90 minutes of hypobaric hypoxia equivalent to 5500 m three times per week for three weeks. The body mass of participants in this study decreased only slightly, though significantly, from 67.1 kg to 65.9 kg. Moreover, Saeed et al., ([2012](#_ENREF_243)) examined the effect of IHE on exercise performance and skeletal muscle strength in patients with chronic heart failure. Patients were exposed to 10 sessions of hypoxia, each 3-4 hours over a period of 22 days. Starting altitude was 1500 m and was increased by 300 m with each subsequent session to a maximum altitude of 2700 m. Peak O2 consumption, exercise time, 6 minute walk test distance, skeletal muscle strength and quality of life scores all improved significantly after completion of altitude sessions, these improvements were sustained one month following conclusion of the sessions. However, it must be noted that both these research studies did not employ a matched control group for comparison and therefore it is unknown whether losses in body mass or improvements in health were due to hypoxia per se. One study which did use a control group was conducted by Workman & Basset ([2012](#_ENREF_297)) – their pilot study examined the post-metabolic response to passive normobaric hypoxia in overweight men. Findings revealed that following either one 3-hour session or seven 3-hour sessions in normobaric hypoxia (arterial O2 saturation; SPO2 80%) there was a shift in substrate utilisation towards lipid sources, which was not observed in the control group. These data suggest that normobaric hypoxia may be used as a new non-pharmacological strategy for the management of obesity, however further evidence is warranted.

Animal studies add support to the role of IHE in weight loss research. Ling et al., ([2008](#_ENREF_164)) exposed female mice to eight intermittent hypoxic exposures (3000 m) a day each of which lasted for 15 minutes and was separated by a five minute interval of normoxia, over 40 days. During the exposures mice were divided in to four groups; (1) normal diet, (2) normal diet and IHE, (3) high fat diet, and (4) high fat diet and IHE. The study found that those mice in Group 4 were the lightest at the end of the study and represented the lowest growth rate despite weighing the same at baseline. Blood sugar, cholesterol and leptin concentrations were also lower in Group 4 compared with those in Group 3, despite a greater food intake in these mice providing positive support for IHE. Martinez et al., ([2010](#_ENREF_182)) also examined the effect of IHE on body weight in mice, albeit at a greater altitude of 7500 m. Following 35 days of IHE or sham IHE, alternating 30 seconds of progressive hypoxia followed by 30 seconds normoxia for 8 hours each day, body weight was significantly reduced in those mice exposed to IHE. Similarly, a two month exposure to two protocols of hypoxia; (1) 24 hours in hypoxia alternated with 24 hours normoxia and, (2) 48 hours hypoxia alternated with 24 hours of normoxia, caused significant reductions in body weight gain in male Wistar rats compared with a control group ([Germack, et al., 2002](#_ENREF_102)). Chen et al., ([2010](#_ENREF_49)) also reported reductions in body mass in male Sprague-Dawley rats after eight weeks of mild intermittent hypoxia treatment (FIO2 0.14-0.15) eight hours per day, six days per week. Additionally plasma glucose and insulin levels in oral glucose tolerance test, and epididymal fat were significantly lower than the pair-matched control group. Although the above studies use prolonged exposures to IHE, the beneficial effects are apparent and research in human studies should explore its use in overall health improvement.

Since animal students have provided good evidence that IHE causes weight loss, it is thought that IHE may have an important role to play within weight loss programmes designed for human individuals. Moreover, as a limited number of human studies using IHT have provided evidence that the combination of exercise and hypoxia may be effective for weight loss ([Haufe, et al., 2008](#_ENREF_122); [Wiesner, et al., 2010](#_ENREF_295)), it is now necessary to establish if IHE alone is sufficient to cause weight loss prior to the inclusion of exercise training. If this method is proven to produce beneficial changes in body mass, it is a method that could be used prior to an exercise intervention to initiate weight loss. In the present study, a protocol which mimicked a typical exercise training programme was used, the aim of the study was to examine the effect of four weeks of IHE (hypoxic dose: 90 min·d-1, 3d·wk-1) on body mass and associated metabolic markers (i.e. blood pressure, aerobic capacity) in sedentary humans. It is hypothesised that the oxidative stress caused by hypoxia (5500 m) will significantly reduce body mass and improve overall health compared with normoxia.

## Methods

### Participants

Seven individuals participated in the study (2 men, 5 women; mean ± 1SD: age 29.3 ± 10.5 years; height 168.9 ± 10.3 cm; body mass 67.9 ± 11.3 kg; BMI 23.9 ± 4.2 kg·m-2). To investigate the effects of IHE a within subjects design was used, participants were therefore required to serve as their own controls following a 12-week wash-out period. Exclusion criteria were coronary heart disease, pulmonary disease, uncontrolled hypertension and poorly controlled diabetes mellitus. Individuals were also excluded if they had been above 1000 m in the six months preceding the study. Following routine medical screening participants were informed of all the procedures involved in the study, and all provided written, informed consent (at Appendix 2). The research conformed to the guidelines laid down in the Declaration of Helsinki (2008) and was approved by the University of Chichester Research Ethics Committee.

### Experimental Design

Participants were instructed to maintain their current physical activity levels and lifestyle throughout the study period. Prior to and following the IHE and control intervention periods anthropometric, metabolic and cardiovascular parameters were measured. During the IHE programme participants were exposed to 4 weeks of IHE (90 min·d-1, 3d·wk-1). During the control period participants were required to remain at SL (< 500 m) at all times[[23]](#footnote-23); participants underwent pre- and post-testing measures four weeks apart.

### Experimental Procedures

Participants reported to the laboratory in the morning (08:00 h) after an overnight fast. Height measured to the nearest 0.1 cm was recorded using a wall-mounted stadiometer (Holtain Ltd, Crymych, UK) and body mass to the nearest 0.05 kg was recorded using weight calibrated scales (Seca Model 873, Seca Ltd, UK). Body composition was measured using the skin-fold thickness technique ([Durnin & Womersley, 1974](#_ENREF_75)). All skin-fold thickness measurements were taken by the same investigator from identical positions for each participant, following anthropometric guidelines of the International Society for the Advancement of Kinanthropometry ([Marfell-Jones et al., 2006](#_ENREF_177)). Skin-fold thickness readings were taken at eight sites: bicep, tricep, subscapular, iliac crest, supraspinale, abdominal, front thigh, and mid-calf, using skin-fold callipers (Harpenden, Baty International, West Sussex, UK). All measures were taken in duplicate from the right side of the body with participants standing in a relaxed position. A third measure was only taken if the difference between the first two measurements was greater than 5% ([Marfell-Jones, et al., 2006](#_ENREF_177)). Following skin-fold measurements, values were entered into Equation 1 to determine gender and age specific[[24]](#footnote-24) body density ([Siri, 1956](#_ENREF_260)); resultant values were then entered into Equation 2 to quantify body fat percentage ([Durnin & Womersley, 1974](#_ENREF_75)).

Equation 1. Body density equation

Body density (men) = 1.1609-[0.0632·LOG (∑ skin-folds)]

Body density (women) = 1.1581-[0.072·LOG (∑ skin-folds)]

Equation 2. Body fat calculation

Body Fat Percentage = [(4.95/body density)-4.5]·100

Waist and hip circumferences were also measured, waist circumference was measured at the torso between the xiphoid process and the umbilicus ([Duncan et al., 2003](#_ENREF_74)), hip circumference was measured at the point yielding the maximum circumference over the buttocks ([Heitmann et al., 2004](#_ENREF_125)) using a flexible tape measure to the nearest 1 cm, both measures were used as an indicator of abdominal fat content.

Following an upright seated 20 minute rest period, O2 consumption and CO2 production were measured for a 10-min period by indirect calorimetry. Research suggests by collecting a 10 minute measurement and eliminating the first 5 minutes, the most accurate reading of resting metabolic rate (RMR) will be given ([Compher et al., 2006](#_ENREF_59)). Gas collection values collected were used to compute resting energy expenditure (REE) and respiratory exchange ratio (RER).

After a resting period of at least 30 min, following expired gas measurements, seated resting heart rate and blood pressure were measured using an automated sphygmomanometer (Omron 705 IT, Medisave, UK). Participants were required to place their hand on a flat surface, palm upward. A blood pressure cuff was then placed on each participant 1-2 cm above the elbow joint with the rubber tubing running down the centre of their arm, the tightness of the cuff was adjusted until firm. Once fitted measurements were taken in triplicate and a mean value was taken.

Finally, fasting venous blood samples were collected in 10 ml heparinized tubes from an antecubital vein for determination of high-density lipoprotein (HDL) and LDL. Samples were immediately chilled for 30 minutes, centrifuged for 15 minutes at 2500 revolutions per minute (rpm (Centurion Scientific, Stoughton, UK)), decanted, and frozen. All samples were stored at -80ºC for future analysis using ELISA kits (Abcam, Cambridge, UK). Haemoglobin and haematocrit were also measured via capillary blood samples taken from the finger-tip using a disposable lancet (HaemoLance+, Prospect Diagnostics, Derbyshire, UK). For the measurement of haemoglobin a finger-tip blood sample was collected directly from the finger by capillary action in to the hemocue microcuvettes and were subsequently analysed using the HemoCue 201+ (HemoCue, Derbyshire, UK). Samples for the measurement of haematocrit were collected in capillary tubes which were subsequently centrifuged at 2500 rpm (Centurion Scientific C2 Series, Centurion Scientific, Stoughton, UK) and analysed using a micro-haematocrit capillary tube reader (Hawksley and Son Ltd, West Sussex, UK).

### Exercise Testing

All exercise tests were performed at ambient room temperature (Tamb 19°C) with a RH equal to 50%. Aerobic exercise capacity was determined using a graded exercise test (GXT) on an electromagnetically braked cycle ergometer (Lode, Excalibur Sport, Cranlea and Co, Bourneville, UK). Each participant was tested at the same time in the morning and by the same investigator on all occasions (pre- and post 4-week IHE or control period). The GXT began at 50 W for two minutes, which was subsequently increased by 20 W for women and 25 W for men every minute thereafter ([Amann et al., 2006](#_ENREF_4)). Participants selected a cadence of between 70 and 90 rpm and maintain this throughout each GXT. Participants were verbally encouraged to give a maximal effort during every visit to the laboratory and were instructed to remain seated throughout the test. During the GXT heart rate, minute ventilation (E), and O2 uptake (O2) (Cosmed K4b2, Cosmed srl, Rome, Italy) were recorded continuously. Directly following the tests participants also gave their rating of perceived exertion (RPE) using the 6-20 point scale ([Borg, 1982](#_ENREF_30)).

Test termination occurred when participants met 3 of the following 4 criteria: (1) O2 reached a plateau (< 150 ml·min-1) despite increasing power output, (2) RER ≥ 1.05, (3) HRpeak ± 10% age predicted maximum, and (4) exhaustion ([Howley et al., 1995](#_ENREF_131)).

### Hypoxic Exposure Programme

Exposure sessions under hypoxia were conducted in a temperature controlled environmental chamber (TISS series 201003-1, TIS Services, UK). Normobaric hypoxia was achieved via a molecular sieve, enclosing microscopic pores which allow only small or mobile molecules to permeate through (i.e. O2, CO2, water vapour); the principle molecule unable to permeate is nitrogen. Compressed air is passed down the fibres and a restriction is applied in order to create pressure in the fibres. The greater restriction applied, the higher the pressure in the fibres leading to lower number of permeated O2 molecules. By varying the restriction the O2 level in the output stream can be varied from around 20% to less than 1%. Room O2 and CO2 concentration within the environmental chamber were continuously monitored by a sensor electrode throughout all exposure sessions.

Participants were assigned to a 4-week exposure programme; exposed 90 min·d-1, 3d·wk-1 to either control or hypoxia (FIO2 0.105; 5500 m) conditions. Participants remained seated throughout each 90 minute period. During exposure sessions SPO2 was continuously monitored using a portable pulse oximeter (Homecare Products, UK) and was recorded every five minutes.

### Statistical Analyses

Data were analysed using SPSS software (release 20.0; SPSS; Chicago, IL). Data were first tested for distribution normality and variance homogeneity using Kolmogorov Smirnov tests (P ≥ 0.05); following mathematical confirmation of a normal distribution parametric statistics were used for data analyses. The estimation of sample size was calculated using G\*Power software (release 3.1.2; Kiel, Germany), a-priori calculations resulted in a total of 8 participants required for the study. Post hoc calculations based on 7 participants results in a power of 0.92. The main outcome measures were body mass, body fat percentage, BMI, REE, RER, DBP, systolic blood pressure (SBP), HDL and LDL.

Two-way repeated measures (time x condition) ANOVA was used to determine if body mass, BMI, REE, RER, heart rate, blood pressure, HDL and LDL were significantly altered over the time course of the exposure programme. Appropriate adjustments to the degrees of freedom were made in cases where the assumptions of sphericity were violated. Effect sizes for ANOVA were calculated using the omega squared (Ω2) method and can be interpreted as small (< 0.06), medium (0.06-0.15) and large (> 0.15) ([Cohen, 1992](#_ENREF_58)). Bonferonni corrected paired t-tests were performed where ANOVA statistics were significant to examine pre- and post-intervention data. Effect size for t-test comparisons were calculated by converting a t-value into an r-value ([Field, 2005](#_ENREF_86); [Rosenthal, 1991](#_ENREF_239); [Rosenthal et al., 2000](#_ENREF_240)), and can be interpreted as small (0.10), medium (0.30), and large [0.50 ([Cohen, 1988](#_ENREF_57))]. All values are presented as mean ± 1SD and for statistical analyses an alpha of P ≤ 0.05 was considered significant unless otherwise stated.

## Results

### Body Composition

The mean body composition responses for both conditions are shown in Table 4.1. Body mass following the control exposure remained unchanged (pre 68.8 ± 12.5; post 68.8 ± 12.0 kg), this was also true following the hypoxic exposures (pre 67.9 ± 11.3; post 68.1 ± 11.6 kg). Thus, BMI was not significantly different pre to post for either condition. Body fat percentage assessed by skin-fold measurement’s also remained unchanged.

Table 4.. Body composition responses to intermittent hypoxic exposures.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Control | | IHE | |
|  | **Pre** | **Post** | **Pre** | **Post** |
| Body Mass (kg) | 68.8 ± 12.5 | 68.8 ± 12.0 | 67.9 ± 11.3 | 68.1 ± 11.6 |
| BMI (kg·m-2) | 24.4 ± 4.4 | 24.2 ± 4.4 | 23.9 ± 4.2 | 23.9 ± 4.3 |
| Body Fat (%) | 25.0 ± 10.4 | 25.6 ± 10.9 | 23.5 ± 10.1 | 23.7 ± 10.9 |
| Sum of 8 Skinfolds (mm) | 121 ± 59 | 122 ± 65 | 103 ± 47 | 103 ± 53 |
| Waist Circumference (cm) | 75 ± 13 | 75 ± 14 | 75 ± 11 | 74 ± 11 |
| Waist-Hip Ratio (WHR) | 0.76 ± 0.09 | 0.77 ± 0.10 | 0.76 ± 0.09 | 0.77 ± 0.09 |

### Fat and Glucose Metabolism

Metabolic responses for both conditions are displayed in Table 4.2. The RMR following the control exposure was reduced (pre 0.25 ± 0.04; post 0.20 ± 0.03); however the reduction was not significant (P ≥ 0.05). The RMR following the hypoxic exposures also showed a slight decrease (pre 0.29 ± 0.11; post 0.24 ± 0.05), but again this difference was not significant (P ≥ 0.05). Post control values however did show a trend towards a reduction [t(6) = 2.063, P = 0.08, r = 0.64], a result which was not observed following the hypoxic exposures. The circulating HDL, LDL, triglycerides (TG), TC and fasting glucose remained unchanged following both the control period and hypoxic exposures. Thus, the LDL/HDL ratio was unchanged, as was HDL/TC ratio.

Table 4.. Metabolic responses to intermittent hypoxic exposures.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Control | | IHE | |
|  | **Pre** | **Post** | **Pre** | **Post** |
| RMR (L·min-1) | 0.25 ± 0.04 | 0.20 ± 0.03 | 0.29 ± 0.11 | 0.24 ± 0.05 |
| REE (kcal·min-1) | 1.27 ± 0.22 | 1.02 ± 0.16 | 1.43 ± 0.55 | 1.18 ± 0.25 |
| RER | 0.83 ± 0.21 | 0.94 ± 0.13 | 0.81 ± 0.31 | 0.85 ± 0.20 |
| Fasting Blood Glucose (mmol) | 3.87 ± 0.29 | 3.93 ± 0.31 | 4.03 ± 0.33 | 3.95 ± 0.26 |
| HDL (mmol·L-1) | 1.6 ± 0.4 | 1.6 ± 0.3 | 1.6 ± 0.4 | 1.6 ± 0.3 |
| LDL (mmol·L-1) | 2.5 ± 0.7 | 2.5 ± 0.4 | 2.3 ± 0.3 | 2.3 ± 0.3 |
| TG (mmol·L-1) | 0.8 ± 0.3 | 0.9 ± 0.4 | 0.8 ± 0.4 | 0.7 ± 0.3 |
| TC (mmol·L-1)] | 4.5 ± 1.0 | 4.5 ± 0.7 | 4.2 ± 0.7 | 4.2 ± 0.5 |
| LDL/HDL ratio | 1.6 ± 0.4 | 1.6 ± 0.3 | 1.5 ± 0.3 | 1.5 ± 0.4 |
| TC/HDL ratio | 2.8 ± 0.4 | 2.9 ± 0.4 | 2.7 ± 0.4 | 2.7 ± 0.4 |
| HDL/TC ratio (%) | 36.1 ± 5.9 | 35.3 ± 4.3 | 37.3 ± 5.2 | 38.1 ± 6.8 |

### Cardiovascular Parameters

The cardio-respiratory responses for both conditions are displayed in Table 4.3. Systolic and diastolic blood pressure remained unchanged throughout the whole study period, as was the resting heart rate. The O2peak reported in absolute (L·min-1) and relative values (mL·kg-1·min-1) was not significantly different following either the control period or hypoxic exposures.

Table 4.. Cardio-respiratory responses to intermittent hypoxic exposures.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Control | | IHE | |
|  | **Pre** | **Post** | **Pre** | **Post** |
| SBP (mmHg) | 118 ± 12 | 119 ± 11 | 112 ± 15 | 114 ± 16 |
| DBP (mmHg) | 76 ± 8 | 76 ± 4 | 72 ± 8 | 73 ± 8 |
| Resting Heart Rate (b·min-1) | 64 ± 8 | 61 ± 9 | 58 ± 7 | 63 ± 7 |
| O2peak (mL·kg-1·min-1) | 41.55 ± 4.87 | 47.14 ± 10.92 | 48.14 ± 7.07 | 45.17 ± 11.52 |
| O2peak (L·min-1) | 2.85 ± 0.60 | 3.17 ± 0.64 | 3.28 ± 0.69 | 3.01 ± 0.71 |

**Note:** All participants met at least 3 of the 4 criteria for test termination on all occasions.

### Haemoglobin and Haematocrit

The pre-exposure haemoglobin values (normoxia 13.4 ± 2.7 g·dL-1, hypoxia 13.8 ± 1.6 g·dL-1) were no different in either of the conditions and both remained unchanged following either a control period or IHE (normoxia 13.9 ± 1.8 g·dL-1, hypoxia 13.1 ± 1.2 g·dL-1). Haematocrit values also remained unchanged, in normoxia values were 39 ± 5% (pre) and 40 ± 5% (post), and in hypoxia values were 46 ± 6% (pre) and 41 ± 3% (post).

## Discussion

The aim of the present study was to examine the effect of intermittent hypoxic exposures (90 min·d-1, 3 d·wk-1) on body composition and associated metabolic risk markers. The main findings show that IHE had no effect on body composition measures (body mass, body fat percentage, BMI, waist circumference, WHR), fat and glucose metabolism (RER, fasting glucose, LDL, HDL), cardiovascular health (DBP, SBP, resting heart rate) or aerobic capacity (O2peak) in this lean, healthy population.

A reduction in body mass with moderate- to high-altitude exposure is well documented, and the likely contributors to the weight loss observed include increased metabolic rate ([Lippl, et al., 2010](#_ENREF_165)), appetite suppression ([Tschop & Morrison, 2001](#_ENREF_274)), increased energy expenditure ([Armellini, et al., 1997](#_ENREF_10)), and reduced protein synthesis ([de Theije, et al., 2013](#_ENREF_68); [Rennie, et al., 1983](#_ENREF_226)). Although the effects of chronic exposure have been well explored, both in lean and obese individuals, the effects of IHE are less known and this concept requires greater investigation in controlled studies. Any positive effects of IHE on body composition would suggest that this method could be used as a non-pharmacological therapy for overweight and obesity without the addition of exercise.

A small number of research studies have demonstrated that IHE may have beneficial effects on body composition and metabolic health, still evidence is sparse. Rodríguez and colleagues (2000), using the same protocol as used within the present study (5500 m; 90min·d-1, 3d·wk-1), but in hypobaric hypoxia demonstrated a small but significant reduction in body mass (≈ 1.2 kg) in lean humans. In rodents, several studies have shown that intermittent hypoxia reduces body mass ([Chen, et al., 2010](#_ENREF_49); [Ling, et al., 2008](#_ENREF_164); [Martinez, et al., 2010](#_ENREF_182)). Together, these studies suggest that IHE could be used as a non-pharmacological therapy within weight control programmes and interventions. However, the majority of research has been conducted in rodents and its application to humans requires caution. The results of the present study do not confirm those observed within rodent studies (i.e. reduced body mass); however this could be due to protocol differences. Often experimental models in rodent studies involve multiple exposures to hypoxia, over several hours, on consecutive days, for a number of weeks, the practicality of this model is inappropriate for use in humans since it would be neither time- or cost-effective. Moreover, although the protocol used in the present study was the same as that used by others demonstrating a small reduction in body mass ([Rodriguez, et al., 2000](#_ENREF_237)), that study utilised hypobaric hypoxia as opposed to normobaric hypoxia. Topical evidence has suggested that there may be differences between the two modalities within several parameters such as, ventilatory responses, fluid balance and performance ([Faiss et al., 2013](#_ENREF_81); [Loeppky et al., 1997](#_ENREF_168); [Millet et al., 2013](#_ENREF_192); [Savourey et al., 2003](#_ENREF_248)). These differences, may therefore account for the discrepancies observed between the findings of the present study and those of Martinez and co-workers.

Dyslipidemia is a risk factor for obesity ([Anderson & Konz, 2001b](#_ENREF_8); [Brown, et al., 2000](#_ENREF_34); [Kopelman, 2000](#_ENREF_152)) and may be important in the relationship of BMI to increased risk of heart disease ([Bray, 2004](#_ENREF_33)). Important indicators in this relationship include HDL and LDL, both a low HDL and high LDL indicate a greater risk of developing cardiovascular problems ([Armstrong et al., 2006](#_ENREF_11)) and thus any improvements are of benefit to an individual. In the present study IHE had no effect on LDL or HDL which is consistent with previous literature reporting no changes in HDL or LDL following IHT ([Haufe, et al., 2008](#_ENREF_122); [Wiesner, et al., 2010](#_ENREF_295)). In addition, no change was seen in the ratio between TC and HDL which has also been shown to be an important predictor of heart disease risk ([Lemieux et al., 2001](#_ENREF_159)). It is possible that no change in the TC/HDL ratio value was observed as initial values were already within the normal range [between 3.5:1 and 5:1 ([Kinosian et al., 1994](#_ENREF_148))].

In an attempt to elucidate the mechanisms which may underpin the benefits of IHE, Workmann and Bassett ([2012](#_ENREF_297)) examined the post metabolic response to passive normobaric hypoxia. In this study they demonstrated that there was a shift in substrate utilisation towards lipid sources with a singular three hour exposure to hypoxia and following seven consecutive normobaric hypoxic exposures in overweight males (BMI ≈ 28 kg·m2). The present study observed no shift in substrate utilisation following the 12 normobaric hypoxic exposures, as demonstrated by respiratory exchange ratio, which may suggest that this response is only sustained short-term. Alternatively, Workmann and colleague may have observed an increase as a direct immediate response to hypoxic exposure since metabolic rate was attained immediately following the final exposure and thus their results could be linked to the increase in respiration rate often observed with acute exposures to hypoxia ([Armstrong, 2000](#_ENREF_12)).

Saeed et al., ([2012](#_ENREF_243)) reported improvements in O2peak and exercise time following 10 three to four hour hypoxic exposures over a 22 day period in patients presenting with chronic heart failure. These improvements were not replicated in the present study where no significant change was observed in SL O2peak values. It is possible that the changes observed by Saeed and colleagues were the result of very poor cardio-respiratory fitness prior to IHE, allowing for greater initial improvements in aerobic capacity. In support of the present study’s findings, no change in 6-minute walk test distance was reported following 14 days of exposure to continuous hypobaric hypoxia ([Lippl, et al., 2010](#_ENREF_165)). Moreover, Rodríguez et al., ([2000](#_ENREF_237)) also showed no significant improvement in cardio-respiratory fitness with intermittent hypobaric hypoxic exposures. They concluded that the short duration of the hypoxic programme may not have been sufficient to allow the cardio-respiratory and metabolic changes to become apparent, which could also explain the results of the present study since a similar protocol was adopted. Additionally, there was no difference between pre values for hematocrit or haemoglobin values providing evidence that the washout period was sufficient between the two trials. More importantly, there was no difference pre to post in either condition, which suggests that there was no shift in the O2 haemoglobin dissociation curve and thus no changes occurred in the O2 delivery system ([Lenfant et al., 1968](#_ENREF_160)). This result demonstrates that acclimatisation did not take place, a result, which for the purposes of the present work is a positive finding since acclimatisation/chronic exposure to hypoxia appears to have many adverse effects including a reduction in fat-free mass ([Boyer & Blume, 1984](#_ENREF_32); [MacDougall, et al., 1991](#_ENREF_171); [Magalhães & Ascensão, 2008](#_ENREF_174); [Rose, et al., 1988](#_ENREF_238)).

Long-term exposure to hypoxic training (≥ 3 weeks) has resulted in mixed findings, with one study reporting significant reductions in both SBP and DBP, two studies reporting increases in both SBP and DBP, and one reporting no change ([Mori et al., 1999](#_ENREF_197); [Siques et al., 2009](#_ENREF_259); [Wee & Climstein, 2013](#_ENREF_284); [Wiesner, et al., 2010](#_ENREF_295)). However, studies using IHE to examine this response are sparse but the evidence that is available suggests that IHE may increase blood pressure ([Fletcher, 2001](#_ENREF_88)), which would not be favourable in a study aiming to improve metabolic health. Consequently, blood pressure was measured pre- and post-IHE. No significant changes were observed in either SBP or DBP suggesting that in healthy lean individuals the use of IHE is safe and has no adverse effect on blood pressure. In support, Lippl et al., ([2010](#_ENREF_165)) provided evidence that DBP can in fact be reduced following one week at moderate-altitude in obese individuals, however whether this was a direct result of weight loss was not explored.

Overall, the use of IHE in healthy lean individuals has no significant metabolic health benefits. However, as no negative risks or adverse effects were identified during and following IHE, it is a method which should be explored further. Future research studies may examine different variations of exposure time, hypoxic insult (O2 fraction) and length of IHE programmes. Exploring the use of IHE programmes in overweight and obese individuals also remains an important area for research since chronic hypoxic exposures have obvious significant benefits. Moreover, greater losses in body mass with chronic hypoxia are often observed in those with higher starting body mass values ([Boyer & Blume, 1984](#_ENREF_32); [Ge, et al., 2010](#_ENREF_101); [Kayser, 1994](#_ENREF_142)), therefore caution should be exercised when interpreting the results of the present study which used lean individuals (BMI < 25 kg·m2).

In Chapter 5, the physiological response to IHE will be explored further. Appetite hormones have been put forward as a key mechanism which underlies the well documented reduction in body mass observed at high-altitude. Therefore, the response of leptin and adiponectin to IHE will be examined in order to identify if there is a link between weight loss and appetite using this method.

# Effect of Intermittent Normobaric Hypoxic Exposures on the Appetite Hormones Leptin and Adiponectin

## Introduction

It is recognised that chronic exposure to high-altitude hypoxia, as a cause or effect of weight loss, alters levels of circulating appetite hormones ([Chaiban, et al., 2008](#_ENREF_45); [Shukla, et al., 2005](#_ENREF_256); [Snyder et al., 2008b](#_ENREF_262)). Leptin and adiponectin are two appetite hormones secreted in white adipose tissue, which act antipodal to each other in the regulation of food intake ([Dridi & Taouis, 2009](#_ENREF_73); [Stanley, et al., 2005](#_ENREF_263)), that have been reported to be altered with hypoxic exposure ([Lippl, et al., 2010](#_ENREF_165); [Shukla, et al., 2005](#_ENREF_256); [Zaccaria et al., 2004](#_ENREF_300)). The primary role of leptin is to circulate to the brain to inhibit food intake ([Klok et al., 2007](#_ENREF_150); [Sierra-Johnson et al., 2008](#_ENREF_258); [Tschop, et al., 2000](#_ENREF_275)), whereas adiponectin circulates to promote feeding ([Stanley, et al., 2005](#_ENREF_263)), thus any interventions that increase leptin or decrease adiponectin could be important for weight management. Although prolonged exposures to hypoxia support this notion, research evidence examining the response of appetite hormones to IHE with or without the combination of exercise is sparse and warrants further exploration before its role as a non-pharmacological therapy in weight loss can be established.

The emerging concept of adipose tissue as an endocrine gland and the related effect of hypoxia on metabolic energy and body mass led researchers Chaiban, Bitar and Azar ([2008](#_ENREF_45)) to examine the effect of chronic continuous hypoxia on appetite hormones leptin and adiponectin in Sprague-Dawley rats ([Chaiban, et al., 2008](#_ENREF_45)). In their study rats were exposed from birth to either 2 or 8 weeks of continuous normobaric hypoxia (FIO2 0.10; 5800 m). After two weeks of hypoxia, there was no change in adiponectin and leptin levels, however following eight weeks of hypoxia adiponectin levels were significantly lowered and leptin levels relative to body weight were increased.

Shukla and colleagues (2005) examined the effect of chronic continuous hypobaric hypoxia on circulating leptin levels in humans. Initially 30 lowlanders were taken to an altitude of 3600 m for 48 hours, after which they ascended to 4300 m for 7 days. After 48 hours at 3600 m there was no significant difference in leptin levels. However, following 48 hours at 4300 m there was a significant increase in leptin levels, which remained elevated for a further 7 days following return to SL ([Shukla, et al., 2005](#_ENREF_256)). The response of leptin to hypobaric hypoxia was also examined by Lippl et al., (2010), who studied the effect of a 7-day stay at 2650 m on the body mass of 20 obese men. In order to understand the mechanisms involved, measurements of leptin were taken at SL and on days 1, 7, 14 and 42. It was reported that participants weighed significantly less on days 14 and 42, and that leptin levels increased at high-altitude. The studies by Shukla et al., (2005) and Lippl et al., (2010), demonstrate leptin increased with 7 days of hypobaric hypoxic exposure and these occurred regardless of the exposure altitude.

Snyder et al., (2008) examined plasma leptin levels in 25 healthy humans who ingested a control meal during normoxia and after 17 h of exposure to normobaric hypoxia (FIO2 0.125; 4100 m). Plasma leptin levels were assessed prior to the control meal, and after at 20 and 40 minutes post-meal. The researchers reported that hypoxia caused a significant elevation in plasma leptin levels 40 minutes post-meal, but no change pre-meal or 20 minutes post-meal. Moreover, there was a significant positive correlation between SPO2 and leptin after the 17 h exposure ([Snyder, et al., 2008b](#_ENREF_262)). These data confirm that leptin increases with hypoxic exposure in humans and may suggest that an earlier onset of satiation occurs with hypoxic exposure.

In contrast to the studies supporting the anorexic effect of leptin, others suggest that it may not be responsible for weight loss at high-altitude. One study supporting this suggestion examined the effect of a prolonged high-altitude exposure on circulating leptin levels and reported a 44% decrease from 1.88 to 1.06 ng·mL-1 ([Zaccaria, et al., 2004](#_ENREF_300)). Blood samples were taken from twelve men at SL, at arrival at 5050 m and following 12-16 days at altitude. In this study leptin levels were significantly suppressed throughout the stay, and this response was correlated with a decrease in body mass. It was concluded by the authors that the changes were linked to hormonal and energy balance variations suggesting that leptin is involved in the endocrine and metabolic adaptations that occur during exposure to high-altitude. A study reporting no change in leptin levels, was conducted by Barnholt and co-workers (2006), they examined the effect of acute and chronic exposure to hypoxia (4300 m) on the modulating effects of caloric restriction ([Barnholt, et al., 2006](#_ENREF_19)). Twenty-six men were divided in to three groups and studied over a 3-week period. Group 1 stayed at SL and received a calorie controlled diet to maintain body mass, whereas groups 2 and 3 were deployed to 4300 m for 21 days. Group 2 was fed adequately to maintain body weight and Group 3 had calorie controlled diet to match Group 1. The SL group experienced an expected calorie restricted induced reduction in leptin. However, despite a significant loss in body mass, adiponectin and leptin did not change from baseline in groups 2 or 3 regardless of energy intake. Therefore, in this study a change in appetite hormones did not account for the weight loss observed.

Although studies examining the effect of continuous chronic hypoxia on leptin and adiponectin are available, studies examining intermittent hypoxic exposure without exercise in humans are sparse. However, animal and cell culture studies have been carried out and their findings are promising, suggesting that adiponectin levels are decreased ([Magalang, et al., 2009](#_ENREF_173); [Zhang, et al., 2010](#_ENREF_302)) and leptin levels increased with IHE ([Polotsky et al., 2003](#_ENREF_215); [Qin, et al., 2007](#_ENREF_221)).

Whilst the combination of exercise and hypoxia is an attractive approach for enhancing weight loss it must first be established how hypoxia per se affects appetite hormones and in turn weight loss. If hypoxia alone was effective it could be used as a preliminary method for weight loss prior to the inclusion of exercise in overweight and obese individuals. Therefore, the aim of the present study was to determine the response of leptin and adiponectin to a 4-week IHE programme (90 mins·d-1, 3 d·wk-1) in humans. It is important to note here that the rationale underlying the chosen exposure time was to mimic the typical duration and frequency of exercise sessions that may be used in a weight reduction intervention programme. It was hypothesised based upon previous findings that four weeks of exposure to intermittent hypoxia would increase leptin and decrease adiponectin levels which may subsequently cause a reduction in body mass.

## Method

### Participants

Seven individuals (2 men, 5 women; mean ± 1SD: age 29.3 ± 10.5 years; height 168.9 ± 10.3 cm; body mass 67.9 ± 11.3 kg; BMI 23.9 ± 4.2 kg·m-2) participated in the study. To investigate the effects of IHE a within subjects design was used, participants were therefore required to serve as their own controls following a 12-week wash-out period. Exclusion criteria were coronary heart disease, pulmonary disease, uncontrolled hypertension and poorly controlled diabetes mellitus. Individuals were also excluded if they had been above 1000 m in the six months preceding the study. Following routine medical screening participants were informed of all the procedures involved in the study, and all provided written, informed consent (at Appendix 2). The research conformed to the guidelines laid down in the Declaration of Helsinki (2008) and was approved by the University of Chichester Research Ethics Committee.

### Experimental Design

Participants were instructed to maintain their current physical activity levels and lifestyle throughout the research study period. Prior to and post IHE and control intervention periods anthropometric parameters as well as serum leptin and adiponectin levels were measured. During the IHE programme participants were exposed to 4 weeks of IHE (90 min·d-1, 3d·wk-1). During the control period participants were required to remain at SL (< 500 m) at all times; participants underwent pre- and post-testing measures four weeks apart.

### Experimental Procedures

Participants reported to the laboratory in the morning (08:00 h) after an overnight fast. Height measured to the nearest 0.1 cm was recorded using a wall-mounted stadiometer (Holtain Ltd, Crymych, UK) and body mass to the nearest 0.05 kg was recorded using weight calibrated scales (Seca Model 873, Seca Ltd, UK). Body composition was measured using the skin-fold thickness technique (Durnin & Wormersley, 1974). All skin-fold thickness measurements were taken by the same investigator from identical positions for each participant, following anthropometric guidelines of the International Society for the Advancement of Kinanthropometry (Marfell-Jones, Olds, Stewart & Carter, 2006). Skin-fold thickness readings were taken at eight sites: bicep, tricep, subscapular, iliac crest, supraspinale, abdominal, front thigh, and mid-calf, using skin-fold callipers (Harpenden, Baty International, West Sussex, UK). All measures were taken in duplicate from the right side of the body with participants standing in a relaxed position. A third measure was taken only if the difference between the first two measurements was greater than 5% ([Marfell-Jones, et al., 2006](#_ENREF_177)). Following skin-fold measurements, values were entered into Equation 1 to determine gender and age specific[[25]](#footnote-25) body density ([Siri, 1956](#_ENREF_260)), resultant values were then entered into Equation 2 to quantify body fat percentage ([Durnin & Womersley, 1974](#_ENREF_75)).

Equation 1. Body density equation

Body density (men) = 1.1609-[0.0632·LOG (∑ skin-folds)]

Body density (women) = 1.1581-[0.072·LOG (∑ skin-folds)]

Equation 2. Body fat calculation

Body Fat Percentage = [(4.95/body density)-4.5]·100

After a resting period of at least one hour, fasting venous blood samples were collected in chilled 10 ml heparinized tubes from an antecubital vein for determination of serum adiponectin and leptin. Samples were immediately chilled for 30 minutes, centrifuged for 15 minutes at 2500 rpm, decanted, and frozen. All samples were stored at -80ºC for future analysis using ELISA kits (Leptin, IBL International, Hamburg, Germany; Adiponectin, Biovendor, Laboratorní medicína a.s).

It must be noted that during venous blood sample collections, complications arose which resulted in two participants not providing samples, therefore those data presented are full sets for 5 individuals.

### Hypoxic Exposure Programme

Exposure sessions under normoxia and hypoxia were conducted in a temperature controlled environmental chamber (TISS series 201003-1, TIS Services, UK). Normobaric hypoxia was achieved via a molecular sieve, enclosing microscopic pores which allow only small or mobile molecules to permeate through (i.e. O2, CO2, water vapour); the principle molecule unable to permeate is nitrogen. Compressed air is passed down the fibres and a restriction is applied in order to create pressure in the fibres. The greater restriction applied, the higher the pressure in the fibres leading to lower number of permeated O2 molecules. By varying the restriction the O2 level in the output stream can be varied from around 20% to less than 1%. Room O2 and CO2 concentration within the environmental chamber were continuously monitored by a sensor electrode throughout all exposure sessions.

Participants were assigned to a 4-week exposure programme; exposed 90 min·d-1, 3d·wk-1 to either control (normoxia FIO2 0.209) or hypoxia (FIO2 0.105) conditions. Participants remained seated throughout each 90 minute period. During exposure sessions SPO2 was continuously monitored using a portable pulse oximeter (Homecare Products, UK) and recorded every five minutes.

### Statistical Analyses

Data were analysed using SPSS software (release 20; SPSS; Chicago, USA). Data were first tested for distribution normality and variance homogeneity using Kolmogorov Smirnov tests (P ≥ 0.05), following mathematical confirmation of a normal distribution parametric statistics were used for data analyses. The estimation of sample size was calculated using G\*Power software (release 3.1.2; Kiel, Germany), a-priori calculations resulted in a total of 8 participants required for the study. Post hoc calculations based on 5 participants results in a power of 0.67.

Two-way repeated measures (time x condition) ANOVA were used to determine if leptin and adiponectin were significantly altered over the time course of the exposure programme. Appropriate adjustments to the degrees of freedom were made in cases where the assumptions of sphericity were violated. Effect sizes were calculated using the Ω2 method and can be interpreted as small (< 0.06), medium (0.06-0.15) and large (> 0.15) ([Cohen, 1992](#_ENREF_58)). All values are presented as mean ± 1SD and for statistical analyses an alpha of P ≤ 0.05 was considered significant unless otherwise stated.

## Results

### Body Composition

All 7 participants completed the entire study protocol. Body mass and thus BMI were unchanged from baseline values following the control period and the IHE programme (Table 5.1). Also body fat percentage, as assessed by skin-fold measurements, did not significantly change over-time or between the two conditions.

Table 5.. Anthropometric measures.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Control | | IHE | |
|  | **Pre** | **Post** | **Pre** | **Post** |
| Body Mass (kg) | 68.8 ± 12.5 | 68.8 ± 12.0 | 67.9 ± 11.3 | 68.1 ± 11.6 |
| BMI (kg·m2) | 24.4 ± 4.4 | 24.2 ± 4.4 | 23.9 ± 4.2 | 23.9 ± 4.3 |
| Body Fat (%) | 25.0 ± 10.4 | 25.6 ± 10.9 | 23.5 ± 10.1 | 23.7 ± 10.9 |
| Sum of 8 Skin Folds (mm) | 121 ± 59 | 122 ± 65 | 103 ± 47 | 103 ± 53 |

### Leptin

The mean leptin response for both conditions is shown in Figure 5.1, with the individual responses shown in Figure 5.2. Leptin levels following the control exposure remained unchanged (pre 4.9 ± 6.4; post 5.2 ± 7.0 ng·mL-1), therefore the difference was not significant (P ≥ 0.05). In contrast, following the hypoxic exposures leptin levels showed a slight decrease (pre 5.6 ± 7.7; post 4.4 ± 5.0 ng·mL-1), but again this difference was not significant (P ≥ 0.05).

|  |
| --- |
| Figure 5.1. Leptin values following intermittent hypoxic exposures (IHE and control (n=5).  **B**  Figure 5.2. Individual responses of leptin to (A) control and (B) intermittent hypoxic exposures (n=5). |

### Adiponectin

**A**

The mean adiponectin response for both conditions is shown in Figure 5.3, with the individual responses shown in Figure 5.4. Adiponectin levels following the control exposure decreased (pre 8.3 ± 3.9; post 6.0 ± 2.9 µg·mL-1), however the difference was not significant. In contrast, following the hypoxic exposures adiponectin levels showed a slight increase (pre 7.9 ± 3.0; post 9.2 ± 2.7 µg·mL-1), but again this difference was not significant.

|  |
| --- |
| Figure 5.3. Adiponectin values following intermittent hypoxic exposures (IHE) and control (n=5).  **A**  **B**  Figure 5.4. Individual responses of adiponectin to (A) control and (B) intermittent hypoxic exposures (n=5). |

### Arterial Oxygen saturation

**B**

Mean SPO2 for the hypoxic exposure sessions are displayed in Table 5.2. The SPO2 remained stable throughout the study period, and no significant differences were found between the weeks of exposure. Individual SPO2 data are in Appendix 8.

Table 5.. Arterial oxygen saturation during intermittent hypoxic expsoures.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| SPO2 (%) | | | | |
| Week 1 | **Week 2** | **Week 3** | **Week 4** | **Mean** |
| 75 ± 3 | 77 ± 3 | 77 ± 1 | 77 ± 2 | 76 ±1 |

## Discussion

The aim of the present investigation was to examine the effect of a 4-week intermittent hypoxic exposure programme on the two appetite hormones, leptin and adiponectin, which are secreted in white adipose tissue. It was hypothesised that IHE would cause a significant increase in leptin and decrease in adiponectin, however the results demonstrated that IHE had no effect on leptin or adiponectin values; these results also coincided with no change in body mass, BMI or percentage of body fat in lean individuals. Upon visual inspection, it was identified that the leptin values of participant 4 differed in response to the results of the other participants (Figure 5.2). Adiponectin values were mainly uniform, the response of participants 3 and 5 demonstrated a small decrease following the control period, but no changes were observed following IHE (Figure 5.4).

Previous research suggests that with continuous chronic hypoxic exposure there is a significant increase in leptin levels, which often coincides with reports of a change in appetite and subsequent losses in body mass ([Shukla, et al., 2005](#_ENREF_256); [Tschop, et al., 2000](#_ENREF_275)). However, the response of leptin to IHE with or without exercise training is substantially understudied in humans. Animal studies examining the response of leptin to IHE however have provided evidence for this approach to weight loss. Two studies examining the role of intermittent hypoxia in mice, albeit using different protocols, observed significant increases in leptin levels. Moreover both studies showed that the increase in leptin levels were concomitant with significant reductions in body mass ([Polotsky, et al., 2003](#_ENREF_215); [Qin, et al., 2007](#_ENREF_221)). The mediated mechanism underlying this response to intermittent hypoxia is thought to be similar to that observed with chronic hypoxia, that leptin gene expression increases via HIF-1α ([Ambrosini et al., 2002](#_ENREF_5)), which is mediated by HIF-1 through a HIF-1 consensus binding site ([Grosfeld et al., 2002](#_ENREF_115)).

In the present study, serum leptin levels remained unchanged following four weeks of IHE (90min·d-1, 3d·wk-1). This result is similar to that shown by an intermittent hypoxic exercise training study (2740 m), in which no changes in leptin or body mass were also observed ([Haufe, et al., 2008](#_ENREF_122)). It is possible that the exposure time or the length of programme was not sufficient enough to disturb homeostasis despite a considerable reduction in SPO2 (76 ± 1%); it is generally accepted that tissue hypoxia develops when O2 saturation drops below 90% ([Urdampilleta, et al., 2012](#_ENREF_276)). However, in two participants, leptin levels were reduced following IHE; the participant with the greatest reduction had a higher BMI (33.2 kg·m2) than the other participants (mean 22.3 kg·m2), however this was not the case for the other participant. In support of this observation, it has been concluded by others that adipose tissue oxygenation is altered in obesity, so such observations on leptin metabolism in lean individuals cannot simply be extrapolated to obese individuals ([Haufe, et al., 2008](#_ENREF_122); [Wiesner, et al., 2010](#_ENREF_295)). Therefore, the response of leptin to intermittent hypoxia without exercise training in an obese population warrants further investigation.

A potential explanation for disparity with previous research findings using animal models is the hypoxic dose. Unlike the research conducted in rodents, the participants in the present study were not exposed to intermittent hypoxia on a daily basis and each hypoxic exposure totalled a maximum of 90 minutes, a substantially shorter exposure time than those used in animal studies. Thus, as the hypoxic stimulus (0.105 FIO2) was similar to that reported within the literature it is thought that hypoxic exposure time is the most important component in altering levels of leptin. Future work should explore different hypoxic doses by manipulating hypoxic exposure times and altering the number of exposures per week. However, it is important not to overlook the rationale underlying the chosen exposure time which was to mimic the typical duration and frequency of exercise sessions that may be used in a weight reduction intervention programme ([Armstrong, et al., 2006](#_ENREF_11); [McQueen, 2009](#_ENREF_189)).

Adiponectin is decreased with continuous hypoxic exposure ([Chaiban, et al., 2008](#_ENREF_45)), however what is still unclear is the response of adiponectin levels to intermittent hypoxia in humans and the implications it may have for weight loss management. Animal studies have demonstrated using models of obstructive sleep apnoea in rodents that adiponectin is reduced following intermittent hypoxic exposures compared with matched controls ([Magalang, et al., 2009](#_ENREF_173); [Zhang, et al., 2010](#_ENREF_302)). It is reported within the literature that the effects of adiponectin are mediated through PPAR-γ activation ([Dridi & Taouis, 2009](#_ENREF_73)) and hypoxia is known to target PPAR-γ subsequent to the activation of HIF-1 ([Maffei, et al., 1995](#_ENREF_172)). Therefore, amplified activation of PPAR-γ with hypoxia may further mediate adiponectin leading to a reduction in expressed levels.

Adiponectin levels in the present study remained unchanged following 4 weeks of IHE. Haufe and colleagues (2008) also reported no change in adiponectin levels following 4 weeks of IHT (60 min·d-1, 3d·wk-1). However, since animal studies reported decreased levels following IHE at rest, the present results may suggest that there may be species-specific variation of adiponectin. In support of this, adiponectin is secreted at lower levels in the cerebrospinal fluid of rodents compared with humans and adiponectin in humans is not thought to cross the blood-brain barrier as observed in rodents ([Dridi & Taouis, 2009](#_ENREF_73)).

The prescribed hypoxic dose is an important consideration in the design of IHE programmes. The use of SPO2 as an indicator of the hypoxic stimulus is an easy and replicable way of assessing and monitoring the impact hypoxia has on each individual. Monitoring of the individuals physiological parameters during hypoxic exposures allows us to avoid the undesirable effects of overdosing ([Urdampilleta, et al., 2012](#_ENREF_276)) and also allows the hypoxic environment to be altered as participants become accustomed. Moreover, the monitoring and reporting of SPO2 allows comparisons to be made between studies. Urdampiletta and colleagues ([2012](#_ENREF_276)) also propose that future studies calculate the hypoxic stimulus charge (HSC = SPO2 \* time in minutes) to provide an objective measure of the hypoxic stress delivered during each session as it compensates for individual variability in scientific studies. Even more recently, Wee and Climstein ([2013](#_ENREF_284)) concluded that although the method proposed by Urdampiletta and colleagues is useful for identifying the level of hypoxic stress delivered in IHE studies, it fails to account for exercise intensity and would therefore not be a good representation of hypoxic stimulus charge in IHT studies. As a result they have proposed that hypoxic load equals average percent heart rate max divided by SPO2 multiplied by time in minutes (hypoxic load = avg % HRmax/SPO2% \* time in minutes) to ensure a consistent relative hypoxic stimulus is achieved. Although, the use of HRmax/peak is controversial in hypoxia, the exercise intensity parameter of this equation could be altered to represent ventilatory threshold.

Sample size in the present study was relatively small, unfortunately despite seven individuals participating in the experiment, due to unforeseen circumstances, only five had a complete set of blood samples due to fainting during sampling and thus this should be considered in the interpretation of the results. However, the individual responses presented in Figure 5.2 demonstrates that leptin remained unchanged in three of the five participants in both conditions which suggests that the response of leptin to IHE is uniform; only in twoparticipants was a reduction observed with IHE. As discussed previously, it is possible that the reduction in leptin in this individual was linked to a higher BMI which further denotes the reason for taking caution when extrapolating data collected from lean individuals to overweight or obese individuals ([Haufe, et al., 2008](#_ENREF_122); [Wiesner, et al., 2010](#_ENREF_295)). The individual responses for adiponectin levels in response to IHE were also uniform, in all participants adiponectin was slightly elevated or remained unchanged. However, following the control period the responses were varied, in two participants adiponectin was reduced, it can only be assumed therefore that these participants did not follow pre-experimental procedures (e.g. overnight fast) in this instance.

Although research demonstrates that continuous exposure to hypoxia increases levels of leptin and decreases adiponectin, the response of these two appetite hormones to intermittent hypoxia in humans is still under debate. The results of the present study suggest that a 4-week IHE programme results in no changes to these important regulators of appetite, which may be due to the combination of large inter-subject variation and the limited sample size. Concomitant with these results no changes in body mass were observed. As the hypoxic stimulus (0.105 FIO2) was similar to that of previous research, exposure time appears to play a significant role in the effects of intermittent hypoxic exposure on appetite and weight loss. Future research should alter the hypoxic dose (i.e. exposure times, days and weeks) in order to identify the optimum protocol for weight loss interventions before combining exercise with hypoxia.

As IHE appeared to have no beneficial effects on body mass or associated health risk markers, it is thought that the results from previous work using IHT ([Haufe, et al., 2008](#_ENREF_122); [Wiesner, et al., 2010](#_ENREF_295)) are a result of hypoxia and exercise combined and not hypoxia per se. However, the determination of exercise intensity in hypoxia presents difficulties due to the hypoxia-induced decrement in aerobic capacity, thus requiring maximal exercise tests to be performed. Often within overweight or obese populations, maximal exercise is contraindicated and therefore an effective method which does not require such methods to determine exercise intensity in hypoxia is required. In chapters 6 and 7, the use of HRpeak and the ventilatory threshold (VT) as methods to determine exercise intensity in normobaric hypoxia will be examined.

# Measurement of Peak Heart Rate to Determine Exercise Intensity in Normobaric Hypoxia

## Introduction

In the last decade intermittent hypoxic exercise training has become increasingly popular among athletes, with the aim to improve SL performance ([Millet, et al., 2010](#_ENREF_193)). However, this method has also been employed within sedentary populations with the aim of improving health ([Bailey, et al., 2000a](#_ENREF_16); [Haufe, et al., 2008](#_ENREF_122); [Netzer, et al., 2008](#_ENREF_202)). An incentive for exposing individuals to hypoxic conditions intermittently rather than for a prolonged period stems from the observation that permanent exposure to severe hypoxia (i.e. 5000m and higher) leads to a considerable deterioration of skeletal muscle tissue ([Hoppeler, et al., 2008](#_ENREF_127)). Furthermore, acute exposure to hypoxic conditions, such as that experienced during IHT or IHE have been shown to serve as supplementary training stimuli for eliciting a compensatory adaptation within the human body ([Cheung, 2010](#_ENREF_52)). At sub-maximal exercise intensities this additional training stimulus is demonstrated by alterations in heart rate, cardiac output, respiratory rate, and net O2 delivery ([Mazzeo, 2008](#_ENREF_185)). Although a hypoxia-induced decrement in aerobic capacity is incurred at altitude, reducing the intensity of exercise that can be performed, research has demonstrated that the same cardiovascular benefits are received ([Cerretelli, 1980](#_ENREF_44); [Friedmann, et al., 2005](#_ENREF_94)). Consequently, IHT appears a viable method of optimising training such that certain individuals (i.e. obese) receive a maximal metabolic and cardiovascular benefit whilst minimising injury risk through a reduction in exercise intensity ([Haufe, et al., 2008](#_ENREF_122)). Moreover, combining exercise training with hypoxia may lead to greater losses in body mass than exercise training at SL ([Quintero, et al., 2010](#_ENREF_223); [Urdampilleta, et al., 2012](#_ENREF_276)).

Consideration of the appropriate training load (intensity, duration, and frequency) is important in a population whose principle objectives are long-term weight loss and its maintenance ([Franckowiak et al., 2011](#_ENREF_92)). Exercise intensity is the primary principle which is manipulated within training programmes, as it can be easily monitored and adjusted to allow for improvements in fitness/health to ensure that progression is maintained, satisfying another principle of training ([Bompa & Haff, 2009](#_ENREF_27)). In hypoxia, the prescription of exercise intensity requires considerable attention due to the reduction in aerobic capacity for a given exercise intensity in hypoxia which is often reported within the literature ([Friedmann, et al., 2005](#_ENREF_94)). It is common for training intensities at SL to be prescribed using a percentage of O2peak or HRpeak, but since both these methods require graded exercise testing to exhaustion, researchers often use age-derived equations to predict peak heart rate (PHRpeak) ([Robergs & Landwehr, 2002](#_ENREF_234)), in specific populations, particularly for those in which maximal exercise is contraindicated — Such equations have been reviewed extensively at SL ([Robergs & Landwehr, 2002](#_ENREF_234)), whilst concluding that there was no acceptable method to estimate maximal heart rate, they suggested that a prediction error of <3 b·min-1 was acceptable for prescribing exercise training intensities. Moreover, due to the decline in aerobic performance capacity with hypoxia, employing these methodologies could be problematic as HRpeak may too be depressed with acute hypoxic exposure. If present such overestimations could lead to the prescription of exercise intensities which are unachievable which may result in decreased adherence to exercise training sessions ([Perri et al., 2002](#_ENREF_192)) and no training effect.

The reduction in measured HRpeak with chronic hypoxic exposure has been consistently observed ([Christensen & Forbes, 1937](#_ENREF_54); [Pugh, 1964](#_ENREF_219); [Reeves et al., 1987](#_ENREF_225); [Richalet et al., 1988](#_ENREF_229)). Contrary to these findings, the reduction in HRpeak with acute exposure to hypoxia is less clear. Several studies have shown that HRpeak changes little or not at all during acute hypoxic exposure ([Lawler et al., 1988a](#_ENREF_155); [Stenberg et al., 1966](#_ENREF_264)) but for others, a significant decrease in HRpeak was observed at and above 3800 m ([Benoit et al., 2003](#_ENREF_23); [Lundby et al., 2001](#_ENREF_170); [Roach et al., 1996](#_ENREF_231)). Discrepancies in findings between studies may be due solely to the altitude used; in general those reporting a decline in HRpeak have used simulated altitudes above 3800 m, whilst those observing no change used lower altitudes ([Benoit, et al., 2003](#_ENREF_23)).

A lowered HRpeak with chronic hypoxia is linked to the severity and the duration of hypoxemia and also contributes to the decrease in O2peak, yet again the situation is less clear in acute hypoxia ([Benoit, et al., 2003](#_ENREF_23)). Many authors have speculated over the cause of the observed decrease in HRpeak with acute hypoxia and direct and indirect evidence points to an activation of the adrenergic system ([Favret & Richalet, 2007](#_ENREF_83)). Plasma and urine norepinephrine concentrations have been found to be elevated in most studies performed in acute hypoxia, at rest or at any given exercise intensity ([Mazzeo et al., 1991](#_ENREF_186); [Richalet et al., 1990](#_ENREF_230)). In addition the direct measurement of the activity of adrenergic fibres has also been linked to an increase in sympathetic activity in response to hypoxia ([Seals et al., 1991](#_ENREF_251)). Collectively these findings provide a strong rationale for the role of the adrenergic system in the reduction of HRpeak in response to acute hypoxia. Alternatively, other research has reported an increase in parasympathetic trafficking with acute hypoxia resulting in a blunting of the cardiac chronotropic function ([Lundby, et al., 2001](#_ENREF_170); [Mollard et al., 2007](#_ENREF_195)). Despite the rationale presented, a single mechanism responsible for the reduction of HRpeak in response to acute hypoxia is yet to be elucidated.

The widespread use of heart rate to prescribe and monitor training intensities means that any reduction in HRpeak could have important implications for altitude training as well as the control of scientific studies. Several studies, which have investigated the effects of altitude training on SL performance, required athletes to perform training in hypoxia at the same relative heart rate at which they or a control group trained in normoxia, suggesting that the authors assumed that exercise intensities would be the same ([Bailey et al., 1998](#_ENREF_18); [Burtscher et al., 1996](#_ENREF_36)). However, Friedmann and colleagues (2005) propose that this may have negative consequences as, if heart rate at the same relative exercise intensities is reduced in hypoxia compared to normoxia the hypoxic groups in these studies would have exercised with higher intensities than the SL control groups which could lead to overreaching or the setting of unachievable goals ([Perri, et al., 2002](#_ENREF_214)). Therefore, the use of age-derived equations in the prediction of HRpeak, may in turn be inappropriate for use in hypoxic exercise training studies as a result of decreased HRpeak with acute hypoxic exposure.

Predicted HRpeak is an important tool for the prescription of exercise intensity for groups in which maximal exercise intensity is contraindicated. Therefore, it is important to establish whether HRpeak is reduced during acute exposure to a hypoxic environment. The aims of the present study were to assess in acute hypoxic conditions if compared to SL, 1) there was a decrease in measured HRpeak with decreasing O2 concentrations, and 2) if selected age-derived equations provide a sufficiently accurate prediction of HRpeak for prescribing exercise intensity within hypoxic exercise training programmes. It was hypothesised that there would be no reduction in HRpeak below 3000 m, as a result of this it was expected that age-derived equations used to predict HRpeak would not accurately estimate HRpeak below 3000 m. Above 3000 m, it was hypothesised that HRpeak would be reduced and that PHRpeak equations would over-estimate HRpeak

## Method

### Participants

Fifteen healthy volunteers (7 women and 8 men; mean ± 1SD: age 22 ± 2 years; height 176.4 ± 10.2 cm; body mass 72.8 ± 14 kg; body fat 23.1 ± 8.1%; O2peak 46.3 ± 7.0 mL·kg-1·min-1) participated in the study. All participants completed a medical history questionnaire (at Appendix 1), stating no contraindications to exhaustive exercise. All were SL residents and had not been above 1000 m in the six months preceding the study. After receiving both written and oral information on the experimental protocol and procedures, participants gave their written informed consent (at Appendix 2). The research conformed to the guidelines laid down in the Declaration of Helsinki (2008) and was approved by the University of Chichester Research Ethics Committee.

### Experimental Design

Each participant visited the laboratory on 6 occasions, including one familiarisation session, with each visit separated by a minimum of 48 hours. To standardise the tests, all participants were instructed not to partake in any vigorous physical training 24 hours prior to each session or consume caffeinated beverages and food for a two-hour period prior to all sessions.

### Experimental Procedures

Following familiarisation, on Visit 2, height measured to the nearest 0.1 cm (Holtain Ltd, Crymych, UK), body mass measured to the nearest 0.05 kg (BC 418 MA, Tanita Ltd, UK), and body composition were measured and recorded (Table 6.1). Body mass index was calculated as measured body mass divided by the square of measured height. All anthropometric measures were repeated on visits 4 and 6 to monitor any changes in body composition over the time course of the study; no significant differences were observed therefore the values from the first visit are reported.

Body composition was assessed using skin-folds taken at four skin-fold sites ([Durnin & Womersley, 1974](#_ENREF_75)); bicep, tricep, subscapular and iliac crest, using standard skin-fold callipers (Harpenden Skin-fold Callipers, Baty International, UK). All skin-fold measures were measured and recorded in duplicate from the right side of the body with participants standing in a relaxed position. A third measure was taken only if the difference between the first two measurements was greater than 5% (Marfell-Jones, Olds, Stewart & Carter, 2006). Following skin fold measurements, values were entered into Equation 1 to determine gender and age specific[[26]](#footnote-26) body density ([Siri, 1956](#_ENREF_260)), resultant values were then entered into Equation 2 to quantify body fat percentage ([Durnin & Womersley, 1974](#_ENREF_75)).

Equation 1. Body density equation

Body density (men) = 1.1609-[0.0632·LOG (∑ skin-folds)]

Body density (women) = 1.1581-[0.072·LOG (∑ skin-folds)]

Equation 2. Body fat calculation

Body Fat Percentage = [(4.95/body density)-4.5]·100

Table 6.. Anthropometric characteristics.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **BMI**  **(kg·m2)** | **Bicep**  **(mm)** | **Tricep**  **(mm)** | **Subscapular**  **(mm)** | **Iliac Crest**  **(mm)** | **Sum of 4 Skin-folds** | **Body Fat (%)** |
| 23.2 ± 2.7 | 9.4 ± 5.4 | 15.7 ± 6.0 | 12.1 ± 4.7 | 13.2 ± 6.4 | 50.4 ± 16.9 | 23.1 ± 8.1 |

### Exercise Testing

All exercise tests were performed in an environmental chamber (TISS series 201003-1, TIS Services, UK), where normobaric hypoxia was achieved via a molecular sieve, enclosing microscopic pores which allow only small or mobile molecules to permeate through (i.e. O2, CO2, water vapour); the principle molecule unable to permeate is nitrogen. Compressed air is passed down the fibres and a restriction is applied in order to create pressure in the fibres. The greater restriction applied, the higher the pressure in the fibres leading to lower number of permeated O2 molecules. By varying the restriction the O2 level in the output stream can be varied from around 20% to less than 1%. Room O2 and CO2 concentration within the environmental chamber were continuously monitored by a sensor electrode throughout all exposure sessions. The tamb, RH and air velocity (v) were controlled for in all sessions (tamb 21.3 ± 1.7°C; RH 50 ± 2%; v 0.00 ± 0.00 m∙s-1). For all tests completed the ambient inspiratory O2 fractions (FIO2) were 0.209, 0.185, 0.165, 0.142 and 0.125 corresponding to SL, 1000 m, 2000 m, 3000 m and 4000 m, respectively. All conditions were randomised to each participant using the Latin Square method ([Keppel, 1983](#_ENREF_147)) and allocated in a single-blind manner. To keep participants uninformed of each condition, when performing at SL (FIO2; 0.209), the compressor attached to the environmental chamber was running at all times to create a ‘sham hypoxia’ environment ([Netzer, et al., 2008](#_ENREF_202)). To monitor participant’s perception of altitude exposure, following each visit participants were asked to report which condition they thought they had been exposed, none of the participants were able to identify the correct condition on every occasion, and only 40% of participants correctly identified the SL condition.

Upon arrival, prior to the GXT, a SL resting heart rate value was obtained using short range telemetry (Polar, Oy, Kemple, Finland); participants were required to rest in a seated position for 5 minutes, the lowest value observed over this time period was recorded. Peak heart rate was determined using a GXT conducted on an electromagnetically braked cycle ergometer (Lode, Excalibur Sport, Cranlea and Co, Bourneville, UK). After resting for 10 minutes inhaling in the corresponding O2 concentration, participants completed a 5 minute warm-up at 50 W. The GXT began at 50 W for two minutes, which was subsequently increased by 20 W for women and 25 W for men every minute thereafter ([Amann, et al., 2006](#_ENREF_4)). Participants selected a cadence between 70 to 90 rpm and were asked to maintain this throughout each GXT. Each participant selected their preferred cadence during their familiarisation session, once selected, the chosen cadence (76 ± 10 rpm) was fixed for each of the 5 exercise tests. Participants were verbally encouraged to give a maximal effort during every visit to the laboratory and were instructed to remain seated throughout the test. During the GXT heart rate, E, O2 and FIO2 were recorded continuously using a breath-by-breath analysis system (Cosmed K4b2, Cosmed srl, Rome, Italy). Arterial O2 saturation (accuracy ± 2%; Model 3800, Datex-Ohmeda Division, Insrumentarium Corp, Finland) was monitored continuously during the GXT and recorded every minute. Pulse oximeter readings were blinded to participants. Participants also gave their RPE ([Borg, 1982](#_ENREF_30); [Shephard et al., 1992](#_ENREF_255)) in the last 10 seconds of every stage.

The GXT protocol used was such that all participants completed the test within 10 ± 2 minutes to reduce the likelihood of potential limitations to exercise e.g. high body temperature, different substrate utilisation or ventilatory muscle fatigue ([Buchfuhrer et al., 1983](#_ENREF_35)). Test termination occurred when participants met the following criteria: (1) O2 reached a plateau despite increasing power output, (2) RER ≥ 1.05, and (3) exhaustion ([Howley, et al., 1995](#_ENREF_131)). Standard criteria (i.e. HRpeak ± 10% of age-predicted maximum) were not used for test termination since the aim of the study was to observe the decline in HRpeak with acute hypoxia, therefore HRpeak could not be used as a judgement criterion ([Benoit, et al., 2003](#_ENREF_23)). The HRpeak was defined as the highest value recorded ([Franckowiak, et al., 2011](#_ENREF_92)), O2peak as the highest 20 second moving average in VO2 ([Posner et al., 1995](#_ENREF_216)) and peak power output (peak) as the highest mechanical power output maintained for one minute ([Bentley & McNaughton, 2003](#_ENREF_24)) during the GXT.

### Prediction of Maximal Heart Rate

Traditional equations frequently used by athletic trainers, coaches and health professionals to prescribe aerobic exercise intensity are multiple ([Robergs & Landwehr, 2002](#_ENREF_234)); however limited evidence is available on the use of these equations in hypoxia. Available research findings suggest that HRpeak is reduced in acute hypoxia, if so the use of age-derived equations is limited. Therefore, the present study was constructed to examine the use of several age-derived equations to predict HRpeak in hypoxia; the equations compared against actual HRpeak response to a GXT performed on a cycle ergometer are presented in Table 6.2. As there are several equations for predicting HRpeak ([Robergs & Landwehr, 2002](#_ENREF_234)) it was decided that equations using only healthy men and women would be analysed in the present study. The majority of the equations compared were derived using treadmill ([Fernhall et al., 2001](#_ENREF_84); [Inbar et al., 1994](#_ENREF_133); [Lester et al., 1968](#_ENREF_161)) and cycling protocols ([Jones, 1988](#_ENREF_137); [Jones et al., 1985](#_ENREF_138); [Ricard et al., 1990](#_ENREF_228); [Tanaka et al., 2001](#_ENREF_272)), with some using a combination of the two modalities ([Fox et al., 1971](#_ENREF_90); [Tanaka et al., 1991](#_ENREF_271)).

Table 6.. Age-derived predictive equations for peak heart rate.

|  |  |  |
| --- | --- | --- |
| Equation | Exercise Mode | Reference |
| 1: 220-age | Treadmill, Cycle ergometer | ([Fox, et al., 1971](#_ENREF_90)) |
| 2: 208-0.7·age | Treadmill | ([Tanaka, et al., 2001](#_ENREF_272)) |
| 3: 205-0.64·age | Treadmill | ([Fernhall, et al., 2001](#_ENREF_84)) |
| 4: 205.8-0.685·age | Treadmill | ([Inbar, et al., 1994](#_ENREF_133)) |
| 5: 202-0.72·age | Cycle ergometer | ([Jones, et al., 1985](#_ENREF_138)) |
| 6: 210-0.65·age | Cycle ergometer | ([Jones, 1988](#_ENREF_137)) |
| 7: 198-0.41·age | Treadmill | ([Lester, et al., 1968](#_ENREF_161)) |
| 8: 209-0.587·age | Cycle ergometer | ([Ricard, et al., 1990](#_ENREF_228)) |
| 9: 200-0.687·age | Cycle ergometer | ([Ricard, et al., 1990](#_ENREF_228)) |
| 10: 211-0.8·age | Treadmill, Cycle ergometer | ([Tanaka, et al., 1991](#_ENREF_271)) |
| 11: 207-0.7·age | Treadmill, Cycle ergometer | ([Tanaka, et al., 1991](#_ENREF_271)) |

### Note: Age in years.

### Statistical Analyses

Statistical analyses were computed by the statistical software package SPSS (release 20.0; SPSS, Chicago, IL). Data were first tested for distribution normality and variance homogeneity using Kolmogorov-Smirnov tests (P ≥ 0.05); following mathematical confirmation of a normal distribution parametric statistics were used for data analyses. The estimation of sample size was calculated a priori using G\*Power software (release 3.1.2: Kiel, Germany) which resulted in a total of 9 participants required for the study for a power of 0.80 (at Appendix 7).

Repeated measures ANOVA were used to compare HRpeak across all five conditions (SL, 1000, 2000, 3000, 4000 m) and to compare HRpeak measured during the GXT and the HRpeak predicted by the equations in Table 6.2. Post-hoc planned comparisons comparing hypoxic measurements against SL and PHRpeak was carried out using Bonferroni corrected t-tests. Effect sizes for ANOVAs were calculated using the Ω2 method, and can be interpreted as small (< 0.06), medium (0.06-0.15) and large (>0.15) ([Cohen, 1992](#_ENREF_58)). Effect size for t-test comparisons were calculated by converting a t-value into an r-value ([Field, 2005](#_ENREF_86); [Rosenthal, 1991](#_ENREF_239); [Rosenthal, et al., 2000](#_ENREF_240)), and can be interpreted as small (0.10), medium (0.30), and large [0.50 ([Cohen, 1988](#_ENREF_57))]. All values are presented as mean ± 1SD and for statistical analyses an alpha of P ≤ 0.05 was considered significant unless otherwise stated.

## Results

### Peak Heart Rate

There was no significant decline in mean HRpeak in hypoxia compared with SL (P ≥ 0.05; Figure 6.1). A significant difference between PHRpeak and measured HRpeak using allequations was observed, results are summarised in Table 6.3; in all instances PHRpeak was higher than measured HRpeak (P ≤ 0.05).

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Figure 6.. Measured heart rate peak response to a graded exercise test in acute hypoxia.

Figure 6.. Individual changes in heart rate peak with hypoxia from sea level.

Table 6.. Predicted heart rate peak and measured heart rate peak comparisons.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | Condition | | | | |
| **SL** | **1000 m** | **2000 m** | **3000 m** | **4000 m** |
| Equation | **PHRpeak**  **(b·min-1)** | **Measured HRpeak (b·min-1)** | | | | |
| 182 ± 13 | 178 ± 11 | 177 ± 9 | 178 ± 9 | 176 ± 11 |
| A | 198 ± 2 | **↑**\* (0.80) | **↑**\* (0.87) | **↑**\*(0.92) | **↑**\*(0.93) | **↑**\*(0.91) |
| B | 192 ± 1 | = | **↑**\*(0.80) | **↑**\*(0.86) | **↑**\*(0.87) | **↑**\*(0.85) |
| C | 191 ± 1 | = | **↑**\*(0.76) | **↑**\*(0.84) | **↑**\*(0.84) | **↑**\*(0.82) |
| D | 190 ± 1 | = | **↑**\*(0.75) | **↑**\*(0.83) | **↑**\*(0.84) | **↑**\*(0.82) |
| E | 186 ± 1 | = | = | **↑**\*(0.71) | **↑**\*(0.69) | **↑**\*(0.70) |
| F | 195 ± 1 | **↑**\*(0.75) | **↑**\*(0.85) | **↑**\*(0.90) | **↑**\*(0.91) | **↑**\*(0.89) |
| G | 189 ± 1 | = | **↑**\*(0.71) | **↑**\*(0.80) | **↑**\*(0.80) | **↑**\*(0.78) |
| H | 196 ± 1 | **↑**\*(0.76) | **↑**\*(0.86) | **↑**\*(0.91) | **↑**\*(0.91) | **↑**\*(0.89) |
| I | 185 ± 1 | = | = | **↑**\*(0.65) | **↑**\*(0.63) | **↑**\*(0.65) |
| J | 193 ± 1 | **↑**\*(0.69) | **↑**\*(0.81) | **↑**\*(0.88) | **↑**\*(0.88) | **↑**\*(0.86) |
| K | 191 ± 1 | = | **↑**\*(0.78) | **↑**\*(0.85) | **↑**\*(0.85) | **↑**\*(0.84) |

**Notes:** PHRpeak predicted heart rate peak. Compared to corresponding altitude: = indicates PHRpeak no different to measured HRpeak; **↑** indicates PHRpeak > measured HRpeak, \* P ≤ 0.01. Effect sizes (r) are displayed in brackets (small < 0.06, medium 0.06-0.15 and large > 0.15).

### Peak Power Output, Peak Oxygen Uptake and Oxygen Saturation

Peak cardio-respiratory responses are shown in Table 6.4. All subjects cycled from 50 W to at least 150 W in each test. Thereafter, the number of participants varied, thus only values at peak are reported. At SL participants achieved an average peak of 247 ± 58 W. In hypoxia there was an overall decline in peak (F(4,56) = 10.126, P ≤ 0.001, Ω2 = 0.44) which was significantly lower than SL at 4000 m (t(14) = 4.795, P ≤ 0.001, r = 0.79). An overall reduction in test time was also observed (F(4,56) = 14.759, P ≤ 0.001, Ω2 = 0.53) which was significantly lower than SL at 2000 (t (14) = 2.902, P = 0.012, r = 0.61), 3000 (t(14) = 3.842, P = 0.002, r = .72) and 4000 m (t(14) = 5.792, P ≤ 0.001, r = 0.84).

Table 6.. Cardio-respiratory responses to graded exercise in acute hypoxia.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **SL** | **1000m** | **2000m** | **3000m** | **4000m** |
| **O2peak (L.min-1)** | 3.39 ± 0.99 | 3.31 ± 1.00 | 3.22 ± 0.90 | 3.09 ± 0.92 | 2.91 ± 0.72\* |
| **O2peak**  **(mL·kg-1·min-1)** | 46.3 ± 7.0 | 46.1 ± 10.4 | 44.3 ± 8.5 | 42.6 ± 9.9 | 40.5 ± 7.4\* |
| **peak** | 247 ± 58 | 243 ± 57 | 237 ± 53 | 232 ± 56 | 218 ± 43\* |
| **Test time (s)** | 556 ± 138 | 538 ± 131 | 518 ± 125\* | 506 ± 133\* | 473 ± 110\* |
| **SPO2 (%)** | 95 ± 5 | 95 ± 2 | 92 ± 2 | 88 ± 3\*§† | 82 ± 4\*§†‡ |

**Note:** \* < SL; § <1000 m; † < 2000 m. ‡ < 3000 m; P ≤ 0.005.

In hypoxia, there was an overall decline in O2peak, whenboth presented absolutely (F(4,52) = 3.443, P = 0.014, Ω2 = 0.34) and relatively to body mass (F(4,52) = 2.819, P = 0.034, Ω2 = 0.45) which were significantly lower than SL at 4000 m (t(13) = 3.240, P = 0.006, r = 0.67; t(13) = 3.465, P = 0.004, r = 0.69) (Table 6.4). The SPO2 was also significantly reduced in hypoxia (F(4,56) = 43.485, P ≤ 0.001, Ω2 = 0.98). Compared with SL SPO2 was lower at 3000 m (t(14) = 5.254, P ≤ 0.001, r = 0.81) and 4000 m (t(14) = 8.434, P ≤ 0.001, r = 0.91). The SPO2 was also significantly reduced at 4000 m compared with 1000 m (t(14) = 11.110, P ≤ 0.001, r = 0.96), 2000 m (t(14) = 7.506, P ≤ 0.001, r = 0.86) and 3000 m (t(14) = 6.108, P ≤ 0.001, r = 0.85).

### Rating of Perceived Exertion

No significant difference was found for RPE between SL (20 ± 0) and any of the hypoxic conditions (1000 m, 20 ± 0; 2000 m, 20 ± 0; 3000 m, 20 ± 0; 4000 m, 20 ± 0), with individuals always reporting maximal exertion on cessation of the GXT.

## Discussion

The first aim of the present study was to identify whether there is a decrease in HRpeak with acute hypoxia and to observe, if evident, the altitude at which the decrement in HRpeak occurs. The results did not demonstrate a decline in HRpeak with exposure to acute normobaric hypoxia at or below 4000 m compared with SL. However, analyses revealed a significant difference in O2peak at 4000 m and also confirmed the well-reported reduction in SPO2 with hypoxia. A second aim was to determine whether age-derived equations to predict HRpeak are viable for prescribing exercise intensity within hypoxia. It was found that most PHRpeak equations significantly overestimated HRpeak in hypoxia. Moreover ratings of perceived exertion indicate that all tests were perceived as maximal with participants giving a rating of 19 or above, with no difference between trials (SL to 4000 m).

Results of the present study confirm those of previous research that have identified little or no change in HRpeak with acute hypoxia below 3800 m ([Lawler, et al., 1988a](#_ENREF_155); [Stenberg, et al., 1966](#_ENREF_264)), however they do not support those reporting a decline in HRpeak with acute hypoxia above 3800 m ([Benoit, et al., 2003](#_ENREF_23); [Friedmann, et al., 2005](#_ENREF_94); [Grataloup et al., 2007](#_ENREF_112); [Lundby, et al., 2001](#_ENREF_170); [Mollard, et al., 2007](#_ENREF_195); [Roach, et al., 1996](#_ENREF_231)). In acute hypoxia, the decline in HRpeak has been attributed to increased parasympathetic traffic resulting in a blunting of the cardiac chronotropic function, rather than a decreased sympathetic function, which is often observed with exhaustive exercise in chronic hypoxia ([Benoit, et al., 2003](#_ENREF_23)). Additionally, it has been hypothesised that the decline in HRpeak with acute hypoxia is due to the direct effect of hypoxia on the cardiac electrophysiological properties i.e. repolarisation length and transmission time on the atrioventricular node ([Roche et al., 2003](#_ENREF_235)), which are coherent with the decrease in SPO2 and change in HRpeak at altitude ([Mollard, et al., 2007](#_ENREF_195)). A decline in SPO2 (88-82%) was observed in the present study, however the reduction was not as great as observed by others [≈ 80% ([Benoit, et al., 2003](#_ENREF_23); [Lundby, et al., 2001](#_ENREF_170))] which may explain the lack of reduction in HRpeak. Therefore, the present results, in addition to previous literature, suggest that a reduction in SPO2 may solely account for the decline in HRpeak with acute normobaric hypoxia. In support, individuals who present greater hypoxemia than others consequently experience a greater reduction in HRpeak with acute hypoxia ([Richalet, et al., 1988](#_ENREF_229)) and larger reductions in SPO2 have been reported to result in greater modifications within cardiac properties such as autonomic changes ([Lundby, et al., 2001](#_ENREF_170); [Ricard, et al., 1990](#_ENREF_228)). Alternatively, the lack of reduction in HRpeak with acute hypoxia observed in the present study could also be due to the lower HRpeak observed at SL (182 ± 13 b·min-1) compared with previous work [196 ± 7 b·min-1 (Benoit, et al., 2003)], which may have been limited by leg fatigue rather than dyspnea (Aliverti et al., 2011), which could be accounted for by the higher pedal cadence chosen in this study using untrained individuals rather than experienced cyclists. This concept should be explored further in future work. Moreover, since HRpeak ranged from 195 to 169 b·min-1 in the SL condition, reporting of the mean values observed in each condition may not accurately reflect values observed on an individual basis, where a reduction in HRpeak may be observed for some. In more detail, the variation observed wasn’t consistent between altitudes as is demonstrated by Figure 6.2, moreover the magnitude of variation appears to differ greatly among participants with some demonstrating small individual variation (e.g. participant 1) and others large individual variation (e.g. participant 15). However, there is no strong motive for removing this participant as the statistical outcome remains the same. Therefore, it is believed that the data set is a true reflection of the individual responses of HRpeak to hypoxic conditions and further adds to support the need to measure HRpeak in hypoxic conditions prior to exercise prescription hypoxic studies/programmes.

Although there was no significant reduction in HRpeak in response to acute normobaric hypoxia, HRpeak was reduced when compared to age-predicted values derived from all eleven equations examined. While it is expected that a greater HRpeak will be observed with treadmill protocols, those equations derived from cycling protocols ([Jones, 1988](#_ENREF_137); [Jones, et al., 1985](#_ENREF_138); [Ricard, et al., 1990](#_ENREF_228)) also overestimated HRpeak. Moreover, despite a moderate sample size of 15, large effect sizes (> 0.15) ([Cohen, 1977](#_ENREF_56)) for all equations were observed suggesting that the over prediction of HRpeak by the age-derived equations is present and of importance. Therefore, the use of current age-derived equations to predict HRpeak would appear to be inappropriate for use in hypoxic conditions and the measurement of normoxic HRpeak during a GXT is necessary. Exercise prescription using age-derived equations may have negative consequences and lead to discrepancies in exercise intensities prescribed between normoxic and normobaric hypoxic training regimes, with hypoxia groups exercising at a greater intensity, this consequently may lead to the setting of unachievable goals, decreased adherence to exercise sessions ([Perri, et al., 2002](#_ENREF_214)) and no observed training effect.

A decrease in O2peak with increasing hypoxia, caused by the reduction in arterial O2 content ([Lawler, et al., 1988a](#_ENREF_155); [Stenberg, et al., 1966](#_ENREF_264)) has been consistently observed in numerous studies examining the effect of acute hypoxia on peak aerobic performance ([Cymerman et al., 1989](#_ENREF_64); [Ferretti et al., 1997](#_ENREF_85)). Results of the present study confirm those reported by others showing a decline in O2peak at 4000 m, which also coincided with a decrease in peak. However, controversy surrounds the interpretation of the effect of the HRpeak modification on O2peak ([Benoit, et al., 2003](#_ENREF_23)). According to the Fick equation [O2 = HR·SV(Cao2-Cvo2)], a fall in HRpeak and SPO2 will consequently have an effect on O2peak via a reduction in peak cardiac output ([Benoit, et al., 2003](#_ENREF_23); [Grataloup, et al., 2007](#_ENREF_112); [Richalet, et al., 1988](#_ENREF_229)). Although a reduction in SPO2 was observed, the lack of decline in HRpeak within the present study is not in support of HRpeak modification on O2peak reduction. Therefore, the decline in HRpeak cannot solely account for the reduction in O2peak. These results suggest that the contribution of reduced SPO2 may be larger than HRpeak in the reduction of O2peak. The results should be interpreted with caution as stroke volume and cardiac output were not measured.

In conclusion, measured HRpeak does not appear to be reduced in response to acute normobaric hypoxia at or below 4000 m, which can be explained by a limited reduction in SPO2 (18-22%) compared with other studies (27-33%) which report a reduction in HRpeak. Age-derived equations overestimate HRpeak in hypoxia despite no observed reduction in measured HRpeak ator below 4000 m; therefore equations appear inappropriate for exercise intensity prescription in hypoxia. Until specific equations are developed to predict HRpeak in hypoxia it is recommended that where possible, HRpeak be measured using a GXT. Until specific equations are developed to predict HRpeak it is recommended that where possible, HRpeak is measured using an incremental exercise test. With the development of appropriate equations exercise physiologists will be able to determine and prescribe safe exercise intensities in hypoxia without the use of a GXT to volitional exhaustion in individuals to which maximal exercise is contraindicated.

Since age-derived equations used to predict HRpeakdo not appear to be appropriate for use in acute normobaric hypoxia, in Chapter 7, a sub-maximal method will be explored. The VT is often used in exercise prescription to determine exercise intensity and due to its sub-maximal nature it is useful for individuals who present with contraindications to exhaustive exercise. Therefore, the response of the VT to acute normobaric hypoxia will be examined, if VT remains unchanged, it could be used as a method to determine exercise intensity in future IHT studies.

# Measurement of the Ventilatory Threshold to Determine Exercise Intensity in Acute Normobaric Hypoxia

Findings from this chapter have been published in: Gallagher, C.A., Willems, M.E.T., Lewis, M.P. & Myers, S.D. (2014). Effects of acute normobaric hypoxia on the ventilatory threshold. European Journal of Applied Physiology, 114(8), 1555-1562.

## Introduction

Exercise intensity, is a key principle of training, and therefore an important tool for prescribing, assessing and monitoring exercise training in all populations ([Bompa & Haff, 2009](#_ENREF_27)). Graded exercise tests to maximal exertion are commonly used to assess training status ([Friedmann et al., 2004](#_ENREF_93); [Subudhi et al., 2006](#_ENREF_268)) and prescribe training intensities. Often HRpeak and O2peak are employed as methods to determine exercise intensity and predict performance. However, some authors have reported that nonlinear increases in physiological variables, such as blood lactate, heart rate and ventilation observed during progressive exercise tests represent “thresholds” that are better predictors of performance([Bosquet et al., 2002](#_ENREF_31); [Subudhi, et al., 2006](#_ENREF_268)). A threshold indicates the upper limit of sensitivity, the point at which further increases in intensity have no typical effect ([Kent, 2007](#_ENREF_146)). Moreover, using O2peak to equate exercise intensity may lead to different physiological responses, for example, 60% O2peak may be above the “threshold” for one individual and below for another which would result in differing training stimuli ([Davis, 1985](#_ENREF_66)). Numerous studies testify to the sensitivity of the VT to endurance training ([Carter et al., 1999](#_ENREF_43); [Jones & Carter, 2000](#_ENREF_136); [Weltman et al., 1992](#_ENREF_287)) and a rightward shift of the VT to a higher power output or running speed is characteristic of a successful training programme ([Jones & Carter, 2000](#_ENREF_136); [Wells & Pate, 1988](#_ENREF_286)). Therefore, the VT is a measurement that is sensitive to change which allows for effective monitoring of training adaptations. The VT can also be reliably detected using sub-maximal exercise tests ([Wasserman & McIlroy, 1964](#_ENREF_283)) and therefore is an ideal measure for populations in which exhaustive exercise may be contraindicated (e.g. sedentary, untrained, obese).

The VT is used widely in exercise prescription and is considered a useful index of functional capacity for patients suffering from cardiovascular and pulmonary conditions ([Wasserman et al., 2005](#_ENREF_282)). It has also been shown to be of prognostic value, and is used as a key outcome measure to assess the effectiveness of exercise interventions ([Ekkekakis et al., 2008](#_ENREF_77); [Wasserman & McIlroy, 1964](#_ENREF_283)). By “definition”, VT is the exercise intensity at which the increase in ventilation becomes disproportional to the increase in power output or speed of locomotion during a GXT ([Svedahl & MacIntosh, 2003](#_ENREF_270)). Moreover, the VT is identified via a continuous rise in the ventilatory equivalent for O2 (E/O2) without a concomitant change in the ventilatory equivalent for CO2 (E/CO2) ([Myers et al., 2008](#_ENREF_199); [Ozcelik & Kelestimur, 2004](#_ENREF_213); [Subudhi, et al., 2006](#_ENREF_268)). The mechanism underlying the VT response has been linked closely with lactate kinetics. Once sufficiently elevated, lactate, or, more specifically, the hydrogen ions (H+) associated with lactate release from skeletal muscle, causes an increase in E through an elevated CO2 output (CO2) as a consequence of the bicarbonate buffer reaction and potentially by H+ directly stimulating the carotid bodies ([Hughson et al., 1995](#_ENREF_132)). Thus, the dissociation between E/O2 and E/CO2 becomes present.

Despite the advantages of VT assessments, there have been relatively few measurements of the VT in response to hypoxia. Evidence suggests that hypoxia causes a further increase in lactate above values observed at SL ([Hughson, et al., 1995](#_ENREF_132); [Mazzeo, et al., 1991](#_ENREF_186)) and the accumulation of H+ associated with lactate ion accumulation in the blood is well correlated during exercise at SL ([Subudhi, et al., 2006](#_ENREF_268)). Thus, it would be expected that the VT would be reduced further during exercise in hypoxic environments compared with SL measurements. Previous research has shown that breathing reduced O2 concentrations (FIO2 0.12-0.14) during graded cycle tests can reduce the VT by up to 33% ([Fukuoka et al., 2003](#_ENREF_95); [Hughson, et al., 1995](#_ENREF_132); [Ozcelik & Kelestimur, 2004](#_ENREF_213)). Subudhi et al., (2006) supported these findings by reporting reduced power output at the VT (41%) at 4300 m compared with SL, as well as reporting an overall reduction in the VT. Running speed, O2 and heart rate at the VT have also been shown to decrease in response to acute hypoxia during treadmill exercise ([Friedmann, et al., 2004](#_ENREF_93)). In contrast, at a lower equivalent altitude of 1500 m there was no reported difference in the VT compared with a SL control condition ([Mateika & Duffin, 1994](#_ENREF_184)). Interestingly, it appears that the reduction in VT may occur at a particular threshold, based on the current findings, a threshold altitude of 3000 m is proposed. Therefore, it is suggested that below this representative altitude, the VT could possibly be utilised as a determinant of exercise intensity within hypoxic training programmes. However, to our knowledge there are no studies within the literature to suggest that this is the case, thus further exploration in this area is warranted.

Since there is little data available in the literature pertaining to the effects of hypoxia on the VT, the primary aim of this study was to investigate the effect of acute hypoxia of varying O2 concentrations (FIO2 0.209-0.125) on the VT response during graded cycle exercise. Since the VT appears to decrease in a linear fashion, or perhaps decreases at a particular threshold somewhere between 1500 m and 4300 m, it was hypothesised that the VT would be reduced at altitudes between this identified range (FIO2 0.197 – 0.12), however at or below 1500 , no reductions in the VT were expected to be observed. Several techniques based on visual inspection of respiratory gas exchange graphical plots have been described to measure the VT ([Santos & Giannella-Neto, 2004](#_ENREF_246)). However, the results are often subjective ([Bosquet, et al., 2002](#_ENREF_31); [Yeh et al., 1983](#_ENREF_298)). Therefore, automatic computerised algorithms have been designed, which may provide more objective and accurate results. To date, these algorithms are yet to be compared during the analysis of data collected in hypoxia. Thus, a secondary aim of this study was to examine the agreement between algorithms used to identify the VT using the computerised software programme Winbreak 3.7 ([Ekkekakis, et al., 2008](#_ENREF_77)).

## Method

### Participants

Fourteen healthy volunteers (7 women, 7 men; mean ± 1SD: age 22 ± 2 years; height 175.8 ± 10.3 cm; body mass 72.2 ± 14.3 kg; BMI 23.2 ± 2.8 kg·m-2; body fat 23.6 ± 8.2%; O2peak 46.3 ± 7.0 mL·kg-1·min-1) participated in the study. Originally 15 participants were recruited but due to corrupted data file errors data from one participant was excluded from the analysis. All participants completed a medical history questionnaire (at Appendix 1), stating no contraindications to exhaustive exercise. All were SL residents and had not been above 1000 m in the six months preceding the study. After receiving both written and oral information on the experimental protocol and procedures, participants gave their written informed consent (at Appendix 2). The research conformed to the guidelines laid down in the Declaration of Helsinki (2008) and was approved by the University of Chichester Research Ethics Committee.

### Experimental Design

Each participant visited the laboratory on 6 occasions, including one familiarisation session, with each visit separated by a minimum of 48 hours. To ensure standardised testing, all participants were instructed not to partake in any vigorous physical training or alcohol consumption 24 hours prior to each session or to consume caffeinated beverages and food for a two-hour period prior to all sessions ([Noonan & Dean, 2000](#_ENREF_207)).

### Experimental Procedures

Following familiarisation, on Visit 2, height was measured to the nearest 0.1 cm (Holtain Ltd, Crymych, UK), body mass to the nearest 0.05 kg (BC 418 MA, Tanita Ltd, Middlesex, UK), and body composition assessed (Table 7.1). Body mass index was calculated as measured body mass divided by the square of measured height. All anthropometric measures were repeated on visits 4 and 6 to monitor any changes in body composition over the time course of the study; no significant differences were observed therefore the values from the first visit are reported.

Body composition was assessed using skin-fold measurements taken at four skin-fold sites ([Durnin & Womersley, 1974](#_ENREF_75)); bicep, tricep, subscapular and iliac crest, using standard skin-fold callipers (Harpenden Skin-fold Callipers, Baty International, UK). All skin-fold measures were measured and recorded in duplicate from the right side of the body with participants standing in a relaxed position. A third measure was taken only if the difference between the first two measurements was greater than 5% (Marfell-Jones et al., 2006). Following skin-fold measurements, values were entered into Equation 1 to determine gender and age specific[[27]](#footnote-27) body density ([Siri, 1956](#_ENREF_260)), resultant values were then entered into Equation 2 to quantify body fat percentage ([Durnin & Womersley, 1974](#_ENREF_75)).

Equation 1. Body density equation

Body density (men) = 1.1609-[0.0632·LOG (∑ skin-folds)]

Body density (women) = 1.1581-[0.072·LOG (∑ skin-folds)]

Equation 2. Body fat calculation

Body Fat Percentage = [(4.95/body density)-4.5]·100

Table 7.. Anthropometric characteristics.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| BMI (kg·m2) | Bicep (mm) | Tricep (mm) | Subscapular (mm) | Iliac Crest (mm) | Sum of 4 Skin-folds | Body Fat (%) |
| 23.2 ± 2.8 | 9.7 ± 5.5 | 15.9 ± 6.2 | 12.2 ± 4.9 | 13.5 ± 6.6 | 51.3 ± 17.2 | 23.6 ± 8.2 |

### Exercise Testing

All exercise tests were performed in an environmental chamber (TISS series 201003-1, TIS Services, UK), where normobaric hypoxia was achieved via a molecular sieve, enclosing microscopic pores which allow only small or mobile molecules to permeate through. Molecules that permeate through the walls of the fibres include O2, CO2 and water vapour. The principle molecule unable to permeate is nitrogen. Compressed air is passed down the fibres and a restriction is applied in order to create pressure in the fibres. The greater restriction applied, the higher the pressure in the fibres leading to lower number of permeated O2 molecules. By varying the restriction the O2 level in the output stream can be varied from around 20% to less than 1%. The tamb, RH and v were controlled for in all sessions (tamb 21.3 ± 1.7°C; RH 50 ± 2%; v 0.00 ± 0.0 0 m.s-1). For all tests completed the ambient FIO2 were 0.209, 0.185, 0.165, 0.142 and 0.125 corresponding to SL, 1000 m, 2000 m, 3000 m and 4000 m, respectively. All conditions were randomised to each participant using a 5 x 5 Latin Square ([Keppel, 1983](#_ENREF_147)) and allocated in a single-blind manner. To keep participants uninformed of each condition, when performing at SL (FIO2; 0.209), the compressor connected to the environmental chamber was running at all times to create a ‘sham hypoxia’ environment ([Netzer, et al., 2008](#_ENREF_202)). To monitor participant’s perception of altitude exposure, following each visit they were asked to report which condition they thought they had been exposed. None of the participants were able to identify the correct hypoxic condition, and only 6 out of 14 participants correctly identified when they were exposed to the SL condition.

Upon arrival, prior to the GXT, a SL resting heart rate value was obtained using short range telemetry (Polar FS1, Oy, Kemple, Finland); participants were required to rest in a seated position for 5 minutes, the lowest value observed over this time period was recorded. Peak heart rate was determined using a GXT on an electromagnetically braked cycle ergometer (Lode, Excalibur Sport, Cranlea and Co, Bourneville, UK). After resting for 10 minutes breathing the corresponding O2 concentration, participants completed a 5 min warm-up at 50 W. The GXT began at 50 W for two minutes, which was increased by 20 W for women and 25 W for men every minute thereafter ([Amann, et al., 2006](#_ENREF_4)). Participants selected a cadence between 70 to 90 rpm and were asked to maintain this throughout each GXT. Participants were verbally encouraged to give a maximal effort during every visit to the laboratory and were instructed to remain seated throughout the test. During the GXT, heart rate, E, O2 and FIO2 were recorded continuously using breath-by-breath analysis (Cosmed K4b2, Cosmed srl, Rome, Italy). Arterial O2 saturation [(SPO2) accuracy ± 2%; Model 3800, Datex-Ohmeda Division, Instrumentarium Corp, Finland) was monitored continuously during the test and recorded every minute. The pulse oximeter readings were blinded to participants. Participants also gave their RPE ([Borg, 1982](#_ENREF_30); [Shephard, et al., 1992](#_ENREF_255)) in the last 10 seconds of every stage.

The GXT protocol used was such that all participants completed the test within 10 ± 2 minutes to reduce the likelihood of potential limitations to exercise e.g. high body temperature, different substrate utilisation or ventilatory muscle fatigue ([Buchfuhrer, et al., 1983](#_ENREF_35)). Test termination occurred when participants met the following criteria: (1) O2 reached a plateau despite increasing power output, (2) RER ≥ 1.05, and (3) exhaustion ([Howley, et al., 1995](#_ENREF_131)).

### Computerised Determination of the Ventilatory Threshold

The VT was identified using a computerised program (WinBreak 3.7, Epistemic Mindworks, USA) that incorporates a number of algorithms that are commonly used to determine this parameter. Data preparation involved three working steps according to the methods of Ekkekakis et al., (2008). Firstly, non-physiological (e.g. negative) values were removed from each data set. Secondly, data were averaged every 20 seconds ([Gaskill et al., 2001](#_ENREF_100)). Lastly, lower and upper boundaries for the VT calculations were set. The lower boundary was always set after the first minute (minute 1). The upper boundary was set either at the end of the test or at the respiratory compensation point (RCP), if one was found. The RCP was determined using a modified version of the method of Beaver et al., (1986). According to Beaver and colleagues the RCP is identified as follows:

“The E versus CO2 data are divided into two linear segments, the intersection of the two segments is the RCP if the change in the slope between them is greater than a preselected amount (15% of initial slope)” (p. 2023).

Beaver and colleagues noted that an RCP does not always occur ([Beaver et al., 1986](#_ENREF_21)). Therefore the data sets were first examined for a significant departure from linearity. In the present study this was the case for 2 out of 70 cases, in this instance the end of the GXT was used as the upper boundary for the ventilatory threshold calculations. If data showed a significant departure, the respiratory compensation point was set at the point of the largest slope difference between the two segments, as using a fixed slope difference (such as the 15% mentioned by Beaver and colleagues) occasionally resulted in untenable results (e.g. points below 50% O2peak). After application of preliminary data processing steps, the VT was estimated using the following five algorithms.

***Algorithm 1.*** The first method consisted of using the “breakpoint” algorithm developed by Jones and Molitoris (1984), in conjunction with CO2 by O2 data, as implemented by Schneider et al., (1993). This algorithm considers two regression equations, one before and one after the breakpoint, y = b0 + b1X and y = b0 + b1X0 + b3 (X-X0). The algorithm then searches for the value of X0 that minimises the residual sum of squares ([Jones & Molitoris, 1984](#_ENREF_139)).

***Algorithm 2.*** The second algorithm consisted of using the “brute force” method proposed by Orr et al., (1982) in conjunction with CO2 by O2 data. This algorithm consists of calculating regression lines through all possible divisions of the data in to two contiguous groups, and finding the pair of lines yielding the least pooled residual sum of squares ([Orr et al., 1982](#_ENREF_211)).

***Algorithm 3.*** The third algorithm consisted of using the “V-slope” method proposed by Beaver et al., (1986) in combination with CO2 by O2 data. The algorithm consists of dividing the CO2 by O2 curve in to two regions, fitting linear regressions through them, and identifying the point at which the ratio of the distance of the intersection point from a single regression line through the data to the mean square error of regression is maximised ([Beaver, et al., 1986](#_ENREF_21)).

***Algorithm 4.*** The fourth algorithm utilised the “Dmax” method of Cheng et al., (1992) in conjunction with CO2 by O2 data. The algorithm involves calculating a third-order polynomial regression curve to fit the data and drawing a straight line connecting the first and last data points. The breakpoint is then defined as the point yielding the maximal distance between the curve and the straight line ([Cheng et al., 1992](#_ENREF_50)).

***Algorithm 5.*** The fifth algorithm consisted of using the “simplified V-slope” method proposed by Sue and colleagues ([1988](#_ENREF_269)), which was later computerised by Dickstein et al., (1990) in conjunction with CO2 by O2 data ([Dickstein et al., 1990](#_ENREF_70); [Sue, et al., 1988](#_ENREF_269)). Similar to the “V-Slope” method this algorithm again calculates regression lines through all possible divisions of the data in to two contiguous groups. This algorithm differs from the “V-slope” method in that it finds a breakpoint at which the first regression has a slope of less than or equal to 1 and the second has a slope higher than 1 rather than identifying the maximal mean square error of regression.

### Statistical Analyses

Statistical analyses were computed by the statistical software package SPSS (release 20; SPSS, Chicago, IL). Data were first tested for distribution normality and variance homogeneity using Kolmogorov-Smirnov tests (P ≥ 0.05); following mathematical confirmation of a normal distribution parametric statistics were used for data analyses. The estimation of sample size was calculated a priori using G\*Power software (release 3.1.2: Kiel, Germany) which resulted in a total of 9 participants required for the study for a power of 0.80 (at Appendix 7). Post-hoc power analyses resulted in a power of 0.99 for a sample size of 14, with a P value of 0.025 and effect size of 0.63.

Repeated measures ANOVA were used to compare the VT across all five conditions (SL, 1000, 2000, 3000, 4000 m) and to compare the VT measured by the different algorithms. Post-hoc planned comparisons comparing hypoxic measurements against SL were carried out using Bonferonni corrected t-tests. Effect sizes for ANOVAs were calculated using the Ω2 method, and can be interpreted as small (< 0.06), medium (0.06-0.15) and large [> 0.15 ([Cohen, 1992](#_ENREF_58))]. Effect size for t-test comparisons were calculated by converting a t-value into an r-value ([Field, 2005](#_ENREF_86); [Rosenthal, 1991](#_ENREF_239); [Rosenthal, et al., 2000](#_ENREF_240)), and can be interpreted as small (0.10), medium (0.30) and large [0.50 ([Cohen, 1988](#_ENREF_57))]. Unless otherwise stated all data are presented as mean ± 1SD and for statistical analyses an alpha of P ≤ 0.05 was considered significant.

## Results

During analysis of the VT some data files did not fit the criteria for the algorithms used; these files were therefore termed indeterminate cases and are presented in Table 7.2. In the discussion, the algorithms referred to will be those that produced the least indeterminate cases, which were algorithms 1, 2 and 4. Data for algorithms 3 and 5 are not presented due a large number of missing data points.

### Indeterminate Cases

As shown in Table 7.2 all VT calculation methods, because of the restrictions they place on the viability of the solutions, resulted in some indeterminate cases. Specifically, algorithms 1, 2 and 4 led to one, Algorithm 3 led to four, and Algorithm 5 to six indeterminate cases (number of cases) in identification of the VT at SL, respectively. Further indeterminate cases were also visible at 1000, 2000, 3000 and 4000 m. Algorithm 1 elicited the least indeterminate cases whilst Algorithm 5 elicited the most. Indeterminate cases were not altitude dependent. Algorithms that demonstrated indeterminate cases equal to or more than 10% of the data set were eliminated from statistical analyses (algorithms 3 and 5). In the case where less than 10% of the values were missing, data values were estimated using the column mean (Table 7.3).

Table 7.. Number of indeterminate cases associated with VT algorithm.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Algorithm | | | | | |
|  | **1** | **2** | **3** | **4** | **5** |
| SL | 1 | 1 | 4 | 1 | 6 |
| 1000 m | 0 | 0 | 1 | 0 | 8 |
| 2000 m | 0 | 1 | 4 | 1 | 6 |
| 3000 m | 1 | 1 | 1 | 1 | 7 |
| 4000 m | 1 | 1 | 2 | 1 | 8 |
| ∑ | **3** | **4** | **12** | **4** | **35** |

**Note:** (1) Jones and Molitoris (1984); (2) Orr et al., (1982); (3) Beaver et al., (1986); (4) Cheng et al., (1992); (5) Sue et al., (1988).

### Ventilatory Threshold

The VT determined by each algorithm are presented in Table 7.3, the VT is expressed as O2 (O2VT), heart rate (HRVT), and power (WVT). The VT is also expressed relative to O2peak (%O2peak) at the corresponding altitude. For example, 1000 m VT is relative to 1000 m O2peak.

Table 7.. Estimates of the ventilatory threshold, expressed as oxygen cost (O2), percentage peak rate of O2 (%O2peak), power and heart rate.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Algorithm | | | | |
|  |  | **1** | **2** |  | **4** |
| SL | **O2VT (L·min-1)**  **HRVT (b·min-1)**  **WVT**  **%O2peak** | 1.98 ± 0.46  128 ± 11  127 ± 36  60 ± 8 | 2.15 ± 0.63  130 ± 20  127 ± 49  65 ± 10 | 1.95 ± 0.47  128 ± 15  119 ± 33  59 ± 9 | |
| 1000 m | **O2VT (L·min-1)**  **HRVT (b·min-1)**  **WVT**  **%O2peak** | 2.03 ± 0.61  127 ± 16  132 ± 40  61 ± 12 | 2.16 ± 0.70  127 ± 16  132 ± 41  65 ± 13 | 2.05 ± 0.61  127 ± 15  130 ± 34  61 ± 11 | |
| 2000 m | **O2VT (L·min-1)**  **HRVT (b·min-1)**  **WVT**  **%O2peak** | 2.27 ± 0.62  139 ± 20  139 ± 50  69 ± 14 | 2.47 ± 0.74  137 ± 24  140 ± 65  74 ± 18 | 2.29 ± 0.55  136 ± 18  148 ± 41  69 ± 13 | |
| 3000 m | **O2VT (L·min-1)**  **HRVT (b·min-1)**  **WVT**  **%O2peak** | 1.84 ± 0.50  135 ± 21  133 ± 40  57 ± 16 | 1.87 ± 0.61  130 ± 17  129 ± 32  56 ± 14 | 1.80 ± 0.50  130 ± 20  115 ± 23  55 ± 15 | |
| 4000 m | **O2VT (L·min-1)**  **HRVT (b·min-1)**  **WVT**  **%O2peak** | 2.29 ± 0.58  141 ± 16\*  150 ± 35  67 ± 14 | 2.36 ± 0.63  139 ± 15  142 ± 33  69 ± 13 | 2.25 ± 0.54  139 ± 12  129 ± 27  66 ± 14 | |

**Note:** \*Greater than SL, P ≤ 0.01. Algorithms (1) Jones and Molitoris (1984); (2) Orr et al., (1982); (4) Cheng et al., (1992).

### Algorithm 1 - Jones and Molitoris (1984)

The WVT during the SL test was 127 ± 36 W (52 ± 15% peak), equating to a O2VT of 1.98 ± 0.46 L·min-1 (58 ± 13% O2peak) and HRVT of 128 ± 11 b·min-1 (71 ± 6% HRpeak). In hypoxia there was an overall significant change in O2VT (F(4,52) = 4.598, P = 0.025, Ω2 = 0.62). A significant change was also observed for HRVT (F(4,52) = 2.886, P = 0.031, Ω2 = 0.66) which was higher than SL at 4000 m (t(13) = -2.915, P = 0.012, r = 0.63). The VT as %O2peak, nor WVT differed in hypoxia compared to SL (Table 7.3).

### Algorithm 2 - Orr et al., (1982)

The WVT during the SL test was 127 ± 49 W (52 ± 20% peak), equating to a O2VT of 2.15 ± 0.63 L·min-1 (63 ± 19% O2peak) and HRVT of 130 ± 20 b·min-1 (72 ± 7% HRpeak). In hypoxia there was an overall significant change in O2VT (F(4,52) = 3.490, P = 0.013, Ω2 = 0.63) and VT as %O2peak (F(4,52) = 3.615, P = 0.011, Ω2 = 0.76). The HRVT nor WVT differed in hypoxia compared to SL (Table 7.3).

### Algorithm 4 - Cheng et al., (1992)

The WVT during the SL test was 119 ± 33 W (49 ± 14% peak), equating to a O2VT of 1.95 ± 0.47 L·min-1 (58 ± 14% O2peak) and HRVT of 128 ± 15 b·min-1 (71 ± 8% HRpeak). In hypoxia there was an overall significant change in O2VT (F(4,52) = 3.893, P = 0.008, Ω2 = 0.68), VT as %O2peak (F(4,52) = 3.453, P = 0.014, Ω2 = 0.72) and WVT (F(4,52) = 2.959, P = 0.028, Ω2 = 0.67). The HRVT did not differ in hypoxia compared to SL (Table 7.3).

### Agreement between Algorithm Determinations of the VT

During tests in all conditions (SL, 1000, 2000, 3000 and 4000 m) O2VT values determined by each algorithm were not significantly different from each other (Figure 7.1).

Figure 7.. The oxygen cost (O2) at the ventilatory threshold identified by each algorithm in each hypoxic condition.

**Note:** (1) Jones and Molitoris (1984); (2) Orr et al., (1982); (4) Cheng et al., (1992).

No significant differences between algorithms were identified for HRVT (Figure 7.2) and WVT (Figure 7.3) in any of the conditions.

Figure 7.. The heart rate at the ventilatory threshold identified by each algorithm in each hypoxic condition.

**Note:** (1) Jones and Molitoris (1984); (2) Orr et al., (1982); (4) Cheng et al., (1992).

Figure 7.. The power at the ventilatory threshold identified by each algorithm in each hypoxic condition.

**Note:** (1) Jones and Molitoris (1984); (2) Orr et al., (1982); (4) Cheng et al., (1992).

### Cardio-respiratory Responses

Peak cardio-respiratory responses are shown in Table 7.4. At SL participants achieved a mean peak of 243 ± 58 W, which was associated with a O2peak of 3.39 ± 0.99 L·min-1, ventilation (Epeak) of 133.90 ± 41.17 L·min-1 and HRpeak of 180 ± 11 b·min-1. In hypoxia, there was an overall decline in peak (F(4,52) = 9.116, P ≤ 0.001, Ω2 = 0.40) and O2peak (F(4,52) = 3.443, P = 0.014, Ω2 = 0.36)which was significantly lower than SL at 4000 m for both measures(O2peak; t(13) = 3.240, P = 0.006, r = 0.67, peak; t(13) = 4.372, P = 0.001, r = 0.77). In contrast, neither HRpeak nor Epeak differed in hypoxia compared to SL. The SPO2 was also significantly reduced overall (F(4,52) = 39.375, P ≤ 0.001, Ω2 = 0.98) which was significantly lower than SL at 3000 (t(13) = 4.825, P ≤ 0.001, r = 0.80) and 4000 m (t(13) = 7.796, P ≤ 0.001, r = 0.91).

Table 7.. Peak cardio-respiratory responses to graded exercise.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | SL | 1000 m | 2000 m | 3000 m | 4000 m |
| peak (W) | 243 ± 58 | 239 ± 57 | 236 ± 55 | 229 ± 57 | 216 ± 43\* |
| O2peak  (mL·kg-1·min-1) | 46.34 ± 7.00 | 44.89 ± 9.78 | 44.93 ± 8.34 | 42.57 ± 10.24 | 40.45 ± 7.41\* |
| Epeak (L·min-1) | 133.90 ± 41.17 | 128.10 ± 34.55 | 132.21 ± 32.52 | 132.63 ± 33.08 | 133.44 ± 27.78 |
| HRpeak (b·min-1) | 180 ± 11 | 178 ± 11 | 178 ± 9 | 178 ± 9 | 175 ± 10 |
| SPO2 (%) | 95 ± 5 | 95 ± 2 | 92 ± 2 | 89 ± 3\* | 83 ± 4\* |

**Note:** \*Significantly lower than SL, P ≤ 0.05.

## Discussion

The primary aim of the present study was to examine the effect of varying inspired O2 concentrations (hypoxia) on the VT response. In respect to the first aim there was no significant change in O2VT with hypoxia. However, a significant change in VT relative to O2peak using algorithms 2 and 4, and a significant reduction in O2peak and peak were identified. The secondary aim was to compare the O2VT, HRVT and WVT identified by the different algorithms (1, 2 and 4), and these were found to be in agreement.

Despite the advantages of VT assessments, in particular for individuals for whom maximal exercise is contraindicated, there have been relatively few measurements of the VT in response to acute hypoxia. This despite exercise in hypoxia offering cardiovascular benefits ([Cerretelli, 1980](#_ENREF_44); [Friedmann, et al., 2005](#_ENREF_94)) and possibly enhancing weight loss in the obese ([Quintero, et al., 2010](#_ENREF_223)). Using the algorithms which produced the least indeterminate cases [1, 2 and 4 ([Cheng, et al., 1992](#_ENREF_50); [Jones & Molitoris, 1984](#_ENREF_139); [Orr, et al., 1982](#_ENREF_211))] a significant overall change in O2VT was identified in hypoxia compared with SL, however post-hoc analyses failed to distinguish the direction of change which differs from previous research ([Fukuoka, et al., 2003](#_ENREF_95); [Hughson, et al., 1995](#_ENREF_132); [Ozcelik & Kelestimur, 2004](#_ENREF_213)) reporting a reduction. It can therefore be concluded that the differences observed were due to individual variation and there was no change in the VT with hypoxia, which can be supported by research conducted at an equivalent altitude of 1500 m ([Mateika & Duffin, 1994](#_ENREF_184)). A reduction in VT with hypoxic exposure is often attributed to increased sympathetic activity and glycolytic flux at a given work rate resulting in an accelerated efflux of lactate and H+ which subsequently increases the ventilatory drive ([Subudhi, et al., 2006](#_ENREF_268)). Consequently, it can be concluded that such an acute bout of hypoxic exposure may not increase sympathetic drive and thus reduce the VT via an increase in ventilatory drive. Moreover, other research suggests that the changes in plasma lactate concentration observed during exercise may not be directly related to FIO2 ([Mateika & Duffin, 1994](#_ENREF_184)) and could therefore explain why a reduction in VT is observed below a FIO2 of 0.14 (3000 m) but not above. Another possible explanation is that lactate concentration is only elevated in more severe hypoxic conditions (i.e. at or above 4000 m). According to others ([Overgaard et al., 2012](#_ENREF_212)) arterial lactate concentrations in severe hypoxia (FIO2 0.10) during exercise is 10 times greater of that observed in normoxia with the same workload ([Amann, et al., 2006](#_ENREF_4)), however at moderate altitudes (FIO2 0.15) similar capillary lactate concentrations between hypoxic and normoxic conditions have been reported during cycling time trial performance. Therefore, from this evidence a reduction in VT would not be expected below 3000 m. As such it is also understood that a lack of lactate and pH measurements is a limitation of the present study and future studies should if feasible look to include these measures.

Previous research has demonstrated that WVT is a more accurate predictor of physical performance (e.g. time trial performance) than O2peak ([Amann, et al., 2006](#_ENREF_4); [Lucia et al., 2004](#_ENREF_169); [Subudhi, et al., 2006](#_ENREF_268)). Research has previously demonstrated that WVT is reduced with hypoxic exposure by up to 41% ([Subudhi, et al., 2006](#_ENREF_268)), in the present study algorithms 1 and 2 did not confirm such findings suggesting that acute hypoxic exposure does not reduce WVT. However, Algorithm 4 produced an overall change in WVT but similar to O2VT the direction of change could not be identified. In summary, it can be concluded that hypoxia had no effect on the VT determined via a GXT. Those studies which reported a significant reduction in O2VT were performed at or above 4000 m ([Fukuoka, et al., 2003](#_ENREF_95); [Ozcelik & Kelestimur, 2004](#_ENREF_213); [Subudhi, et al., 2006](#_ENREF_268)) which may possibly explain the disparity between results. However, it is difficult to clarify whether SPO2 were similar between studies as previous research failed to report these data. Moreover, methodological differences in acclimatisation status, training status, hypoxic exposure duration and the method used to identify the VT may contribute to the differences in findings.

Identification of the VT was similar with all algorithms thus no significant differences between algorithms were observed in any of the conditions (SL, 1000, 2000, 3000 and 4000 m). These results suggest that when prescribing exercise intensity using the VT, algorithms 1, 2 and 4 can be used interchangeably. Interestingly, both algorithms 3 and 5 were excluded due to the number of indeterminate cases, as both these algorithms use a similar method of identifying the VT this could be a possible explanation for their inability to do so in hypoxia. Both algorithms 3 and 5 fit linear regressions through all possible divisions of the data in to two contiguous groups. The only difference between these two algorithms is the point at which the ratio of the distance of the intersection point from a single regression line through the data to the mean square error of regression is identified. Algorithm 3 identifies the VT where the ratio is maximised whereas Algorithm 5 scans the data from left to right and stops as soon as its criterion was satisfied [the slope of the second regression through the CO2 by O2 data exceeding 1.0 ([Sue, et al., 1988](#_ENREF_269))]. Therefore, the method used by Algorithm 5 seems inappropriate for the data provided in this study, and the pattern of searching for a solution (i.e. not considering all possibilities) probably accounted for the number of indeterminate cases present ([Ekkekakis, et al., 2008](#_ENREF_77)). It is therefore recommended that thresholds determined using algorithms 3 and 5 should be avoided or interpreted with caution.

Our findings of reduced O2peak and peak are in line with several other investigations performed at and above 4000 m ([Ferretti, et al., 1997](#_ENREF_85); [Lawler, et al., 1988a](#_ENREF_155); [Ozcelik & Kelestimur, 2004](#_ENREF_213); [Stenberg, et al., 1966](#_ENREF_264)). The reduction in O2peak is often attributed to a reduction in SPO2 content, caused by desaturation ([Benoit, et al., 2003](#_ENREF_23); [Calbet et al., 2003](#_ENREF_39); [Stenberg, et al., 1966](#_ENREF_264)). However, at altitudes above 4000 m the reduction in O2peak and exercise capacity is substantially larger than that expected only from a reduction in arterial O2 content. Thus, it is important to note that the reduction in O2peak may also be due to an impairment of pulmonary gas exchange, a reduction in maximal cardiac output and a reduction in peak leg blood flow ([Calbet, et al., 2003](#_ENREF_39)). It is also important to highlight that the lack of change in O2peak at 2000 m and 3000 m found in the present work is surprising since previous authors have noted that performance is reduced by 1% every 100 m ascended above 1500 m ([Amann & Kayser, 2009](#_ENREF_3)). However, it is believed that training status may account for the results shown, it has been identified by previous research that the detrimental effects of hypoxia are greater in individuals with higher aerobic capacity ([Chapman et al., 1999](#_ENREF_47); [Ferretti, et al., 1997](#_ENREF_85); [Gore et al., 1996](#_ENREF_111); [Roach et al., 2000](#_ENREF_233); [Wehrlin & Hallen, 2006](#_ENREF_285)) due to greater hypoxemia in highly trained individuals. Therefore, the untrained status of the individuals used in the present study could explain why the reduction in O2peak and peak were not observed at or below 3000 m.

A limitation within the present study was that samples were not measured on two separate occasions for each hypoxic condition; primarily this was due to agreement of algorithms to identify the VT being a secondary aim of the study. Duplicate tests in each condition would have allowed for the determination of the tendency for certain pairs of methods to yield consistent estimates regardless of the data set, suggesting a systematic agreement and to also identify whether there was a tendency for certain methods to consistently (regardless of data set) yield higher or lower estimates compared with other methods ([Ekkekakis, et al., 2008](#_ENREF_77)). Future research should take this direction.

In conclusion, findings from the present study suggest that below 4000 m there is no change in the VT with exposure to acute normobaric hypoxia, this was the case for all algorithms analysed, supporting that the VT as a measure of endurance can be utilised within training programmes prescribed in hypoxia. Moreover, there was no significant difference between algorithms for O2, HRVT, WVT or VT relative to O2peak. Therefore, results of the present study suggest that the VT is a more suitable measure than HRpeak (Chapter 6) for exercise prescription in hypoxia. Moreover due to its sub-maximal nature use of the VT is ideal for use in populations in which maximal exercise may be contraindicated. This method should be used instead of HRpeak when prescribing exercise intensities in acute hypoxia.

# General Discussion

## Introduction

Obesity, worldwide is an ever increasing epidemic, and as the percentage of individuals who are classified as obese increases at a rapid rate, obesity and its associated health problems now represent a major health burden to society. Over the last decade, there has been a compelling demand for innovative weight loss programmes which appoint several methods (e.g. dietetic and nutritional managements, physical activity programmes, pharmacological managements and bariatric surgery) yet effective therapies for obesity still remain elusive. Original investigations have reported that hypoxia may produce changes in body composition ([Armellini, et al., 1997](#_ENREF_10); [Boyer & Blume, 1984](#_ENREF_32); [Lippl, et al., 2010](#_ENREF_165)), which have been further supported by reports of a reduction in body mass in lowlanders staying at altitude ([Boyer & Blume, 1984](#_ENREF_32); [de Glisezinski, et al., 1999](#_ENREF_67); [Fulco, et al., 1985](#_ENREF_96); [Lippl, et al., 2010](#_ENREF_165); [Pugh, 1962](#_ENREF_218); [Rose, et al., 1988](#_ENREF_238)). If correct, this would have important implications for overweight/obese individuals, as the use of hypoxia within weight loss programmes may stimulate reductions in body mass. As chronic exposure to hypoxia is not a practical nor feasible method for most humans, research in to the role of intermittent hypoxia within weight loss management programmes is required.

The purpose of the research described in this thesis was to investigate the role of hypoxia as a non-pharmacological therapy for weight loss. It was established in the first experimental chapter of this thesis (Chapter 2) that acute physiological hypoxia (FIO2 0.05) had no detrimental effects on the growth and development of C2C12 satellite cell myoblasts. However, in Chapter 3 acute hypoxia (FIO2 0.05, 24 h) up-regulated the mRNA expression of atrogenes MURF-1 and MAFbx in myotubes, which have been associated with skeletal muscle atrophy. Moreover, myostatin, a gene involved in muscle hypertrophy was down-regulated. Therefore, hypoxia appears to have differential effects on myoblasts and myotubes. In chapters 4 and 5, the role of IHE was explored in humans at a whole-body level. The IHE protocol chosen for this study was based upon a typical exercise programme which would be used within weight loss programmes. It was demonstrated that a 4-week programme of IHE had no beneficial effects for body composition or associated metabolic health risk markers [(e.g. LDL, HDL, TC) Chapter 4], nor did it have any effect on serum levels of adiponectin and leptin (Chapter 5). These results suggest that data from previously published IHT studies are a result of the combined hypoxia and exercise, not intermittent hypoxia per se. This finding therefore led to the requirement to identify an appropriate method to determine exercise intensity and monitor exercise training progression in acute normobaric hypoxia. In Chapter 6 the use of age-derived equations to predict HRpeak were examined and in Chapter 7 the VT method was explored. As a result of this exploration it was concluded that HRpeak is not an accurate method for the prescription of exercise intensity in acute hypoxia. However, the VT does not change with acute hypoxia and therefore could be used to determine exercise intensity in future IHT studies.

## Acute Hypoxia and Skeletal Muscle

Despite previous reports that hypoxia causes an increase in myoblast proliferation ([Chakravarthy, et al., 2001](#_ENREF_46); [Csete, et al., 2001](#_ENREF_61); [Zhao, et al., 2003](#_ENREF_303)), the results reported in this thesis demonstrate that exposure to physiological hypoxia (FIO2 0.05) for 24 hours does not elicit a different response in C2C12 satellite cell myoblast growth and development to that observed in normoxic conditions (FIO2 0.209). Total cell count was 50%, 20%, 8% and 4% lower in hypoxia compared with normoxia at 24, 48, 72 and 96 hours, respectively (Table 8.1). As a result, the greatest reduction was observed in the earliest stages of C2C12 myoblast development. Moreover, cell viability was not significantly reduced following 24 hours of hypoxia compared with normoxia at any growth phase. It was concluded from these results that unlike the cascade of events which occur under severe hypoxia leading to cell apoptosis it appears that during acute hypoxia myoblasts are able to sense and adapt to the low O2 environment. This is achieved through the cells ability to activate a number of genes which are involved in metabolic adaptation, and further stimulate cell proliferation allowing for increased oxygenation of the tissue ([Greijer & van der Wall, 2004](#_ENREF_114)). Therefore, suggesting that the effect of hypoxia on skeletal muscle is both dependent upon the length of exposure and the O2 concentration, which appear to play a crucial role in the maintenance of cell viability.

Table 8.. Summary of findings (Chapter 2).

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Time (h) | | | | | | | |
|  | **24** | | **48** | | **72** | | **96** | |
|  | N | H | N | H | N | H | N | H |
| Cell count | 7 | 3 | 5 | 4 | 27 | 23 | 52 | 51 |
| Total cell count | 130000 | 60000 | 90000 | 70000 | 530000 | 450000 | 104000 | 1020000 |
| Cell viability (%) | 53 ± 19 | 88 ± 18 | 100 ± 0 | 67 ± 47 | 96 ± 6 | 87 ± 19 | 95 ± 7 | 95 ± 7 |
| Myf5 mRNA expression | 1.42 ± 0.82 | 1.60 ± 0.47 | 1.76 ± 0.33 | 1.66 ± 0.52 | 2.04 ± 0.28 | 1.79 ± 0.64 | 2.35 ± 0.61 | 1.87 ± 0.71 |
| MyoD mRNA expression | 0.88 ± 0.28 | 1.35 ± 0.74 | 0.94 ± 0.13 | 0.94 ± 0.11 | 0.95 ± 0.49 | 0.77 ± 0.29 | 1.04 ± 0.36 | 1.05 ± 0.24 |
| Myogenin mRNA expression | 2.32 ± 0.92 | 5.40 ± 3.76 | 1.07 ± 0.80 | 0.80 ± 0.18 | 2.32 ± 0.22 | 0.70 ± 0.46 | 1.69 ± 0.60 | 1.86 ± 0.85 |

**Note:** N; Normoxia, H; Hypoxia.

In addition to the maintenance of cell viability following hypoxic exposure, it was also demonstrated that exposure to physiological hypoxia (F O2 0.05) did not significantly change the expression of genes associated with the proliferation of C2C12 myoblasts (Chapter 2; Table 8.1). Although a change in proliferative capacity with low O2 concentrations has been associated with both up- and down-regulation of genes associated with cell proliferation ([Csete, et al., 2001](#_ENREF_61); [Di Carlo, et al., 2004](#_ENREF_69); [Zhao, et al., 2003](#_ENREF_303)), the results presented in Chapter 2 of this thesis do not support either response. It is thought that the disparity between results of the present study and of others may be related to the number of passages the cells have undergone prior to the experiments; cells used in the present study cells were at passage 7. It has been shown that at low passages (0-5) an increase in proliferative capacity is observed, however at higher passages (6-8) the response is diminished ([Koning, et al., 2011](#_ENREF_151)). Thus, further experiments should establish the response of myoblast proliferation at both low and high passages. To summarise, acute exposure to hypoxia (≤ 24 hours) does not result in reduced myoblast growth and development and thus does not appear to be the cause of the reduction in lean muscle mass often reported with high-altitude exposure.

In Chapter 3, both 90 minutes and 24 hours of hypoxic exposure (FIO2 0.05) had a significant effect on the mRNA expression of MURF-1, and MAFbx. Following 24 hours hypoxia this was demonstrated alongside an increase in myostatin and a decrease in IGF-1 in C2C12 myotubes (Table 8.2). Following 24 hours of exposure, the increase in MURF-1 and MAFbx mRNA expression is thought to be directly linked to the increase in myostatin and decrease in IGF-1 mRNA which was also observed. Under normal cell culture conditions, IGF-1 signalling is dominant and blocks the myostatin pathway; therefore under conditions of skeletal muscle atrophy IGF-1 is inhibited allowing for increased expression of myostatin ([Amirouche, et al., 2009](#_ENREF_6); [Elkina, et al., 2011](#_ENREF_78)). Thus, this cascade of events inhibits the activation of Akt phosphorylation which activates foxO transcription and subsequently leads to increased expression of MURF-1 and MAFbx resulting in protein degradation ([McFarlane, et al., 2006](#_ENREF_188)). It is thought that this pathway is responsible for the hypoxia-induced loss in fat-free mass. The results presented in Chapter 3 suggest that 24 hours of hypoxia (FIO2 0.05) may lead to increased protein degradation and skeletal muscle atrophy in myotubes via the IGF-1/PI3k/Akt pathway. Following 90 minutes exposure, myostatin was not measured but IGF-1 was and remained unchanged. This disparity between 90 minutes and 24 hours may be exposure time dependent. However, it is possible that IGF-1 had started to be secreted but was not yet expressed at the mRNA level and thus an increase in MURF-1 and MAFbx was still observed, albeit in smaller amounts. Since higher levels of mRNA were expressed following 24 hours exposure compared with 90 minutes in can be concluded that hypoxia-induced protein degradation may be time-dependent which indicated that smaller time exposures are necessary for human studies.

**Table 8.2. Summary of findings (Chapter 3).**

|  |  |  |  |
| --- | --- | --- | --- |
| Time |  | Condition | |
|  |  | **Normoxia** | **Hypoxia** |
| 90 mins | MURF1 | 1.31 ± 0.11 | 1.56 ± 0.05\* |
| MAFbx | 1.09 ± 0.13 | 1.54 ± 0.31\* |
| IGF-1 | 0.91 ± 0.23 | 0.91 ± 0.20 |
| HIF-1α | 1.11 ± 0.09 | 1.17 ± 0.03 |
| 24 h | MURF-1 | 2.79 ± 1.04 | 4.22 ± 1.31\* |
| MAFbx | 1.11 ± 0.20 | 2.79 ± 0.85\* |
| Myostatin | 2.14 ± 1.10 | 2.77 ± 1.10\* |
| IGF-1 | 4.60 ± 0.74 | 3.81 ± 0.93† |
| HIF-1α | 0.63 ± 0.14 | 0.67 ± 0.18 |

**Note:** \*P ≤ 0.05, significantly higher than normoxia; †P ≤ 0.05, significantly lower than normoxia.

In summary, Chapter 2 of this thesis demonstrates that acute hypoxia (FIO2 0.05) does not negatively disturb cell homeostasis. It appears that the growth and development of C2C12 satellite cell myoblasts was maintained at levels similar to those observed in normoxia (FIO2 0.209). Moreover, genes associated with cell proliferation remained stable which suggest that the cell is able to adapt to this level of hypoxia. In Chapter 3, the results suggest that increased protein degradation is present in C2C12 myotubes following both 90 minutes and 24 hours of hypoxic exposure. The idea supporting this experimental work was that with chronic exposure to high-altitude, a significant loss in skeletal muscle mass is observed ([Boyer & Blume, 1984](#_ENREF_32); [Fulco, et al., 1985](#_ENREF_96); [Hoppeler, et al., 2008](#_ENREF_127); [Sergi, et al., 2010](#_ENREF_253)); however the acute response is substantially under investigated. Therefore, these results are of significant importance to humans who are to be exposed to acute bouts of hypoxia such as those used within IHE programmes. It is suggested that when designing IHE programmes that the length of each exposure is no more than 90 minutes until further research has been carried out. Further studies are required to establish an optimal exposure length such that individuals receive maximal physiological benefits while maintaining lean muscle mass.

## Intermittent Hypoxic Exposure and Weight Loss

Intermittent hypoxic exposures, with no prescribed physical activity for 4 weeks did not significantly change parameters associated with body composition when compared with a normoxic control period (Chapter 4). Body mass was 68.1 kg post-IHE and 68.8 kg post-control, the pattern was similar for BMI, and also the percentage of body fat. In addition, there was no significant change in parameters associated with fat and glucose metabolism (RER, REE, RMR, fasting glucose, LDL, HDL, TC, TG), cardiovascular health (DBP, SBP, resting heart rate) and aerobic capacity (O2peak). Given that the findings demonstrated no significant effects, the results observed were attributed to the chosen protocol. However, this was chosen to mimic the typical duration and frequency of exercise sessions which may be used in a weight reduction intervention programme ([Armstrong, et al., 2006](#_ENREF_11); [McQueen, 2009](#_ENREF_189)). Moreover, the protocol has been used by others previously reporting a small but significant reduction in body mass in lean individuals ([Rodriguez, et al., 2000](#_ENREF_237)). Therefore, it was expected that significant changes would be observed, future work should establish an effective hypoxic dose (duration x O2 concentration).

The hypoxic dose for IHE programmes designed for weight loss is a new concept which requires further exploration. Since prolonged exposure to altitude (2650 m) at rest has resulted in significant losses in body mass ([Lippl, et al., 2010](#_ENREF_165)) it is thought that rather than simulated altitude height, it is the duration of exposure, and the numbers of exposures per day and per week that are most important for establishing an effective non-pharmacological weight loss therapy. Other research conducted using animal models has demonstrated significant losses in body mass with exposure times ranging from 30 seconds to 48 hours for up to two months ([Germack, et al., 2002](#_ENREF_102); [Ling, et al., 2008](#_ENREF_164); [Martinez, et al., 2010](#_ENREF_182)). However, although these are very promising they require prolonged periods of hypoxic exposure time which are not feasible for human individuals to partake in. Thus, the hypoxic dose used in this thesis to examine the effects of IHE on weight loss was chosen for its practicality and to mimic a typical exercise programme designed for weight loss ([Armstrong, et al., 2006](#_ENREF_11); [McQueen, 2009](#_ENREF_189)).

Moreover, one study has reported a small but significant reduction in body mass in lean individuals exposed to IHE, however, this research was conducted using a hypobaric hypoxic chamber ([Rodriguez, et al., 2000](#_ENREF_237)). It has been suggested that there are differences in the physiological responses to normobaric and hypobaric hypoxia ([Millet, et al., 2013](#_ENREF_192); [Savourey, et al., 2003](#_ENREF_248)). For example, Loeppky et al., ([1997](#_ENREF_168)) observed that at equivalent partial pressures of O2, participants showed a reduced ventilatory response to hypobaric hypoxia, with minute volume being 26% lower. This has been confirmed since by others ([Faiss, et al., 2013](#_ENREF_81); [Savourey, et al., 2003](#_ENREF_248)). Therefore, differences could extend further to other physiological responses (e.g. changes in body mass).

Overall, 4 weeks of IHE, ninety minutes three times weekly breathing an oxygen fraction of 0.105 did not elicit any obvious benefits for weight loss or metabolic health. Therefore it can be concluded that intermittent hypoxia alone does not stimulate the same responses that are observed when exercise training and hypoxia are combined. Thus, the results in this thesis suggest that to observe positive health benefits, exercise and hypoxia should be combined, however the optimal exercise modality, intensity and duration remains to be identified. Yet, as this is the first study to our knowledge to explore the use of IHE as a non-pharmacological weight loss therapy it could be that the optimal hypoxic dose was not used. However, it was shown that the protocol used was safe and had no apparent adverse effect, this is particularly important for the development of a protocol which is designed for use in unconditioned humans. Exploring alternative exposure times, hypoxic doses and programme lengths however could still play an important role in hypoxia and weight loss research, and the combination of exercise could prove fruitful in establishing an effective programme.

## Intermittent Hypoxic Exposure and Appetite Hormones

In Chapter 5, the effect of IHE on two key appetite hormones, leptin and adiponectin was examined. The results demonstrated that IHE reduced leptin by 22% compared with a 6% increase following the control period. The results followed a similar pattern for adiponectin, but in this instance adiponectin was increased by 17% following IHE and decreased by 28% following control. Although both leptin and adiponectin changed as a result of IHE the results were not significant due to the combination of large inter-subject variation and the limited sample size. In addition, although the response of adiponectin was uniform, leptin values were skewed by an anomaly in the data, where in 3 of the 5 participants leptin remained unchanged, but for one individual it was reduced by 33% following IHE. This anomaly result may be attributable to this individuals higher BMI. A previous study suggests that caution should be taken when data collected from lean individuals is extrapolated to overweight or obese individuals due to differences in tissue oxygenation ([Haufe, et al., 2008](#_ENREF_122)). Therefore, it cannot be firmly concluded from the data presented in this thesis that IHE does not reduce leptin in overweight or obese individuals.

Previous studies in rodents have suggested that IHE significantly alters both leptin ([Polotsky, et al., 2003](#_ENREF_215); [Qin, et al., 2007](#_ENREF_221)) and adiponectin ([Magalang, et al., 2009](#_ENREF_173); [Zhang, et al., 2010](#_ENREF_302)). Additionally, in humans, chronic hypoxia has been shown to produce a significant increase in leptin levels, which is often concomitant with reductions in body mass ([Lippl, et al., 2010](#_ENREF_165); [Shukla, et al., 2005](#_ENREF_256)). As previously mentioned, no change in serum leptin or adiponectin levels was observed. It is therefore concluded that since it is difficult to discern the cause and effect relationship between appetite hormones and weight loss, no changes were observed in leptin and adiponectin as a result of no change in body mass, since the two responses act simultaneous to each other. However, it was important to study these parameters within this thesis in order to establish the efficacy of the approach used since it is a relatively new method which has yet to be used within human research.

## Acute Hypoxia and Exercise Intensity

In this thesis it was reported that whilst IHE did not have any positive effects on body mass or associated metabolic health risks, it did not appear to have any detrimental effects either. The findings reported in this thesis therefore suggest that in order for IHE to be effective it must be combined with exercise. In order for any exercise training to be successful, appropriate exercise intensities need to be prescribed and monitored to ensure progress and training adaptations occur. Since exhaustive exercise is often contraindicated in overweight/obese individuals, the prescription of exercise intensity often uses predictive equations (e.g. percentage of HRpeak) or sub-maximal exercise tests (e.g. VT). Therefore in chapters 6 and 7 these methods were explored to ensure their accuracy in a range of normobaric hypoxic conditions.

The findings of Chapter 6 demonstrate that using age-derived HRpeak equations, as a method of determining exercise intensity in hypoxia, is inaccurate. It was shown that although measured HRpeak was not significantly reduced with acute hypoxia, the range of age-derived predictive equations utilised significantly overestimated HRpeak, in most instances at the equivalent altitudes of 1000, 2000, 3000, and 4000 m. Therefore, the use of age-derived equations to predict HRpeak would appear inappropriate for use in hypoxic conditions and the measurement of HRpeak during a GXT would be deemed necessary for accurate determination of exercise intensity. Consequently, this method is not suitable for those individuals in which exercise may be contraindicated (i.e. overweight/obese).

The finding of Chapter 6 led to the exploration of the VT identification as an alternative sub-maximal method to determine exercise intensity in acute hypoxia, these results are presented in Chapter 7. As the VT can be identified at sub-maximal exercise intensities ([Wasserman & McIlroy, 1964](#_ENREF_283)) it provides a better measure of exercise intensity for individuals who are unaccustomed to high-intensity exercise or for which exhaustive exercise is contraindicated. It was shown that acute hypoxia does not significantly affect the VT during a GXT performed on a cycle ergometer up to a simulated altitude of 4000 m. Consequently, these results suggest that the VT is a suitable method to determine exercise intensity in hypoxic training programmes. However, at present this application only applies to acute hypoxic exposures such as those used in IHE programmes. Chronic exposure to hypoxia may result in a different response, in fact it has been shown to reduce the VT for up to 12 days and after acclimatisation it was shown to be decreased further (Myers et al., 2008). Therefore, acute and chronic exposures to hypoxia should always be explored both separately and in tandem.

The efficacy of five different algorithms to identify the VT was also explored in Chapter 7 to determine whether they produced similar results. Although two of the algorithms did not satisfy the assumptions placed upon them for all the data sets, the remaining 3 algorithms produced similar results (Table 8.3). The data also followed a related pattern for other parameters measured at the VT (i.e. power, heart rate). These data suggest that these algorithms can be used interchangeably for identifying the VT in normoxia and normobaric hypoxia.

Table 8.. Estimates of the ventilatory threshold, expressed as oxygen cost (O2VT), determined by 3 algorithms.

|  |  |  |  |
| --- | --- | --- | --- |
| Algorithm | | | |
|  | **1** | **2** | **4** |
| SL | 1.98 ± 0.46 | 2.15 ± 0.63 | 1.95 ± 0.47 |
| 1000 m | 2.03 ± 0.61 | 2.16 ± 0.70 | 2.05 ± 0.61 |
| 2000 m | 2.27 ± 0.62 | 2.47 ± 0.74 | 2.29 ± 0.55 |
| 3000 m | 1.84 ± 0.50 | 1.87 ± 0.61 | 1.80 ± 0.50 |
| 4000 m | 2.29 ± 0.58 | 2.36 ± 0.63 | 2.25 ± 0.54 |

**Note:** Algorithms (1) Jones and Molitoris (1984); (2) Orr et al., (1982); (4) Cheng et al., (1992).

Overall, those data presented in chapters 6 and 7 suggest that when determining exercise intensity in studies which are to utilise acute normobaric hypoxic exposes (e.g. IHE), HRpeak will not give an accurate comparison between the two conditions. However, the use of the VT appears to be a suitable method and it is recommended that this parameter is used to determine exercise intensity in normobaric hypoxia, especially for those individuals in which exhaustive exercise is contraindicated.

## Conclusions

The aims of this research were designed to assess the use of hypoxia as a non-pharmacological therapy for overweight/obesity. It is well documented that exposure to high-altitudes causes a reduction in body mass ([Armellini, et al., 1997](#_ENREF_10); [Boyer & Blume, 1984](#_ENREF_32); [Lippl, et al., 2010](#_ENREF_165)). Although a number of contributors to this observed weight loss have been put forward, the identification of a single cause, if any, remains elusive. Nevertheless it was rationalised that since chronic hypoxia contributes to losses in body mass, intermittent hypoxia may in fact cause a similar response, and this has been supported by research using animal models ([Germack, et al., 2002](#_ENREF_102); [Ling, et al., 2008](#_ENREF_164)). The research presented in this thesis provides a valuable insight into how hypoxia may be used within weight loss research and lays the foundations for future research in this area. Cell culture studies laid the foundations for the data collected in the later stages of the programme of study. In these studies it was demonstrated that acute normobaric hypoxia does not have any negative effects on skeletal muscle myoblasts. However, the response was different in skeletal muscle myotubes where data suggest that protein degradation may be present or at least stimulated. This latter finding suggests an IHE approach might be the best to take in order to enhance fat loss. Of course, before including exercise in any in vivo study it was necessary to establish if IHE alone was sufficient to cause weight loss. Unfortunately, on this occasion the findings demonstrate that IHE alone did not result in weight loss. Additionally, no obvious benefits were observed for improvements in metabolic health risk markers. However, as previously discussed it is expected that by altering the hypoxic dose, different responses may be identified. These findings provide justification for combining exercise with hypoxia, with those data presented in this thesis demonstrating that the VT is an appropriate way to determine exercise intensity and monitor progress within any IHT programmes prescribed.

## Recommendations for Future Research

The studies provided in this thesis provide a basis from which to continue further research in this area, particularly regarding the effect of IHE with or without exercise training on weight loss and parameters associated with metabolic health. The main topics in need of further exploration are described below.

**Cell Lines:** All in vitro experimental work presented in this thesis used the mouse myoblast C2C12 cell line which provided a good model for the studies conducted. Future experimental work should continue to explore different hypoxic doses to understand the mechanisms which underpin protein synthesis and protein degradation in response to hypoxia-induced skeletal muscle atrophy. Although the use of this cell line has many benefits (e.g. cheap, easily accessible and a strong evidence base), alongside the use of this method, any new research produced should be supported with cells obtained from human skeletal muscle using biopsy techniques. This work will strengthen the conclusions produced and will provide greater ecological validity.

**Skeletal Muscle PIO2:** Oxygen concentration in skeletal muscle cells in vivo is documented to be between 1 and 10% ([Sato, et al., 2011](#_ENREF_247)) however although these values are widely accepted as values observed in normal O2 conditions (FIO2 0.209), the physiological range under hypoxic conditions are poorly documented. To gain an understanding of skeletal muscle responses to hypoxia, it is imperative that research identifies and documents skeletal muscle O2 concentration under a range of O2 concentrations. Results from such studies would provide researchers with an important insight in to the effects of hypoxia on skeletal muscle under both in vivo and in vitro comparisons. Thus, allowing a direct comparison between the two methods which would provide a strong evidence based background for future work in this area.

**Hypoxic Dose:** Although the hypoxic dose (FIO2 0.105) used in chapters 4 and 5 were suitable to expose individuals for 90 minutes and equally sufficient enough to elicit temporary changes in SPO2, no prolonged adaptations in body composition parameters were observed. Since, to our knowledge, this was the first study of its kind in a human population, research is required to determine the optimal dose required to stimulate prolonged changes in body composition. This work will involve making changes to session exposure time, the number of exposures per week, the length of IHE programme and stimulated altitude height.

**Exercise Modality:** For the purpose of the thesis it was necessary that intermittent hypoxic exposures were examined at rest, therefore more work is needed to identify the additional effects of exercise. Previous work published in the literature has explored some exercise modalities, such as the treadmill, cycle ergometer and stepper ([Haufe, et al., 2008](#_ENREF_122); [Netzer, et al., 2008](#_ENREF_202); [Wiesner, et al., 2010](#_ENREF_295)). However, anecdotal evidence has highlighted that for overweight/obese individuals an upright cycle ergometer is uncomfortable and the excess fat carried during treadmill exercise is problematic for the knee and ankle joints. Therefore, it is proposed that the recumbent cycle ergometer is trialled as an alternative exercise modality in hypoxia.

**Exercise Intensity:** Previous studies which have examined the use of IHT as a non-pharmacological therapy for obesity have used exercise intensities which can be classified as moderate [60 - 65% HRpeak/O2peak ([Netzer, et al., 2008](#_ENREF_202); [Wiesner, et al., 2010](#_ENREF_295))]. Therefore, there is still research to be conducted which examines the role of high intensity interval training (HIIT). The use of HIIT as a weight loss management method has become increasingly popular in recent years due to its ability to illicit positive changes in body mass with short training session durations. The health benefits of HIIT include reduced blood glucose, increased GLUT4 levels, reduced central body fat, and increased O2peak ([Gaesser & Angadi, 2011](#_ENREF_98)). It is thought that performing HIIT under hypoxic conditions may produce the same benefits of HIIT under normoxic conditions but at a lower exercise intensity which provides an additional benefit for those with a lower exercise capacity (e.g. overweight/obese individuals). Moreover, HIIT overcomes the “lack of perceived time” barrier to exercise which is one of the most frequently cited barriers ([Gaesser & Angadi, 2011](#_ENREF_98); [Godin et al., 1994](#_ENREF_108)).

**Outcome Measures:** Outcome measures are integral part of any exercise intervention as it allows the researcher/physiologist to monitor both positive and negative responses/adaptations to training. Individuals who carry excess body mass/fat experience a reduced quality of life ([Lean et al., 1998](#_ENREF_157)) and thus a primary goal for any weight loss intervention is to improve everyday lifestyle tasks such as stair walking, standing from a seated position, shopping, lifting etc. In future studies, the effect of IHE and IHT on quality of life measures should be examined in order to provide a broader application for the use of this method in overweight/obese individuals.

In summary, this thesis provides a base for which future research can expand upon, it explores how hypoxia can be used as a method for weight loss at rest and describes the additional benefits of exercise to this programme. Future work in this area should aim to examine the effect of differing hypoxic doses and the application of exercise and hypoxia.

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Appendices

**Appendix 1:** Health History Questionnaire

**Appendix 2:** Consent Form

**Appendix 3:** Information Sheet - IHE Study

**Appendix 4:** Information Sheet - Exercise Intensity Study

**Appendix 5:** Trypan Blue Exclusion Method

**Appendix 6:** RT-PCR Methods

**Appendix 7:** G\*Power Results

**Appendix 8:** SPO2 Data

**Appendix 9:** VT Protocol Data

**Appendix 10:** Normal Values for Biochemistry

**Appendix 11:** Publications

Appendix 1: Healthy History Questionnaire

[http://t1.gstatic.com/images?q=tbn:yjltLKaie6J7FM:http://www.graduatejobsouth.co.uk/cms/site/images/Uni%2520Chi%2520logo.jpg](http://www.google.co.uk/imgres?imgurl=http://www.graduatejobsouth.co.uk/cms/site/images/Uni%20Chi%20logo.jpg&imgrefurl=http://www.graduatejobsouth.co.uk/employers/courses-at-university-.aspx&usg=__FuQpDS4gAdELFUAJUgHnF-YevbU=&h=811&w=2741&sz=479&hl=en&start=2&zoom=1&um=1&itbs=1&tbnid=yjltLKaie6J7FM:&tbnh=44&tbnw=150&prev=/images?q=university+of+chichester&um=1&hl=en&sa=N&tbs=isch:1)Bishop Otter Campus,

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Before we can carry out any physiological tests on you, we have to check that you are in a reasonably healthy condition to undergo strenuous exercise. PLEASE complete the following questionnaire about yourself. ALL information will be treated as strictly confidential.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Name:** | | | **Date of Birth:** | |  | | | |
| **Specialist Sport:** | | | **Age:** | |  | **Sex: M F** | |
|  | | | | | | | |
| How best would you describe your current level of ‘activity’ in both work and recreation? | Sedentary | Moderate Activity | | Active | | | Highly Active |
| In terms of ‘fitness’ how would you best describe your present level? | Very Unfit | Medium Fitness | | Trained | | | Highly Trained |
| How do you view your ‘current bodyweight’? Are you? | Underweight | Ideal Weight | | Slightly Overweight | | | Very Overweight |
| Are you, or, have you ever been a ‘smoker’? If yes, how many did you smoke? | YES | | | NO | | | |
| Do you drink ‘alcohol’? If yes, do you consider yourself to be...? | YES | | | NO | | | |
| Have you had to consult your doctor in the last 6 months? If so, say briefly why? | YES | | | NO | | | |
| Have you suffered from a bacterial/viral infection in the last 2 weeks? If so, say briefly why? | YES | | | NO | | | |
| Are you taking any form of medication? If YES, give details: | YES | | | NO | | | |
| Are you a ‘diabetic’? If YES, give details: | YES | | | NO | | | |
| Are you a current or past’ Asthmatic’? If YES, give details: | YES | | | NO | | | |
| Have you ever suffered from ‘Bronchitis’? If YES, give details: | YES | | | NO | | | |
| Do you suffer from any form of ‘Heart Disease’? If YES, give details: | YES | | | NO | | | |
| Is there any ‘history’ of Heart Disease in your family? If YES, give details: | YES | | | NO | | | |
| Do you currently have any Muscular or Joint Injury? If YES, give details: | YES | | | NO | | | |
| Have you ever suffered from ‘Hepatitis’? If YES, give details: | YES | | | NO | | | |
| Have you ever had a ‘Blood Transfusion’? If YES, give details: | YES | | | NO | | | |
| Are you or have you ever been considered as at ‘risk’ from AIDS – [Acquired Immune Deficiency Syndrome]? | YES | | | NO | | | |
| Have you had to suspend your ‘normal’ training routine, for any reason in the last 2 weeks? If YES, give details: | YES | | | NO | | | |
| LASTLY, is there anything to your knowledge that could prevent you from completing the ‘tests’ that have been outlined to you? If YES, give details: | YES | | | NO | | | |
| **SIGN:** | | | **DATE:** | | | | |

Appendix 2: Consent Form

**Consent Form**

**Department of Sport, Exercise and Health Sciences**

PLEASE READ THE FOLLOWING CAREFULLY

Study title:..........................................................................................................

Please read the following statements carefully and tick the appropriate answer:

|  |  |  |
| --- | --- | --- |
| 1) I have read and understood the information sheet dated............... for this research project. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. | Yes | No |
| 2) I understand that my participation in the activity is voluntary and that I am therefore free to withdraw my involvement at any stage, without giving a reason. | Yes | No |
| 3) I confirm that I understand that there becomes a point in time after which it would be impractical to withdraw from the study and that the researcher has made this clear to me at the outset of the study. | Yes | No |
| 4) I understand that all information will be anonymised and that my personal information will not be released to any third parties. | Yes | No |
| 5) I consent to taking, use and storage of my blood and tissue samples as stated in the information sheet. | Yes | No |
| 6) I agree to the use of my data or results which will arise from my participation in this study and understand that my data or results will be limited to the use described in the information sheet. | Yes | No |
| 7) I am happy to participate in this research. | Yes | No |

Your name (please print).................................................................................

Your signature......................................................................... Date..................................

Principal investigator name.............................................................................

Principal investigators signature.............................................. Date..................................

**Thank you for your time**

Appendix 3: Participant Information Sheet

**PARTICIPANT INFORMATION SHEET**

[http://t1.gstatic.com/images?q=tbn:yjltLKaie6J7FM:http://www.graduatejobsouth.co.uk/cms/site/images/Uni%2520Chi%2520logo.jpg](http://www.google.co.uk/imgres?imgurl=http://www.graduatejobsouth.co.uk/cms/site/images/Uni%20Chi%20logo.jpg&imgrefurl=http://www.graduatejobsouth.co.uk/employers/courses-at-university-.aspx&usg=__FuQpDS4gAdELFUAJUgHnF-YevbU=&h=811&w=2741&sz=479&hl=en&start=2&zoom=1&um=1&itbs=1&tbnid=yjltLKaie6J7FM:&tbnh=44&tbnw=150&prev=/images?q=university+of+chichester&um=1&hl=en&sa=N&tbs=isch:1)

**Research Title:** The effect of short-term acute normobaric hypoxia at rest on metabolic markers

**Research Coordinators:**

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PhD Researcher **Email:** [C.Gallagher@chi.ac.uk](mailto:C.Gallagher@chi.ac.uk)

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Senior Lecturer Exercise Physiology **Email:** [S.Myers@chi.ac.uk](mailto:S.Myers@chi.ac.uk)

**Invitation to take part**

You are invited as a volunteer to take part in a research investigation. Before you decide to take part it is important for you to understand why the research is being conducted and what will be required of you should you agree to be involved. Please take time to read the following information carefully and discuss it with the investigators. Ask us if there is anything that is not clear or if you would like more information.

**Do I have to take part?**

This is entirely your decision. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. **If you decide to take part, you are still free to withdraw at any time without giving a reason**. If you decide not to participate or you wish to withdraw during the study, your decision will not affect your relationship with the University of Chichester, or any of the investigators involved in the study.

All information collected during the study will be coded and treated confidentially. The information will be kept in a locked filing cabinet and on the research coordinator’s password secured computer for 5 years before being confidentially destroyed. If you withdraw from the study, no new information about you will be collected by study personnel, although information about you that has already been collected may continue to be used in order to preserve the value of the study. If you have any questions or concerns about this, it is recommended that you contact the research co-coordinators listed above.

**Am I eligible to take part?**

To take part in this research study you must be a clinically normal weight presenting with a body mass index (your weight divided by the square of your height) above 19m·kg2. You will be excluded from the research study if you are hypertensive (high blood pressure), diabetic or report any symptoms of heart or breathing problems. Health status of all individuals will be determined via direct questioning and the completion of a health history questionnaire.

You will also be excluded if you are on specific types of medication or supplements (i.e. insulin, Beta Blockers), if you have been above 2500 m in the last month or have been a participant in other studies involving intermittent hypoxic/altitude exposure within the last three months.

**Background**

Although weight loss research findings are vast, effective treatments remain elusive. Pioneering investigations have reported that low oxygen concentrations (i.e. hypoxia) may produce changes in body composition. In support of investigations, reductions in weight have been consistently reported in lowlanders staying at high-altitude and in fact hypoxia has recently been used to induce weight loss in moderately obese individuals. This means that the hypoxic stimulus associated with this environment could be used as a useful tool to improve the effectiveness of weight loss programmes. Therefore, the purpose of the present study is to examine the effect of acute hypoxic exposure on metabolic changes and associated markers.

**Location**

This study will be carried out at the Bishop Otter Campus, University of Chichester, College Lane, Chichester, PO19 6PE. All testing will take place in a specialised environmental chamber located within the sport and exercise physiology laboratories. You will be informed of the room number as soon as room bookings have taken place and been confirmed.

**What will I be asked to do if I take part?**

If you decide to take part in this study you will be asked to schedule seventeen visits to the laboratory consisting of:

1. Project briefing,
2. Fitness testing (O2peak test),
3. Baseline measurements,
4. Twelve experimental sessions,
5. Fitness testing (O2peak), and
6. Post-test measurements

Prior to baseline and post-test measurements you will have to fast (not have anything to eat and only drink plain water) overnight for at least 8 hours before you arrive for your study visits.

The sessions are summarised below.

**Visit 1: Project briefing with question and answer session (1 hour)**

At this session, you will meet the lead investigator and be fully briefed about the requirements of the project. You will be talked through the subject information sheet by an investigator and given the opportunity to ask any questions. You will be given this information sheet to keep and given time to review the information and discuss your involvement in the study with significant others (family/friends) if you wish. This will also allow you additional time to think of questions regarding the study. Once you are fully satisfied with the information and on verbal agreement to take part in the study you will be asked to complete an informed consent form, medical questionnaire, physical activity questionnaire and arrange for further visits. A demonstration of any non-invasive techniques you are unsure of can be given during this time period if you are unclear or unsure of what is required.

**Visit 2: O2peak test (30 minutes)**

The O2peak test provides a quick and simple measure of an individual’s ability to perform aerobic exercise (exercise lasting more than 30 seconds). The O2peak test will be performed in a laboratory setting on a stationary cycle bike. Firstly you will complete a five minute warm up, for this you will cycle at 60 revolutions per minute against a 1.0kg load. Once warmed up you will perform an incremental test to exhaustion. Work load will start at 80 watts and will be increased by 30 watts (males) and 25 watts (females) every 90 seconds; you will cycle at 80 revolutions per minute. Once you are no longer able to maintain the required power output the test will be terminated. Measurements will be made of your heart rate, arterial oxygen saturation (level of oxygen in your blood) level of perceived exertion pre- and post-test.

**Note:** Measurement of the level of oxygen in your blood does not require a blood sample. Oxygen saturation is measured using a non-invasive device designed to measure levels of oxygen in the blood.

**Visit 3: Baseline measurements (2 hours)**

It is important that prior to baseline measurements you arrive well hydrated. All measurements will be taken following an overnight fast. No food or drink should be consumed for at least 8 hours prior to this session. Snacks and drinks will be provided following the session. It is also important that you do no strenuous physical exercise in the 24 hours before this session.

Baseline testing will include skin-fold measurements for the assessment of body fat, expired gas sampling for the determination of resting metabolic rate (RMR), measurements of resting heart rate and blood pressure, appetite ratings, six minute walk test (6MWT), blood sampling and muscle biopsies. All measures will be completed in normoxia between 8:00 and 12:00 a.m. following an overnight fast of at least 8 hours.

**Resting Metabolic Rate (RMR) and Respiratory Exchange Ration (RER):** Both RMR and RER will be assessed by what is known as indirect calorimetry performed in a seated upright position. As a participant you will be rested in a seated position 20 minutes prior to the expired gas collection. A face mask covering you mouth and nose will be positioned on the face by the investigator to assure an air tight seal. Expired gas will then be collected into a large bag (Douglas bag) via a low resistance valve and tube for 10 minutes (2 x 5 minute measurements).

**Resting cardiovascular parameters:** Heart rate and blood pressure will be recorded immediately following the RMR and RER measurements using an automated blood pressure cuff. You will be required to place your palm on a flat surface facing up. The blood pressure cuff will be placed 1-2cm above the elbow joint with the rubber tub running down the centre of the arm. The cuff will then be tightened till the tightness of the cuff is firm. The measurement will be taken from the left arm three times and the average value will be used as your resting value.

**Skinfold measurement:** Skinfold measurement’s are a measure of skinfold thickness and require an investigator to pinch the skin at appropriate sites to raise a double layer of skin and the underlying adipose (fat) tissue, but not the muscle. In this study skinfold measures will be taken from eight sites (2 x upper arm (front and back), 1 x upper back (just below shoulder blade as in image), 2 x hip, 1 x stomach (5cm from belly button), 1 x upper leg, 1 x mid lower leg)) using skinfold callipers. All measures will be taken in duplicate from the right side of the body with you as the participant standing in a relaxed position. Waist, hip, arm and mid-calf circumferences will also be recorded.

**Blood sampling:** A 10 ml intravenous blood sample will be taken from a vein in your arm on two occasions (baseline testing and post-testing). These samples will be taken at the same time of day, after an overnight fast (approximately 8 hours). Both samples will be taken by a qualified investigator, hygiene and protection procedures will be in place.

Following baseline measurements you will either be assigned to a low oxygen (hypoxia) group or normal oxygen (normoxia) group.

**Visits 4 to 15: Experimental sessions (1.5 hours per visit)**

Hypoxia or normoxia exposures will begin 5-7 days after baseline testing. You will be required to visit the laboratory three times a week each separated by 48 hours over a 4-week period. Each exposure will consist of 90 minutes of seated exposure to either normobaric hypoxia or normoxic conditions in an environmental chamber. If you are assigned to the hypoxia group the hypoxic environment will replicate an altitude of 5500 m. During the experimental sessions heart rate and arterial oxygen saturation will be monitored continuously using a portable pulse oximeter. The chamber will be set at a comfortable temperature and whilst you are seated you can read or work on a laptop etc.

**Note:** additionally, during the experimental period, once a week, measurements of body composition, heart rate and blood pressure will be taken following procedures outlined above under baseline measurements. Appetite ratings will also be recorded once a day, five times a week during the period of experimental sessions.

**Visit 16: O2 max test**

All procedures as above, as performed on visit 2.

**Visit 17: Post-test measurements**

All procedures as above, as performed on visit 3.

**Summary of time commitment:**

Project Briefing - 1 hour maximum

O2peak x 2 1 hour

Baseline Measurements - 2 hours

Experimental Trials - 22 hours

Post-test Measurements - 2 hours

**Total time commitment** **27 hours**

**Advantages of taking part**

A benefit of taking part in this study is that you will receive comprehensive feedback, with full explanations, on your body composition (e.g. % body fat). Additionally you will also receive feedback on blood pressure, heart rate and metabolism. Full reports on any data collected will be available on request. A further benefit is that you will be contributing to a wider research programme designed to optimise weight loss programmes.

**Disadvantages of taking part**

Time commitments to the study are relatively large; to complete all aspects of the study you will be required to attend the lab on fifteen occasions which accumulates to about twenty-six hours of your time. We understand that making such a commitment to attend and will try to be very efficient in our work and keep lab time succinct.

**What are the risks for taking part?**

It is expected that providing intravenous blood samples may cause slight bruising, some soreness to the skin, fainting and possibly psychological anxiety for those who have not experienced blood sampling previously. As with all blood samples there is a very minimal risk of infection at the site of needle insertion; however hygiene procedures will be in place to reduce this risk. However, blood sampling is a well established procedure and all procedures will be carried out by the principal investigator, Dr Stephen Myers or Dr Charles Minter, all of whom are qualified to take venous blood samples.

For any concerns or other questions about this study please contact the research coordinators names on page 1 of this information sheet.

Before you sign the informed consent form, you should ask questions about anything that you do not understand. The study staff, Carla Gallagher and Dr Stephen Myers will answer any questions before, during and after the study.

Appendix 4: Participant Information Sheet

**PARTICIPANT INFORMATION SHEET**

[http://t1.gstatic.com/images?q=tbn:yjltLKaie6J7FM:http://www.graduatejobsouth.co.uk/cms/site/images/Uni%2520Chi%2520logo.jpg](http://www.google.co.uk/imgres?imgurl=http://www.graduatejobsouth.co.uk/cms/site/images/Uni%20Chi%20logo.jpg&imgrefurl=http://www.graduatejobsouth.co.uk/employers/courses-at-university-.aspx&usg=__FuQpDS4gAdELFUAJUgHnF-YevbU=&h=811&w=2741&sz=479&hl=en&start=2&zoom=1&um=1&itbs=1&tbnid=yjltLKaie6J7FM:&tbnh=44&tbnw=150&prev=/images?q=university+of+chichester&um=1&hl=en&sa=N&tbs=isch:1)

**Research Title:** Determination of peak heart rate in normobaric hypoxia

**Research Coordinators:** Carla Gallagher **Tel:** 07970820277

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**Additional Investigators:** Dr Richard Clements ([R.Clements@chi.ac.uk](mailto:R.Clements@chi.ac.uk); 01243 816455)

Dr Marc Willems ([M.Willems@chi.ac.uk](mailto:M.Willems@chi.ac.uk); 01243 816468)

**Invitation to take part**

You are invited as a volunteer to take part in a research investigation. Before you decide to take part it is important for you to understand why the research is being conducted and what will be required of you should you agree to be involved. Please take time to read the following information carefully and discuss it with the investigators. Ask us if there is anything that is not clear or if you would like more information.

**Do I have to take part?**

This is entirely your decision. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time without giving a reason. If you decide not to participate or withdraw during the study, your decision will not affect your relationship with the University of Chichester, or any of the investigators involved in the study.

All information collected during the study will be coded and treated confidentially. The information will be kept in a locked filing cabinet and on the research coordinator’s password secured computer for 5 years before being confidentially destroyed.

**Background**

It has been demonstrated in previous research that an environment which reduces oxygen (O2) delivery (e.g. high altitude) reduces maximal heart rate. In recent years athletes have begun to train at altitude in order to boost sea-level performance. In the prescription of exercise training under conditions which reflect the altitude environment, it is important that the reduction in heart rate at maximal intensities is taken in to consideration in order to optimise training. Therefore the current research aims to examine the difference between peak heart rate in conditions with lowered oxygen availability.

**Location**

Bishop Otter Campus, University of Chichester, College Lane, Chichester, PO19 6PE. All testing will take place in a specialised environmental chamber located within the sport and exercise physiology laboratories. You will be informed of the room number as soon as room bookings have taken place and been confirmed.

**What will be expected of you?**

If you decide to take part in this study you will be asked to schedule six visits to the laboratory as outlined below.

1. **Project briefing**

* At this meeting you will be fully briefed about the requirements of the project.
* Provide written informed consent.
* Complete a health history questionnaire.

1. **The day prior to commencing each trial**, you will be expected to:

* Avoid participation in any strenuous exercise for 24 hours before you arrive for testing
* Avoid drinking alcohol, and caffeinated drinks (i.e. coffee, tea, cola)
* In the two hours before the testing session consume no food or energy drinks and drink only **plain** water

1. **Whilst completing the five trials** you will be expected to:

* Arrive hydrated
* Wear shorts, t-shirt and appropriate footwear
* Provide your early morning resting heart rate
* Have your height, weight and body composition recorded
* Perform a maximal cycle test
* Have your heart rate, respiratory measures and rating of perceived exertion continuously recorded throughout exercise

You will be excluded if you are a smoker or if you are on specific types of medication or supplements (i.e. Insulin, Beta Blockers), have any respiratory or pulmonary disorders, have been above 1000 m in the last month or have been a participant in other studies involving intermittent hypoxic/altitude exposure.

**Summary of time commitment:**

Project Briefing - 1 hour maximum

Experimental Trials - 5 hours

**Total time commitment** **6 hours**

**Summary of visits**

**Visit one: Project briefing with question and answer session (1 hour)**

At this meeting, you will be fully briefed about the requirements of the project. You will be talked through the subject information sheet by an investigator and given the opportunity to ask any questions. You will then leave with the information sheet to allow you time to discuss your involvement in the study with significant others. This will also allow you additional time to think of questions regarding the study. Once you are fully satisfied with the information and on verbal agreement to take part in the study you will be asked to complete an informed consent form, medical questionnaire and arrange for further visits.

**Visits two to six: Experimental trials (1 hour per visit)**

The procedures for each visit including the familiarisation session will be the same. On arrival at the laboratory anthropometric measures of height, weight and body composition will be obtained. Body composition will be assessed using various methods; skin-folds, circumference measures and bioelectrical impedance analysis.

Once anthropometric measures have been recorded, you will be fitted with a heart rate monitor, a low resistance face mask, a pulse oximeter and a blood pressure cuff. Following this preparation you will complete a five minute warm up on an electronically braked cycle ergometer, for this you will cycle at 60 revolutions per minute against a 1.0kg load. Once warmed up you will perform an incremental test to exhaustion. Work load will start at 80 watts and will be increased by 30 watts (males) and 25 watts (females) every 90 seconds; you will cycle at 80 revolutions per minute. Once you are no longer able to maintain the required power output the test will be terminated. Heart rate, gas exchange (expired air), arterial saturation, and rating of perceived exertion will be recorded throughout the test.

Each test will be performed under a different environmental condition (FIO2 0.209, 0.170, 0.150, 0.130), conditions will randomised and you will not know the order until completion of the research study.

**Advantages of taking part**

A benefit of taking part in this study is that you will receive comprehensive feedback, with full explanations, on your body composition (e.g. % body fat). Additionally you will also receive feedback on your cardio-respiratory fitness (e.g. maximal heart rate, maximal oxygen uptake).

**Disadvantages of taking part**

The disadvantage of taking part in this study you are probably most concerned about is time commitment. To complete all aspects of the study you will be required to attend the lab on five occasions which accumulates to about six hours of your time, one hour weekly. We understand that making such a commitment to attend and will try to be very efficient in our work and keep lab time succinct.

Carla Gallagher, Dr Stephen Myers or any of the additional investigators will happily answer any further questions.

Appendix 5: Trypan Blue Exclusion Methods

The Trypan blue exclusion method is used to identify live cells within media. If cells take up Trypan blue, they are considered non-viable.

**Protocol:**

1. Add 10 µl Trypan blue to small tube
2. Add 10 µl cells to Trypan blue
3. Mix well
4. Add 10 µl of solution to haemocytometer (top chamber)
5. Add 10 µl of solution to haemocytometer (bottom chamber)
6. Count cells using haemocytometer, each of the 4 squares
7. Calculate the average of the 4 squares of cell counts
8. Multiply value by 10,000
9. Multiply value by 2
10. Multiply by original amount of solution

Appendix 6: Real-Time Quantitative Chain Reaction Methods

The polymerase chain reaction technique is a molecular biology technique which is often employed for in vitro amplification of mRNA and is used in the comparison of the expression of target sequences of mRNA between different experimental conditions.

The first step in the process is the extraction of mRNA from the tissue or cell of interest as described previously (chapters 2 and 3) which is followed by the reverse transcription of mRNA in to cDNA. Reverse transcription is carried out using an oglio primer that anneals to the 3’polyA tail of the mRNA molecule, and the enzyme reverse transcriptase uses random nucleotides (dNTP’s) to copy a complementary sequence of DNA. This process is carried out at temperatures between 40 and 60°C. Following cDNA synthesis, PCR can be conducted.

PCR involves three steps; denaturation, annealing and extension, each relying on the use of different temperatures to fulfil their role.

1. *Denaturation.* Involves the heating of the reaction tubes to 95 °C, this allows for the separation of hydrogen bonds of the complementary strands of DNA.
2. *Annealing.* During the annealing stage, the temperature is lowered to allow primers[[28]](#footnote-28) to attach to their complementary dissociated DNA template strands. DNA polymerase then begins to add deoxynucleotides to the 3’OH group of the primers which produces new duplex molecules. It is important to note here that the temperatures used in this step are dependent upon the primers used but is usually between 40 and 72°C.
3. *Extension.* New double-stranded molecules are once again denatured and each single strand provides a primer binding site which acts as a template for DNA synthesis. During this step the temperature is adjusted to 72°C which is optimal for DNA polymerase activity.

When coupled together these 3 PCR steps are termed a complete cycle, to amplify the target DNA it is necessary to cycle through these steps several times (i.e. 20-40), which results in millions of copies of the specific sequence of DNA that is of interest.

Real-time qPCR uses fluorescence to detect the amount of target DNA which has been amplified in real-time during PCR. In the work presented in this thesis, SYBR green (a fluorescent dye) was used to monitor DNA amplification, although other methods are available (e.g. Taqman). SYBR green specifically binds to double-stranded DNA by intercalating between base pairs and upon doing so emits fluorescence; the amount of which is quantified by the PCR instrument. Detection of the fluorescent signal is measured at the end of the annealing step of each cycle when the greatest amount of double-stranded DNA product is present.

The amount of fluorescence is quantified by detecting the cycle at which the fluorescence produced by the reaction exceeds the background fluorescence (threshold cycle; CT). The CT is used to quantify the amount of the target gene which is present in the sample at the start of the reaction. At this point the mean CT values can be normalised to a housekeeping gene and relative gene expression can be calculated using the 2(-ΔΔCT)method ([Livak & Schmittgen, 2001](#_ENREF_167); [Schmittgen & Livak, 2008](#_ENREF_249)).

Appendix 7: G\*Power Results – Sample Size Calculations

**Chapters 4 and 5**

|  |  |  |  |
| --- | --- | --- | --- |
| **Test Family**  F-tests | **Statistical test**  ANOVA: Repeated measures, within factors | | |
| **Type of Power Analyses**  A Priori: Compute required sample size – given α, power and effect size | | | |
| **Input Parameters** | | **Output Parameters** | |
| **Effect size f (U)** | 1.6162441 | **Nonconcentrality parameter ʎ** | 15.6734699 |
| **α error prob** | 0.05 | **Critical F** | 5.9873776 |
| **Power (1-β error prob)** | 0.9 | **Numerator df** | 1 |
| **Number of groups** | 2 | **Denominator df** | 6 |
| **Number of measurements** | 2 | **Total sample size** | 8 |
| **Nonsphericity correction Ɛ** | 1 | **Actual power** | 0.9063746 |

**Distributions Plot**

**Chapters 6 and 7**

|  |  |  |  |
| --- | --- | --- | --- |
| **Test Family**  F-tests | **Statistical test**  ANOVA: Repeated measures, within factors | | |
| **Type of Power Analyses**  A Priori: Compute required sample size – given α, power and effect size | | | |
| **Input Parameters** | | **Output Parameters** | |
| **Effect size f (U)** | 1.2712835 | **Nonconcentrality parameter ʎ** | 25.8585878 |
| **α error prob** | 0.05 | **Critical F** | 3.0069173 |
| **Power (1-β error prob)** | 0.9 | **Numerator df** | 4 |
| **Number of groups** | 1 | **Denominator df** | 16 |
| **Number of measurements** | 5 | **Total sample size** | 5 |
| **Nonsphericity correction Ɛ** | 1 | **Actual power** | 0.9561459 |

**Distributions Plot**



Appendix 8: Chapter 5 Individual Weekly SPO2 Data

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Exposure 1** | **Exposure 2** | **Exposure 3** | **Exposure 4** | **Exposure 5** | **Exposure 6** | **Exposure 7** | **Exposure 8** | **Exposure 9** | **Exposure 10** | **Exposure 11** | **Exposure 12** | **Mean ± 1SD** |
| **SPO2 (%)** | 75 ± 4 | 75 ± 10 | 76 ± 7 | 75 ± 7 | 78 ± 9 | 77 ­± 2 | 76 ± 2 | 76 ± 4 | 77 ± 2 | 76 ± 1 | 78 ± 5 | 77 ± 6 | 76 ± 3 |
| **SPO2 \* Time** | 6711 ± 395 | 6724 ± 863 | 6827 ± 609 | 6750 ± 610 | 7050 ± 786 | 6904 ± 192 | 6870 ± 147 | 6870 ± 385 | 6930 ± 164 | 6866 ± 124 | 7020 ± 450 | 6930 ± 525 | 6871 ± 251 |

**Note:** These data suggest that participants did not acclimatise to hypoxia during the 4-week intermittent hypoxic exposure programme. No significant difference was observed over time (F(11,22) = 0.705, P = 0.721). These results were confirmed by the results of haemoglobin, this parameter also did not show any change over the 4-week time period (13.8 ± 1.6 g·dL-1 versus 13.1 ± 1.2 g·dL-1).

Appendix 9: Methods

In order to determine whether protocol design could impact up on the results observed within a VT test, a progressive GXT and an increment GXT were compared. Both tests started at a load of 50 W, which was attained for two minutes however the increment design differed between the two tests. During the progressive GXT a load of 20 W for women and 25 W for men was applied over a 60 second period for each 1 minute stage whereas during the increment GXT the load was applied at the onset of the stage (see figure below). The comparison between the two protocols was only conducted at SL with 4 participants.

**Results**

**Ventilatory Threshold**

The mean O2VT determined by algorithms 1-4 for both GTX protocols are shown in the table below. Algorithm 3 produced the lowest O2VT values in both conditions and Algorithm 2 the highest, again for both GTX protocols.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| O2VT | Algorithm 1 | Algorithm 2 | Algorithm 3 | Algorithm 4 |
| Progressive GXT | 3.37 ± 0.39\* | 3.66 ± 0.45\* | 3.26 ± 0.41\* | 3.35 ± 0.53 |
| Increment GXT | 2.65 ± 0.13 | 2.91 ± 0.61 | 2.64 ± 0.43 | 2.83 ± 0.17 |

**Note:** \*P < 0.05; progressive GXT > increment GXT.

When comparing the results of each algorithm between the two protocols, the O2VT determined by the progressive GXT was significantly higher (P ≤ 0.05) with all algorithms except Algorithm 4, in which there was no significant difference.

**Peak Oxygen Uptake (L·min-1)**

There was no significant difference observed between the O2peak determined via a progressive GXT or increment GXT (t(3) = 2.429, P = 0.093).

**Discussion**

In order to determine the ventilatory threshold in acute normobaric hypoxia, it is suggested that conclusions drawn are only appointed to the methods used and not generalised to the identification of the ventilatory threshold using all GTX protocols. In the work presented in this thesis, the incremental protocol was chosen. It was concluded that the O2VT did not change with hypoxic exposure, however, these results should not be applied to other protocols which may be used (i.e. progressive GTX). It is likely that a higher VT is achieved using the progressive protocol due to the much smaller increments in power carried over each one minute stage, whether this represents an overestimation of the VT is unknown and goes beyond the aims of this thesis, however this result does appear to be consistent.

In summary, the identification of the VT when compared in several acute normobaric hypoxic conditions should be measured using the same GTX protocol each time, this will allow for an accurate determination during each test and therefore conclusions drawn will be accurate to the degree of the test used.

Appendix 10: Normal Biochemistry Values

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | BMI < 25 kg·m-2 | BMI 25-30 kg·m-2 | BMI > 30 kg·m-2 |
| Leptin[[29]](#footnote-29) | **Men** | 3.8 ± 1.8 | x | x |
| **Women** | 7.4 ± 3.7 | x | x |
| Adiponectin[[30]](#footnote-30) | **Men** | 10.9 ± 4.0 | 8.8 ± 4.0 | 8.3 ± 2.8 |
| **Women** | 13.6 ± 5.4 | 13.9 ± 8.6 | 11.4 ± 3.8 |
| Category |  | **Low** | **Normal** | **High** |
| Fasting blood glucose (mg·dL-1) |  | <60 | 60 – 99 | > 99 |
| HDL (mg·dL-1) |  | < 40 | x | ≥ 60 |
| LDL (mg·dL-1) |  | x | < 100 | 160 - 189 |
| TG (mg·dL-1) |  | x | < 150 | 200-499 |
| TC (mg·dL-1) |  | x | < 200 | > 240 |
| LDL/HDL ratio |  | x | x | x |
| HDL/TC ratio |  | < 3 | 3 – 3.5 | > 3.5[[31]](#footnote-31) |

Appendix 11 – Publications

**Authors:** Carla A. Gallagher1, Mark Willems1, Mark P. Lewis2 & Stephen D. Myers1

**Title:** The application of maximal heart rate predictive equations in hypoxic conditions

**Affiliation:** 1University of Chichester, Chichester, West Sussex, UK

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**ABSTRACT**

Purpose: Peak heart rate (HRpeak) is a common tool used in exercise prescription for groups in which maximal exercise intensity is contraindicated; however the application of this method in normobaric hypoxia is unknown. Therefore, this study investigated the response of HRpeak and the application of predictive HRpeak equations to prescribe exercise intensity in acute normobaric hypoxia. Results were used to examine whether age-derived HRpeak predictive equations are valid in hypoxic conditions. Methods: Fifteen untrained (8 men) volunteers (age 22±2 years; O2peak 46.3±7.0 ml·kg-1·min-1) completed incremental cycle ergometer tests (randomised order) to measure HRpeak at sea-level (SL (FIO2 0.209)) and four normobaric hypoxic conditions FIO2: 0.185, 0.165, 0.142, 0.125 (≈1000, 2000, 3000, 4000 m). Results: HRpeak was similar across all conditions (SL, 182±13; 0.185, 178±11; 0.165, 177±9; 0.142, 178±9; 0.125, 175±10 b·min-1) despite a reduction in oxygen saturation (SPO2) with increasing hypoxia (SL, 95±5; 0.185, 95±2; 0.165, 92±2; 0.142, 88±3; 0.125, 82±4%; P≤0.05). The HRpeak was overestimated by all equations compared to the measured value (P<0.05). Four equations overestimated HRpeak in all conditions (P<0.01); two in 4 conditions (0.185, 0.165, 0.142, 0.125; P<0.01); and two in 3 conditions (0.165, 0.142, 0.125; P<0.01). Conclusion: The overestimation of HRpeak by commonly used age-derived predictive equations in normobaric hypoxic conditions suggests that despite possible contraindications researchers should directly measure HRpeak whenever possible if it is to be used to prescribe exercise intensities.

**Key words:** NORMOBARIC HYPOXIA; EXERCISE PRESCRIPTION; EXERCISE INTENSITY; CYCLE ERGOMETRY.

**INTRODUCTION**

In the last decade intermittent hypoxic exercise training has become increasingly popular among athletes, with the aim to improve sea-level (SL) performance ([Millet, et al., 2010](#_ENREF_193)). However, this method has also been employed within sedentary populations with the aim of improving health ([Haufe, et al., 2008](#_ENREF_122); [Netzer, et al., 2008](#_ENREF_202)). Although a hypoxia-induced decrement in aerobic capacity is incurred at altitude, reducing the intensity of exercise that can be performed, research has demonstrated that the same cardiovascular benefits are received ([Cerretelli, 1980](#_ENREF_44); [Friedmann, et al., 2005](#_ENREF_94)). Consequently, intermittent hypoxic training appears a viable method of optimising training such that certain individuals (e.g. untrained, sedentary, obese) receive the maximal metabolic and cardiovascular benefit whilst minimizing injury risk through a reduction in exercise intensity ([Haufe, et al., 2008](#_ENREF_122)). Moreover, combining exercise training with hypoxia may lead to greater losses of body mass than exercise training at SL ([Quintero, et al., 2010](#_ENREF_223)).

The reduction in measured HRpeak with chronic hypoxic exposure has been consistently observed ([Christensen & Forbes, 1937](#_ENREF_54); [Pugh, 1964](#_ENREF_219); [Richalet, et al., 1988](#_ENREF_229)). Contrary to these findings, the reduction in HRpeak with acute exposure to hypoxia is less clear. Several studies have shown that HRpeak changes little or not at all during acute hypoxic exposure ([Lawler et al., 1988b](#_ENREF_156); [Stenberg, et al., 1966](#_ENREF_264)) but for others, a significant decrease in HRpeak has been observed at and above 3800 m ([Benoit, et al., 2003](#_ENREF_23); [Lundby, et al., 2001](#_ENREF_170); [Roach, et al., 1996](#_ENREF_231)). Discrepancies in findings between studies may be due solely to the altitude used; in general those reporting a decline in HRpeak have used simulated altitudes above 3800 m (e.g. 4000 m or FIO2 0.125), whilst those observing no change used lower altitudes [e.g. 3500 m or FIO2 0.136 ([Benoit, et al., 2003](#_ENREF_23))]. Moreover, the differences could be due to exercise time, modality (e.g. treadmill, cycling), and participant characteristics (e.g. trained, untrained, sedentary), which should be taken into account.

Consideration of the appropriate training load (intensity, duration and frequency) is important in a population whose principle objectives are long-term weight loss and its maintenance ([Franckowiak, et al., 2011](#_ENREF_92)). In hypoxia, the prescription of exercise intensity requires considerable attention due to the reduction in aerobic capacity for a given exercise intensity in hypoxia which is often reported within the literature ([Friedmann, et al., 2005](#_ENREF_94)). It is common for training intensities at SL to be prescribed using a percentage of power or speed at peak oxygen uptake (O2peak) or peak heart rate (HRpeak), but since both these methods require maximal exercise testing, researchers often use age-derived equations to predict peak heart rate (PHRpeak) ([Robergs & Landwehr, 2002](#_ENREF_234)), in specific populations, particularly for those in which maximal exercise is contraindicated. Such predictive equations have been reviewed extensively at SL by Robergs and Landwehr, whilst concluding that there was no acceptable method to estimate maximal heart rate, they suggested that a prediction error of < 3 b·min-1 was acceptable for prescribing exercise training intensities ([Robergs & Landwehr, 2002](#_ENREF_234)). Moreover, due to the decline in aerobic performance capacity with hypoxia, employing predictive methods could be problematic as HRpeak may too be depressed with acute hypoxic exposure. If present such overestimations could lead to the prescription of exercise intensities which are unachievable and may result in decreased adherence to exercise training sessions ([Perri et al., 2002](#_ENREF_192)) and therefore no training effect.

The widespread use of heart rate to prescribe and monitor training intensities means that any reduction in HRpeak could have important implications for altitude training as well as the control of scientific studies. Several studies, which have investigated the effects of altitude training on SL performance, required athletes to perform training in hypoxia at the same relative heart rate at which they or a control group trained in normoxia, suggesting that the authors assumed that exercise intensities would be the same ([Bailey, et al., 1998](#_ENREF_18); [Burtscher, et al., 1996](#_ENREF_36)). However, Friedmann and colleagues (2005) propose that this may have negative consequences as, if heart rate at the same relative exercise intensities is reduced in hypoxia compared to normoxia the hypoxic groups in these studies would have exercised with higher intensities than the SL control groups which could lead to overreaching or the setting of unachievable goals ([Perri, et al., 2002](#_ENREF_214)). Therefore, the use of age-derived equations in the prediction of HRpeak, may be inappropriate for use in hypoxic exercise training studies as a result of decreased HRpeak with acute hypoxic exposure.

Predicted HRpeak is an important tool for the prescription of exercise intensity for groups in which maximal exercise intensity is contraindicated. Therefore, it is important for research to establish whether HRpeak is reduced during acute exposure to a hypoxic environment. The aims of the study were to assess in acute hypoxic conditions if compared to SL, 1) there was a decrease in measured HRpeak with decreasing oxygen (O2) concentrations and 2) if selected age-derived equations provide a sufficiently accurate prediction of HRpeak for prescribing exercise intensity within hypoxic exercise training programmes. It was hypothesised that there would be no reduction in HRpeak below a FIO2 0.142 (3000 m) as a result of this it was expected that age-derived equations used to predict HRpeak would not accurately estimate HRpeak below this. Above a FIO2 0.142, it was hypothesised that HRpeak would be reduced and that PHRpeak equations would over-estimate HRpeak

**Key words:** NORMOBARIC HYPOXIA; EXERCISE PRESCRIPTION; EXERCISE INTENSITY; CYCLE ERGOMETRY.

**METHODS**

**Participants**

Fifteen healthy volunteers (7 women and 8 men; mean ± SD: age 22 ± 2 years; height 176.4 ± 10.2 cm; body mass 72.8 ± 14.0 kg; body mass index 23.2 ± 2.7 kg·m2; O2peak 46.3 ± 7.0 ml·kg-1·min-1) participated in the study. All participants completed a medical history questionnaire, stating no contraindications to exhaustive exercise. All were SL residents and had not been above 1000 m in the six months preceding the study. After receiving both written and oral information on the experimental protocol and procedures, participants gave their written informed consent. The research conformed to the guidelines laid down in the Declaration of Helsinki (2008) and was approved by the University of Chichester Research Ethics Committee.

**Experimental procedure**

Each participant visited the laboratory on six occasions, including one familiarisation session, with each visit separated by a minimum of 48 h. All participants were instructed not to partake in any vigorous physical training 24 h prior to each session and to avoid the consumption of caffeinated beverages and food for a two-hour period prior to all sessions.

Height was measured to the nearest 1.0 mm (Holtain Ltd, Crymych, UK), and body mass to the nearest 0.05 kg (BC 418 MA, Tanita Ltd, UK) prior to exercise testing. All exercise tests were performed in an environmental chamber (TISS series 201003-1, TIS Services, UK), where normobaric hypoxia was achieved via a molecular sieve. The ambient temperature (tamb), relative humidity (RH) and air velocity (*v*) were controlled for in all sessions (tamb 20°C; RH 50%; *v* 0.00 m∙s-1). For all tests completed the ambient inspiratory O2 fractions (FIO2) were 0.209, 0.185, 0.165, 0.142 and 0.125 corresponding to SL, 1000 m, 2000 m, 3000 m and 4000 m, respectively. All conditions were randomised to each participant in a single-blind manner. To keep participants uninformed of each condition, when performing at SL, the compressor attached to the environmental chamber was running at all times to create a ‘sham hypoxia’ environment ([Netzer, et al., 2008](#_ENREF_202)). To monitor participant’s perception of altitude exposure, following each visit they were asked to report which condition they thought they had been exposed, none of the participants were able to identify the correct condition on every occasion, and only 40% of participants correctly identified the SL condition.

Peak heart rate was determined using an incremental exercise protocol conducted on an electromagnetically braked cycle ergometer (Lode, Excalibur Sport, Cranlea and Co, Bourneville, UK). All participants were seated in the chamber for 10 min prior to the exercise test. The protocol began at 50 W, which was subsequently increased by 20 W for women and 25 W for men every minute thereafter ([Amann, et al., 2006](#_ENREF_4)). Participants selected a cadence between 70 to 90 revolutions per minute (rpm) and were required to maintain this throughout each test. Each participant selected their preferred pedal cadence during their familiarization session, once selected, the chosen cadence (76 ± 10 rpm) mean was fixed for each of the 5 exercise tests. During the incremental test heart rate (beats per minute; b·min-1), minute ventilation (E), O2 uptake (O2) and FIO2 (Cosmed K4b2, Cosmed srl, Rome, Italy) were recorded continuously. Arterial O2 saturation (SPO2) was monitored continuously during the incremental test using infrared pulse oximetry (accuracy ± 2%; Datex-Ohmeda 3800, Datex-Ohmedia Division, Instrumentarium Corp, Finland) and recorded every minute. Pulse oximetry was blinded to participants. Participants also gave their rating of perceived exertion [RPE ([Borg, 1982](#_ENREF_30))] in the last 10 seconds of every stage ([Shephard, et al., 1992](#_ENREF_255)). The protocol used was such that all participants completed the test within 10 ± 2 min to reduce the likelihood of potential limitations to exercise e.g. high body temperature, different substrate utilisation or ventilatory muscle fatigue ([Buchfuhrer, et al., 1983](#_ENREF_35)).

Test termination occurred when participants were unable to maintain the required power output. Standard criteria (i.e. HRpeak ± 10% of age-predicted maximum) was not used for test termination since the aim of the study was to observe the decline in HRpeak with acute hypoxia, therefore HRpeak could not be used as a judgement criterion ([Benoit, et al., 2003](#_ENREF_23)). The HRpeak was defined as the highest 10 sec moving average in HR ([Franckowiak, et al., 2011](#_ENREF_92)), O2peak as the highest 20 sec moving average in VO2 ([Posner, et al., 1995](#_ENREF_216)) and peak power output (peak) as the highest mechanical power output maintained for one minute ([Bentley & McNaughton, 2003](#_ENREF_24)) during the incremental exercise test.

**Prediction of maximal heart rate**

Traditional equations frequently used by trainers and health professionals to prescribe aerobic exercise intensity are multiple; however limited evidence is available on the use of these equations in hypoxia. Available research findings suggest that HRpeak is reduced in acute hypoxia, if so the use of age-derived equations is limited. Therefore, the present study was constructed to examine the use of several age-derived equations to predict HRpeak (PHRpeak) in hypoxia; the equations compared against actual HRpeak response to incremental cycle ergometer exercise are presented in Table 1. As there are several equations for predicting HRpeak ([Robergs & Landwehr, 2002](#_ENREF_234)) it was decided that equations using only healthy men and women would be analysed in the present study. The equations compared used mainly cycling protocols ([Jones, 1988](#_ENREF_137); [Jones, et al., 1985](#_ENREF_138); [Ricard, et al., 1990](#_ENREF_228)), with some using a combination of the treadmill and cycling protocols ([Fox, et al., 1971](#_ENREF_90); [Tanaka, et al., 1991](#_ENREF_271); [Tanaka, et al., 2001](#_ENREF_272)).

**[INSERT TABLE 1 HERE]**

**Statistical Analysis**

Statistical analyses were computed by the statistical software package SPSS (release 20.0; SPSS Inc., Chicago, USA). Following mathematical confirmation of a normal distribution, repeated measures analysis of variance (ANOVA) was used to compare HRpeak across all five conditions (FIO2 0.209, 0.185, 0.165, 0.142 and 0.125) and to compare HRpeak measured during the incremental exercise test and the HRpeak predicted by the equations in Table 1. Post-hoc planned comparisons comparing hypoxic measurements against SL and PHRpeak were carried out using Bonferroni corrected t-tests. Effect sizes for ANOVAs were calculated using the omega squared (Ω2) method, and can be interpreted as small (< 0.06), medium (0.06-0.15) and large [> 0.15([Cohen, 1992](#_ENREF_58))]. Effect size for t-test comparisons were calculated by converting a *t*-value into an *r*-value ([Field, 2005](#_ENREF_86); [Rosenthal, et al., 2000](#_ENREF_240)). Sample size was calculated a priori using G\*Power software (release 3.1.2: Kiel, Germany) which resulted in a total of 9 participants required for the study for a power of 0.80. Post-hoc power analyses resulted in a power of 0.86 for a sample size of 15, with a P value of 0.167 and effect size of 0.26. All values are presented as mean ± 1SD and for statistical analyses an alpha of P ≤ 0.05 was considered significant unless otherwise stated.

**RESULTS**

**Peak heart rate**

There was no significant decline in HRpeak in hypoxia compared with SL (P = 0.17, Ω2 = 0.26; Figure 1). A significant difference between PHRpeak and measured HRpeak using allequations was observed, the results are summarised in Table 2, showing in all instances PHRpeak was higher than measured HRpeak (P < 0.05).

**[INSERT FIGURE 1 HERE]**

**[INSERT FIGURE 2 HERE]**

**[INSERT TABLE 2 HERE]**

**Peak power output, peak oxygen uptake and oxygen saturation**

Peak cardiorespiratory responses are shown in Table 3. All subjects cycled from 50 W to at least 150 W in each test. Thereafter, the number of participants varied, thus only values at peak are reported. At SL participants achieved an average peak of 247 ± 58 W. In hypoxia there was an overall decline in peak (P ≤ 0.001, Ω2 = 0.44) which was significantly lower than SL at FIO2 0.125(P ≤ 0.001, *r* = 0.79). An overall reduction in test time was also observed (P ≤ 0.001, Ω = 0.53) which was significantly lower than SL at FIO2 0.165 (P = 0.012, *r* = 0.61), 0.142 (P = 0.002, *r* = 0.72) and 0.125 (P < 0.001, *r* = 0.84).

**[INSERT TABLE 3 HERE]**

In hypoxia, there was an overall decline in O2peak, whenboth presented absolutely (P = 0.014, Ω2 = 0.34) and relative to body mass (P = 0.034, Ω2 = 0.45), which were significantly lower than SL at FIO2 0.125 (P = 0.006, *r* = 0.67; P = 0.004, *r* = 0.69) (Table 3). The SPO2 was also significantly reduced in hypoxia (P ≤ 0.001, Ω2 = 0.98). Compared with SL SPO2 was lower at FIO2 0.142 (P ≤ 0.001, *r* = 0.81) and 0.125 (P ≤ 0.001, *r* = 0.91). The SPO2 was also significantly reduced at FIO2 0.125 compared with 0.185 (P ≤ 0.001, *r* = 0.96), 0.165 (P ≤ 0.001, *r* = 0.86) and 0.142 (P ≤ 0.001, *r* = 0.85).

**Rating of perceived exertion**

No significant difference was found for RPE between SL (20 ± 0) and any of the hypoxic conditions (FIO2 0.185, 20 ± 0; 0.165, 20 ± 0; 0.142, 20 ± 0; 0.125, 20 ± 0), with individuals always reporting maximal exertion on cessation of the incremental cycle test.

**DISCUSSION**

The first aim of the present study was to identify whether there is a decrease in HRpeak with acute hypoxia and to observe, if evident, the altitude at which the decrement in HRpeak occurs. The results did not demonstrate a decline in HRpeak with exposure to acute normobaric hypoxia at or below a FIO2 0.125 (4000 m) compared with SL. However, analyses revealed a significant difference in O2peak at a FIO2 0.125 and also confirmed the well-reported reduction in SPO2 with hypoxia. A second aim was to determine whether age-derived equations to predict HRpeak are viable for prescribing exercise intensity within hypoxia. It was found that most PHRpeak equations significantly overestimated HRpeak in hypoxia. Moreover, ratings of perceived exertion indicate that all participants perceived the tests as maximal (20), with no difference between trials.

Results of the present study confirm those of previous research that have identified little or no change in HRpeak with acute hypoxia below 3800 m ([Lawler, et al., 1988b](#_ENREF_156); [Stenberg, et al., 1966](#_ENREF_264)), however results do not support those reporting a decline in HRpeak with acute hypoxia above 3800 m ([Benoit, et al., 2003](#_ENREF_23); [Friedmann, et al., 2005](#_ENREF_94); [Grataloup, et al., 2007](#_ENREF_112); [Lundby, et al., 2001](#_ENREF_170); [Mollard, et al., 2007](#_ENREF_195); [Roach, et al., 1996](#_ENREF_231)). Discrepancies between results can be accounted for by exercise modality and participant characteristics, as well as the amount of hypoxia induced. In acute hypoxia, the decline in HRpeak has been attributed to increased parasympathetic traffic resulting in a blunting of the cardiac chronotropic function, rather than a decreased sympathetic function, which is often observed with maximal exercise in chronic hypoxia ([Benoit, et al., 2003](#_ENREF_23)). Additionally, it has been hypothesised that the decline in HRpeak with acute hypoxia is due to the direct effect of hypoxia on the cardiac electrophysiological properties i.e. repolarisation length and transmission time on the atrioventricular node ([Roche, et al., 2003](#_ENREF_235)), which are coherent with the decrease in SPO2 and change in HRpeak at altitude ([Mollard, et al., 2007](#_ENREF_195)). A decline in SPO2 (88-82%) was observed in the present study however, the reduction was not as great as observed by others [≈80% ([Benoit, et al., 2003](#_ENREF_23); [Lundby, et al., 2001](#_ENREF_170))] which may explain the lack of reduction in HRpeak. Therefore, the present results, in addition to previous literature, suggest that a reduction in SPO2 is a key attributor to the decline in HRpeak often observed with acute normobaric hypoxia. In support, individuals who present greater hypoxemia than others consequently experience a greater reduction in HRpeak with acute hypoxia ([Richalet, et al., 1988](#_ENREF_229)) and larger reductions in SPO2 have been reported to result in greater modifications within cardiac properties such as autonomic changes ([Lundby, et al., 2001](#_ENREF_170); [Ricard, et al., 1990](#_ENREF_228)). Alternatively, the lack of reduction in HRpeak with acute hypoxia observed in the present study could also be due to the lower HRpeak observed at SL (182 ± 13 b·min-1) compared with previous work [196 ± 7 b·min-1 ([Benoit, et al., 2003](#_ENREF_23))], which may have been limited by leg fatigue rather than dyspnea ([Aliverti et al., 2011](#_ENREF_1)), which could be accounted for by the higher pedal cadence chosen in this study using untrained individuals rather than experienced cyclised. This concept should be explored further in future work. Moreover, since HRpeak ranged from 195 to 169 b·min-1 in the SL condition, reporting of the mean values observed in each condition may not accurately reflect values observed on an individual basis, where a reduction in HRpeak may be observed for some. In more detail, the variation observed wasn’t consistent between altitudes as is demonstrated by Figure 2, moreover the magnitude of variation appears to differ greatly among participants with some demonstrating small individual variation (e.g. participant 1) and others large individual variation (e.g. participant 15). However, there is no strong motive for removing this participant as the statistical outcome remains the same. Therefore, it is believed that the data set is a true reflection of the individual responses of HRpeak to hypoxic conditions and further adds to support the need to measure HRpeak in hypoxic conditions prior to exercise prescription hypoxic studies/programmes.

Although there was no significant reduction in HRpeak in response to acute normobaric hypoxia, HRpeak was reduced when compared to age-predicted values derived from all eight equations examined. While it is expected that greater HRpeak is observed with treadmill protocols, those equations solely derived from cycling protocols ([Jones, 1988](#_ENREF_137); [Jones, et al., 1985](#_ENREF_138); [Ricard, et al., 1990](#_ENREF_228)) also overestimated HRpeak. Moreover, despite a moderate sample size of 15, large effect sizes (> 0.15) ([Cohen, 1992](#_ENREF_58)) for all equations were observed suggesting that the over prediction of HRpeak by the age-derived equations is present and of importance. Therefore, the use of current age-derived equations to predict HRpeak would appear to be inappropriate for use in hypoxic conditions and the measurement of normoxic HRpeak during an incremental exercise test is necessary. Exercise prescription using age-derived equations may have negative consequences and lead to discrepancies in exercise intensities prescribed between normoxic and normobaric hypoxic training regimes, with hypoxia groups exercising at a greater intensity, this consequently may lead to the setting of unachievable goals and decreased adherence to exercise sessions ([Perri, et al., 2002](#_ENREF_214)) and no observed training effect. On the contrary, a greater understanding of the effect of acute hypoxia in individuals who have contraindications to exhaustive exercise is still warranted since previous authors have reported that the difference between predictive heart rate equations and measure HRpeak at SL are minimal ([Franckowiak, et al., 2011](#_ENREF_92)). Therefore, the same equations used in the present study may not over- or under-estimate HRpeak in these individuals.

In conclusion, it appears that measured HRpeak is not reduced in response to acute normobaric hypoxia at or below a FIO2 0.125 (4000 m), which can be partially explained by a reduction in SPO2 (18-22%). Age-derived equations appear to overestimate HRpeak in hypoxia despite no observed reduction in measured HRpeak ator below a FIO2 0.125 (4000 m); therefore equations appear inappropriate for exercise intensity prescription in hypoxia. However, taken together, based on the results observed in this study, no firm conclusions can be drawn in regards to the prediction of HRpeak in response to acute hypoxia. However, based on the literature, the limitations of these equations are most likely increased during incremental exercise in hypoxic conditions. Until specific equations are developed to predict HRpeak in hypoxia it is recommended that where possible, HRpeak is measured using an incremental exercise test. With the development of appropriate equations exercise physiologists will be able to determine and prescribe safe exercise intensities in hypoxia without the use of incremental exercise tests to volitional exhaustion in individuals to which maximal exercise is contraindicated.

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**Abbreviations:**

ANOVA Analysis of variance

FIO2 Ambient inspiratory oxygen fraction

HRpeak Peak heart rate

O2 Oxygen

PHRpeak Predicted peak heart rate

RH Relative humidity

RPE Rating of perceived exertion

SL Sea-level

SPO2 Arterial oxygen saturation

tamb Ambient temperature

*v,* Velocity

E Minute ventilation

O2 Oxygen uptake

O2peak Peak rate of oxygen consumption

peak Peak power output

Ω2 Omega squared

**Tables**

**Table 1.** Age-derived predictive equations for peak heart rate

|  |  |
| --- | --- |
| Equation | Reference |
| 1: 220-age | ([Fox, et al., 1971](#_ENREF_90)) |
| 2: 208-0.7·age | ([Tanaka, et al., 2001](#_ENREF_272)) |
| 3: 202-0.72·age | ([Jones, et al., 1985](#_ENREF_138)) |
| 4: 210-0.65·age | ([Jones, 1988](#_ENREF_137)) |
| 5: 209-0.587·age | ([Ricard, et al., 1990](#_ENREF_228)) |
| 6: 200-0.687·age | ([Ricard, et al., 1990](#_ENREF_228)) |
| 7: 211-0.8·age | ([Tanaka, et al., 1991](#_ENREF_271)) |
| 8: 207-0.7·age | ([Tanaka, et al., 1991](#_ENREF_271)) |

**Table 2.** Predicted heart rate peak and measured heart rate peak comparisons

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | Fraction of inspired oxygen (equivalent altitude m) | | | | |
| **0.209**  **(Sea level)** | **0.185**  **(1000 m)** | **0.165**  **(2000 m)** | **0.142**  **(3000 m)** | **0.125**  **(4000 m)** |
| Equation | **PHRpeak**  **(b·min-1)** | **Measured HRpeak (b·min-1)** | | | | |
| 182 ± 13 | 178 ± 11 | 177 ± 9 | 178 ± 9 | 176 ± 11 |
| A | 198 ± 2 | **↑**\* (0.80) | **↑**\* (0.87) | **↑**\*(0.92) | **↑**\*(0.93) | **↑**\*(0.91) |
| B | 192 ± 1 | = | **↑**\*(0.80) | **↑**\*(0.86) | **↑**\*(0.87) | **↑**\*(0.85) |
| C | 186 ± 1 | = | = | **↑**\*(0.71) | **↑**\*(0.69) | **↑**\*(0.70) |
| D | 195 ± 1 | **↑**\*(0.75) | **↑**\*(0.85) | **↑**\*(0.90) | **↑**\*(0.91) | **↑**\*(0.89) |
| E | 196 ± 1 | **↑**\*(0.76) | **↑**\*(0.86) | **↑**\*(0.91) | **↑**\*(0.91) | **↑**\*(0.89) |
| F | 185 ± 1 | = | = | ↑\*(0.65) | **↑**\*(0.63) | **↑**\*(0.65) |
| G | 193 ± 1 | **↑**\*(0.69) | **↑**\*(0.81) | **↑**\*(0.88) | **↑**\*(0.88) | **↑**\*(0.86) |
| H | 191 ± 1 | = | **↑**\*(0.78) | **↑**\*(0.85) | **↑**\*(0.85) | **↑**\*(0.84) |

**Notes:** PHRpeak predicted heart rate peak. Compared to corresponding FIO2: = indicates PHRpeak no different to measured HRpeak; ↑ indicates PHRpeak > measured HRpeak, \* P ≤ 0.01. Effect sizes (*r*) are displayed in brackets.

**Table 3. Cardiorespiratory responses**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Fraction of inspired oxygen (equivalent altitude m) | | | | |
|  | 0.209 (sea-level) | 0.185 (1000 m) | 0.165 (2000 m) | 0.142 (3000 m) | 0.125 (4000 m) |
| O2peak (L·min-1) | 3.39 ± 0.99 | 3.31 ± 1.00 | 3.22 ± 0.90 | 3.09 ± 0.92 | 2.91 ± 0.72\* |
| O2peak  (ml·kg-1·min-1) | 46.3 ± 7.0 | 46.1 ± 10.4 | 44.3 ± 8.5 | 42.6 ± 9.9 | 40.5 ± 7.4\* |
| peak | 247 ± 58 | 243 ± 57 | 237 ± 53 | 232 ± 56 | 218 ± 43\* |
| Test time (s) | 556 ± 138 | 538 ± 131 | 518 ± 125\* | 506 ± 133\* | 473 ± 110\* |
| SPO2 (%) | 95 ± 5 | 95 ± 2 | 92 ± 2 | 88 ± 3\*§† | 82 ± 4\*§†‡ |
| VE (L·min-1) | 143.6 ± 41.3 | 138.6 ± 35.5 | 138.3 ± 36.1 | 144.2 ± 31.9 | 143.5 ± 29.7 |
| VCO2 (ml.min-1) | 3685 ± 1057 | 3479 ± 1023 | 3476 ± 1077 | 3224 ± 977 | 3051 ± 929 |
| RER | 1.13 ± 0.16 | 1.07 ± 0.11 | 1.07 ± 0.08 | 1.03 ± 0.16 | 1.02 ± 0.13 |

**Note:** \* < SL; § < 0.185; † < 0.165. ‡ < 0.142 m; P ≤ 0.005

**Illustrations**

Fig 1. Measured HRpeak response to incremental exercise

1. A measurement of metabolic rate by the ingestion of accurately weighed isotopes followed by urine sample collections; often used to determine physical activity levels in humans [↑](#footnote-ref-1)
2. An increase in water formation as a result of carbohydrate, fat and/or protein oxidation [↑](#footnote-ref-2)
3. Marker of cellular oxidative damage [↑](#footnote-ref-3)
4. A condition in which a person’s body tissues have a lowered response to insulin [↑](#footnote-ref-4)
5. An increase in cell number by division [↑](#footnote-ref-5)
6. The process by which a less specialised cell develops to possess a more distinct form/function [↑](#footnote-ref-6)
7. Group of risk factors that raise the risk of heart disease, includes abdominal obesity, high triglyceride levels, low HDL cholesterol levels, hypertension and high fasting blood glucose [↑](#footnote-ref-7)
8. Expressed in adipose tissue GLUT4 is responsible for insulin regulated glucose storage [↑](#footnote-ref-8)
9. A naturally occurring amino acid found in blood plasma [↑](#footnote-ref-9)
10. A method used to quantify an individual’s contribution of insulin resistance and β-cell function to fasting hyperglycaemia [↑](#footnote-ref-10)
11. Agents that induce mitosis (division of chromosomes in cell nucleus) [↑](#footnote-ref-11)
12. Cells enter a quiescent state remaining in the early phases of the cell cycle [↑](#footnote-ref-12)
13. Cells enter a quiescent state remaining in the early phases of the cell cycle [↑](#footnote-ref-13)
14. Optical density at 260 nm equals 1.0 for a 40 µg/mL solution [↑](#footnote-ref-14)
15. A ratio of ≈2.0 is accepted as “pure” for RNA. If the ratio is substantially lower, it may indicate the presence of protein, phenol or other contaminants that absorb at/near 280 nm [↑](#footnote-ref-15)
16. Methods outlined at Appendix 6 [↑](#footnote-ref-16)
17. Complementary deoxyribonucleic acid [↑](#footnote-ref-17)
18. Phosphatidylinositide 3-kinase [↑](#footnote-ref-18)
19. Protein kinase B [↑](#footnote-ref-19)
20. Forkhead box protein [↑](#footnote-ref-20)
21. A technique used to quantify mRNA; involves separation of RNA by electrophoresis, transfer of RNA from gel to nitrocellulose and detection with a suitable probe [↑](#footnote-ref-21)
22. Methods outlined at Appendix 6 [↑](#footnote-ref-22)
23. Residential altitude of participants was 27 above SL [↑](#footnote-ref-23)
24. Two women were in a different age category (40-49 years) to other participants, in this instance body density was calculated using the following equation: 1.1333 – [0.0612·LOG (∑ skin-folds)] [↑](#footnote-ref-24)
25. Two women were in a different age category (40- 49 years) to other participants, in this instance body density was calculated using the following equation: 1.1333 – [0.0612·LOG (∑ skin-folds)] [↑](#footnote-ref-25)
26. All participants were in the 20-29 year age bracket and so only one equation for males and one equation for females is presented. [↑](#footnote-ref-26)
27. All participants were in the 20-29 year age bracket and so only one equation for males and one equation for females is presented. [↑](#footnote-ref-27)
28. A single stranded sequence of nucleotides known as an oglionucleotide, each is complementary to one of the original DNA strands, to either the left (5’) or right (3’) side of the sequence of interest. [↑](#footnote-ref-28)
29. Values as reported by IBL International®, Hamburg, Germany [↑](#footnote-ref-29)
30. Values as reported by Biovendor®, Laboratorní medicína a.s [↑](#footnote-ref-30)
31. Values as reported by American College of Sports Medicine [↑](#footnote-ref-31)