Doctoral Thesis

Erythropoietin's impact on endurance performance under normoxic condition and upon acclimatization to moderate altitude

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Erythropoietin’s impact on endurance performance under normoxic condition and upon acclimatization to moderate altitude

A dissertation submitted to

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for the degree of

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2009
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1. Summary

Hypoxia is the main stimulus of erythropoietin (Epo) expression. The oxygen (O₂)-sensing protein termed hypoxia-inducible factor (Hif) has been identified as a key regulator of Epo. At normoxia, Hif-α, a subunit of Hif, is degraded but stabilized under hypoxic condition, in which it enhances Epo gene expression resulting in increased erythrocyte production and in an increased O₂ transport capacity of blood. Within the normal physiological range, maximal O₂ uptake (\(\dot{V}O_{2\text{max}}\)) increases in parallel with increasing blood O₂ transport capacity at normoxia. Therefore, some endurance athletes live at moderate altitude and train at low altitude to improve sea level performance. Others do misuse recombinant human Epo (rhEpo), originally developed for the treatment of anemia. An unwanted side effect of an Epo-induced increase in the blood’s O₂ transport capacity is an increased blood viscosity due to the elevated hematocrit (Htc) levels. The higher blood viscosity strains the cardiovascular system and may limit endurance performance. On the other hand, our transgenic mice line (tg6) overexpressed Epo constitutively and therefore reached Htc levels between 0.8 and 0.9 and interestingly, there were no signs of pathological alterations in three month old mice. To study the impact of various Htc levels on exercise performance and the cardiovascular system, telemetry and indirect calorimetry in exercising mice were combined and improved in a first study. Telemetry offers the possibility to monitor arterial blood pressure from conscious, freely moving laboratory mice; however, its use has been limited because of high morbidity and mortality particularly in genetically modified animals. Often, either a weak telemetric signal is observed during exercise or even none at all is observed. Here, we show an optimized transmitter implantation technique to improve the telemetric signal in exercising mice. Moreover, a new postoperative intensive care regime and analgesia were used to reduce morbidity and mortality. The new tool is useful for investigators who plan to measure cardiovascular function in mice using telemetry, but the surgical procedure remains challenging.

The aim of the second study was to investigate the effect of varying Htc levels on exercise performance and the vascular system. To this end, wild type mice (wt) and tg6 mice were injected with the novel erythropoiesis stimulating protein (NESP; wtNESP), or the hemolysis inducing compound phenylhydrazine (PHZ; tg6PHZ), respectively. Highest \(\dot{V}O_{2\text{max}}\) and best time to exhaustion were reached at Htc values of 0.58 and 0.57 for wtNESP mice, and 0.68 and 0.66 for tg6PHZ, respectively. Maximal stroke volume was observed at similar Htc levels. Interestingly, \(\dot{V}O_{2\text{max}}\) of wtNESP was most closely related to whole body hemoglobin in an Htc range from 0.4 to 0.55. \(\dot{V}O_{2\text{max}}\) was correlated with blood viscosity.
We conclude that (1.) tgPHZ adapt better to varying Htc levels than wtNESP do, (2.) endurance performance is primarily limited by O₂ delivery and (3) the general observation that \( \dot{V}O_{2\text{max}} \) is strongly correlated with whole body hemoglobin is only valid within the physiological Htc range and when Htc levels are increased.

Acute hypoxia induces a reduction in \( \dot{V}O_{2\text{max}} \). During altitude acclimatization above 4100 m, arterial O₂ content increases due to the increasing hemoglobin concentration without effecting \( \dot{V}O_{2\text{max}} \). The data on moderate altitude are controversial. Thus, a third study investigated the hypothesis that \( \dot{V}O_{2\text{max}} \) and performance increase upon altitude acclimatization to 2340 m. Therefore, eight elite cyclists trained during a period of 21 days according to the “live high-train low” approach. Performance parameters were mainly improved within the first fourteen days, whereas in the following week, only a slight improvement was observed. These results suggest that athletes who plan to compete around this altitude have to arrive at least fourteen days before the beginning of the competition in order to be prepared optimally for the competition day.
2. Zusammenfassung


Das Ziel der zweiten Studie war es den Einfluss verschiedener Htc-Werte auf die sportliche Leistungsfähigkeit sowie kardiovaskuläre System zu untersuchen. Für diesen Zweck wurden Wildtyp (wt)- und tg6-Mäuse mit dem Erythropoiese stimulierenden Protein
NESP; wtNESP) respektive, dem Hämolyse induzierenden Phenylhydrazin (PHZ; tg6PHZ) behandelt. Höchster \( \text{\`{O}}_{2\text{max}} \) und beste Zeit bis zur Erschöpfung wurden bei Htc-Werten von 0.58 und 0.57 für wtNESP, bzw. 0.68 und 0.66 für tg6PHZ gemessen. Maximaales Herzschlagvolumen wurde bei ähnlichen Htc-Werten beobachtet. Interessanterweise korrelierte \( \text{\`{O}}_{2\text{max}} \) nur bei wtNESP-Mäusen in einem Htc-Bereich von 0.4 bis 0.55 am stärksten mit der gesamten Hämoglobinmenge. Zudem wurde eine Abhängigkeit zwischen \( \text{\`{O}}_{2\text{max}} \) und Blutviskosität beobachtet. Wir schlossen daraus, dass (1.) sich tg6PHZ-Mäuse besser an die veränderten Htc-Werte anpassen konnten als die wtNESP-Mäuse, (2.) die Ausdauerleistung hauptsächlich von der O\(_2\)-Verfügbarkeit limitiert ist und (3.) \( \text{\`{O}}_{2\text{max}} \) stark mit der gesamten Hämoglobinkonzentration korreliert. Allerdings gilt letzteres nur innerhalb des physiologischen Htc-Bereichs und sofern die Htc-Werte erhöht wurden.

Akute Hypoxie verursacht eine Reduktion von \( \text{\`{O}}_{2\text{max}} \). Während der Höhenakklimatisierung oberhalb von 4100 m, steigt der arterielle O\(_2\)-Gehalt im Blut aufgrund der Zunahme der Hämoglobinkonzentration auf oder über Meeresöhneniveau an, wobei \( \text{\`{O}}_{2\text{max}} \) davon unbeeinflusst ist. Doch sind die Daten auf mittlerer Höhe kontrovers. Während in einigen Studien \( \text{\`{O}}_{2\text{max}} \) nicht anstieg, berichten andere von einer minimalen Zunahme. Darum wurde in der dritten Studie die Hypothese untersucht, ob \( \text{\`{O}}_{2\text{max}} \) und Leistung während der Höhenakklimatisation an 2340 m ansteigen. 8 Eliteradfahrer trainierten während 21 Tagen gemäss dem „live-high-train low“ Prinzip. Die leistungsbezogenen Parameter verbesserten sich hauptsächlich in den ersten 14 Tagen während in den darauf folgenden 7 Tagen nur noch ein leichter Anstieg beobachtet wurde. Diese Resultate legen nahe, dass Sportler, die auf etwa dieser Höhe einen Wettkampf betreiben möchten, sich mindestens 14 Tage vor Wettkampfbeginn auf diese Höhe begeben müssen, um am Wettkampftag bestmöglichst vorbereitet zu sein.
3. General introduction

3.1. Hypoxia

Adequate oxygen (O₂) supply is essential to the aerobic metabolism of most eukaryotic organisms. O₂ participates in the cellular metabolism to ensure energy production in the cell as substrate for an optimal oxidation. Even a slight reduction in O₂ availability (hypoxia) seriously impairs this energy metabolism in humans. O₂ may become limited by anemia or cardiovascular, pulmonary or haematological diseases, but also by exercise and exposure to high altitude.

While the percentage of O₂ in the atmosphere below 10,000 m altitude is constant at 20-21%, O₂ partial pressure (pO₂) falls exponentially with increasing altitude. With decreasing pO₂, inspiratory and alveolar partial pressure (pO₂) is also reduced. For instance, at sea level the mean alveolar pO₂ is about 100 mmHg, whereas it drops to about 46 mmHg at an altitude of 5000 m. As a response to hypoxic environment, several hypoxic sensors are activated to maintain homeostasis. Tab. 3.1 shows altitude related to Patm as well as pO₂.

<table>
<thead>
<tr>
<th>Altitude [m]</th>
<th>Patm [mmHg]</th>
<th>pO₂ [mmHg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>760</td>
<td>100</td>
</tr>
<tr>
<td>2000</td>
<td>596</td>
<td>82</td>
</tr>
<tr>
<td>3000</td>
<td>526</td>
<td>67</td>
</tr>
<tr>
<td>4000</td>
<td>462</td>
<td>50</td>
</tr>
<tr>
<td>5000</td>
<td>405</td>
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<tr>
<td>6000</td>
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<tr>
<td>7000</td>
<td>308</td>
<td>35</td>
</tr>
<tr>
<td>8000</td>
<td>267</td>
<td>32</td>
</tr>
<tr>
<td>9000</td>
<td>231</td>
<td>30</td>
</tr>
</tbody>
</table>

Tab. 3.1. Altitude related to the atmosphere pressure [Patm] as well as the alveolar oxygen partial pressure [pO₂] (modified from West, 1995)
3.2. Adaptational mechanism to hypoxic environment

The physiological adjustments to hypoxia occur at systemic and cellular levels. When $pO_2$ falls, homeostatic mechanisms of the respiratory and cardiovascular systems, such as an increase in ventilation and heart rate, are immediately activated to deliver adequate $O_2$ to the organism. When impaired $O_2$ supply is prolonged, the response includes changes in gene expression in the cell. Altitudes between 3500 and 5000 m induce the required enzyme synthesis for increased glycolysis, Krebs cycle, respiratory chain and several glucose membrane transporters (Rynafarje et al., 1962; Ou and Tenney, 1970; West and Mangan, 1970). Above 5000 m, enzyme activity is reduced and the organism undergoes a loss of muscle mass and body weight (Hoppeler et al., 1990; Kayser et al., 1993; Steinacker et al., 1996).

A key molecular global regulator of hypoxia is hypoxia-inducible factor-1 (Hif-1). Hif-1 is a transcription factor which regulates many genes influencing angiogenesis, erythropoiesis, glycolysis, iron metabolism, cell survival and growth (Semenza, 2001). More than 100 genes are known to be directly or indirectly regulated by Hif-1 (Kotch et al., 1999; Wang et al., 1995). Table 3.2 shows the most abundant of the identified Hif-1 target genes. It has been shown that Hif-1 induces broad hypoxia at systemic and cellular level in order to compensate for the energy deficit in the cell. Moreover, the Hif-1 pathway is critical in development, physiology and disease (Jelkmann, 2007; Krishnan et al., 2008; Soliz et al., 2005; Tovari et al., 2008).

Hif-1 is a heterodimeric transcription factor composed of Hif-1α and Hif-1β subunits (Hopfl et al., 2004) and is expressed in all tissue of many species such as drosophila, fish, C. elegans and mammals (Abbrecht and Littell, 1972; Epstein et al., 2001; Soitamo et al., 2001).

Whereas the β subunit is a non-responsive nuclear protein and thus, stable, the accumulation of α subunit is controlled by cellular $O_2$ concentration (Hopfl et al., 2004; Wang et al., 1995). At normoxia, Hif-1α is immediately degraded, but this process is inhibited under hypoxic condition and therefore, it causes a response to hypoxia. As a consequence of hypoxia induced stabilization, Hif-1α translocates to the nucleus and heterodimerizes with Hif-1β to form a function at Hif-1 complex, which interacts with the hypoxia response element located within the promoter and enhancer of the $O_2$-dependent target gene expression (Hopfl et al., 2004). These genes are particularly relevant for homeostasis at the cellular and systemic levels. The most of the identified Hif-1 target genes are listed in Tab. 3.2. One of the best studied Hif target genes is erythropoietin (Epo).
| - Adenylate kinase 3 (AK-33) | - Intestinal trefoil factor |
| - Adrenomedullin (ADM) | - Kreatin 14, 18, 19 (KRT14, 18, 19) |
| - Aldolase A (ALDA) | - Lactate dehydrogenase A (LDHA) |
| - Aldolase C (ALDC) | - LDL-receptor-related protein (LRP1)Leptin (LEP) |
| - ANF/GPI | - LDL-receptor-related protein 1 (LRP1) |
| - Autocrine mobility factor/ (AMF/GPI), | - Metalloproteinase (MMP2) |
| - \(\alpha_{1B}\)-adrenergic receptor (\(\alpha_{1B}-\)AR) | - MIC2 |
| - Carbonic anhydrase 9 (CA-9) | - Multidrugresistance (MDR1) |
| - Cathepsin D (CATHD) | - NIP3 |
| - Ceruloplasmin | - Nitric oxide synthase 2 (NOS2) |
| - c-MET | - NIX |
| - Collagen type V (\(\alpha_1\)) | - NUR77 |
| - Cyclin G2 | - Phosphofructokinase L (PFKL) |
| - Differentiated embryo-chondrocyte expressed gene 1,2 (DEC1,2) | - Phosphoglyceratekinase 1 (PGK1) |
| - Ecto-5'-nucleotidase | - 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKBF3) |
| - Endocrine-gland-derived VEGF (EG-VEGF) | - Plasminogen-activator inhibitor 1 (PAI1) |
| - Endoglucin (ENG) | - Prolyl-4-hydroxylase \(\alpha\) |
| - Endothelin-1 (ET-1) | - Pyruvate kinase (PKM) |
| - Enolase 1 (ENO1) | - p35srj |
| - Erythropoietin (EPO) | - RTP801 |
| - ETS-1 | - Transferin |
| - Ferrochelatase (FECH) | - Transferin receptor |
| - Fibronectin 1 (FN1) | - Transforming growth factor- \(\alpha\) (TGF-\(\alpha\)) |
| - Glucose transporter 1,3 (GLUT1,3) | - Transforming growth factor- \(\beta 3\) (TGF- \(\beta 3\)) |
| - Glyceraldehyde-3-P-dehydrogenase (GAPDH) | - Transglutaminase 2 |
| - Haem oxygenase-1 (HO-1) | - Triosephosphate isomerase (TPI) |
| - Hexokinase 1, 2 (HK1, 2) | - Urokinase plasminogen activator receptor (UPAR) |
| - Inhibitor of differentiation/DNA binding 2 (ID2) | - Vascular growth factor (VEGF) |
| - Insulin-like growth-factor (IGF2) | - Vimentin (VIM) |
| - Insulin-like growth-factor-binding-protein 1, 2, 3 (IGF-BP1, 2, 3) | - WAF-1 |

Tab. 3.2. Some HIF-1 target genes, adapted from Kotch et al. (1999), Wang et al. (1995) and own update
More recently, further Hif-α isoforms have been identified, namely Hif-2α and Hif-3α (Luo et al., 1997). These subunits are also O₂-labile and can dimerise with Hif-1β under hypoxic condition but are different with respect to their tissue-specific mRNA expression pattern (Wiesener et al., 2002). Recent studies show that Hif-2α controls Epo gene expression (Warnecke et al., 2004).

### 3.3. Erythropoietin

#### 3.3.1. Structure of erythropoietin

The secreted human Epo protein is a glycoprotein hormone 34 kDa in weight and consists of 165 amino acids (60% of the total molecule) and 4 carbohydrate chains (40% of the total molecule) (Egrie and Browne, 2001; Jelkmann, 2004; Wen et al., 1993). The carbohydrate chains are essential to the biological stability and affinity of the circulating protein (Egrie et al., 2003). However, several investigators have studied its tertiary structures (Bazan, 1990; Cheetham et al., 1998; Syed et al., 1998). All these models are based on two antiparallel pairs of α-helical bundles with interconnecting variably sized loops. Human and mouse Epo are 80% identical in amino acid sequences (McDonald et al., 1986; Shoemaker and Mitsock, 1986; Wen et al., 1993). The Epo gene in human beings is located on chromosome 7 between 7q21 and 7q22 as a single copy and contains 5 exons (Koury and Bondurant, 1992; Powell et al. 1986; Wang et al., 1995). Epo is expressed in most tissue. This gene is activated by a variety of stressors, including hypoxia (Jelkmann, 2004). When O₂ supply drops, Epo expression induces an exponential increase in Epo plasma levels (Jelkmann, 2003), but this inducibility is tissue specific with the strongest effect in kidney and brain (Koury and Bondurant, 1992; Siren et al., 2001).

#### 3.3.2. Structure of erythropoietin receptor

The Epo protein binds to specific receptors present in the membrane of the target cells (Jelkmann et al., 2008). In general, the number of Epo receptors ranges from approximately 1000-3000/cell (Broudy et al., 1988; D’Andrea and Zon, 1990; Koury and Bondurant; 1992). The human gene is located on chromosome 7 and has 8 exons (Budarf et al., 1990; Maouche et al., 1991). Exons 1-5 encode the extracellular domain. Exon 6 encodes the transmembrane and exons 7, 8 encode the cytoplasmatic domain. Interestingly, the gene is transcribed continuously (Wickrema et al., 1992), but hypoxia may have an impact on its up-regulation. The resulting protein has a molecular mass of 66 kDa and is a 484 amino-acids glycoprotein of the cytokine receptor superfamily which is characterized by
ligand-inducible dimerization (Bailey et al., 1993; D’Andreas et al., 1989; Jelkmann, 2005; 2008). Two of the membrane-spanning Epo receptor molecules form a dimer to which the Epo molecule binds (Jelkmann, 2004).

3.3.3. Function
Epo is a cytokine whose main function is producing erythrocytes by stimulating the proliferation, differentiation and maturation of the progenitors in the bone marrow, and preventing the apoptosis of these erythroid progenitors, by binding to and activating the Epo receptor on the surface of the cell (Jelkmann et al., 2008). It is rapidly up-regulated when pO2 is reduced in the venous blood and tissue. Hypoxia leads to reduced pO2, which results in Hif-1 induced up-regulation of the Epo gene expression, mainly occurring in the kidney. Accordingly, the Epo plasma level is elevated and finally, the number of circulating erythrocytes in the bloodstream is increased.

Apart from the kidney, Epo is produced in (fetal) liver, brain, lung, spleen, bone marrow, male and female reproductive organs and also in numerous cancer cells (Fandrey and Bunn, 1993; Hermine et al., 1991; Jelkmann et al., 2008; Maxwell et al., 1993, 1997; Tan et al., 1992; Yasuda et al., 1998). Additionally, Epo receptor expression has been observed in the brain, retina, and heart and in fractions of skeletal muscle fibers (Grimm et al., 2002; Junk et al., 2002; Lundby et al., 2008a; Wu et al., 1999). The non-hematopoietic functions of Epo are associated with these tissues. Thus, Epo is involved in the modulation of responses to injuries such as cerebral ischemia, cardiac infarction and retina degeneration (Gassmann et al., 2003; Marzo et al., 2008; Wiessner et al., 2001, Wright et al., 2004). Nevertheless, the physiological impact of Epo remains largely unclear. A recently published paper showed that Epo-levels in plasma and brain are involved in the ventilatory response to acute and chronic hypoxia (Soliz et al., 2005) and recombinant human Epo (rhEpo) treated humans feel improvements in mood (Miskowiak et al., 2008) as well as perceived physical conditioning (Ninot et al., 2006). Furthermore, it was reported that Epo promotes the enhancement of the vascular endothelial growth factor (VEGF) expression in tissue (Alvarez et al., 1998), resulting in increased skeletal muscle capillary growth. In a recent published study performed on rodents, it was reported that muscle fiber types change toward a more oxidative phenotype when Epo treatment occurs during training (Cayla et al., 2008). Interestingly, no alterations in skeletal muscle angiogenesis or VEGF mRNA levels were found after long time Epo treatment, nor was a shift in muscle fiber type found (Lundby et al., 2008a).
3.3.4. Exercise

Endurance performance, normally characterized as maximal O₂ uptake (\(\dot{V}O_{2\text{max}}\)), is affected by a series of steps in the transport of O₂ from the atmosphere to the mitochondria of the muscle. Inhibition of each step in the O₂ transport system potentially leads to an impediment of the O₂ flux. These steps include central factors such as O₂ diffusion from the lung into the blood (pulmonary system), cardiac output and O₂ carrying capacity of the blood, and peripheral factors such as skeletal muscle characteristics such as muscle capillary density, O₂ diffusion gradient from the surface of red blood cells to sarcolemma and mitochondrial enzyme activity (Fig. 3.1). The issue of which factors limit \(\dot{V}O_{2\text{max}}\) remains a subject to intensive debate.

![Fig. 3.1. Potential factors which limit maximal oxygen uptake (\(\dot{V}O_{2\text{max}}\))](image)

The difficulty to identify limiting factors is the fact that some variables can easily be influenced by endurance training while others can not be modified. Thus, there is no single variable limiting endurance performance, but there are a number of components reflecting a cascade mechanism.
However, the blood’s O₂ transport capacity is of special interest in this thesis. According to Fick’s law, $\dot{V}O_{2\text{max}}$ depends on cardiac output and the arteriovenous O₂ difference. This implies that all factors influencing these physiological quantities may exert endurance limiting effects. The most important related to blood supply are the blood volume and whole body hemoglobin. Several studies have shown that there is a strong correlation between $\dot{V}O_{2\text{max}}$ and blood volume as well as whole body hemoglobin, but not hemoglobin concentration [Hb] (Åstrand, 1952, 1977; Ekblom and Hermansen, 1968; Kanstrup and Ekblom, 1984; Heinicke et al., 2001). Note that most human studies are conducted with subjects reaching hematocrit (Htc) levels up to 0.5. Thus, it is not known whether the relationships exist at Htc values higher than 0.5, too.

If $\dot{V}O_{2}$ is maintained during endurance exercise, it is equal to the product of the athlete’s $\dot{V}O_{2\text{max}}$ and the percent of $\dot{V}O_{2\text{max}}$ that can be maintained during performance (Coyle et al., 1995). The percentage of $\dot{V}O_{2\text{max}}$ is related to the $\dot{V}O_{2}$ at the lactate. Artificially raised plasma volume, which increases whole blood volume but keeps red cell numbers constant and lower [Hb], has no effect on $\dot{V}O_{2\text{max}}$ in trained subjects (Kanstrup and Ekblom, 1982), while submaximal exercise duration may be prolonged (Coyle et al., 1990; Luetkemeier and Thomas, 1994). Note hypervolemic plasma expansion in untrained subjects induces significantly an increment in $\dot{V}O_{2\text{max}}$ due to increased stroke volume (Warburton et al., 2004). Otherwise, an increment of whole body hemoglobin mainly affects $\dot{V}O_{2\text{max}}$. Endurance training at sea level improves cardiac output and muscular vascularisation (Gaudard et al., 2003), but its impact on the stimulation of erythropoiesis is controversial. Some studies performed in untrained and trained athletes from different disciplines showed that submaximal and maximal exercise have no impact on circulating plasma levels (Berglund et al., 1988; Gareau et al., 1991, Ricci et al., 1990; Schmidt et al., 1991). In contrast, other studies found a slight increase in the Epo plasma level after several hours of exercise (Ricci et al., 1988; Schobersberger et al., 2000; Schwandt et al., 1991). One explanation for the increasing Epo plasma level might be that these changes are due to changes in plasma volume and another explanation might be that hormones in the blood show circadian rhythm with a nadir in the morning. Thus, measurements have to be done at the same time of day (Jelkmann, 2003; Schmidt et al., 1991, 1993). However, the number of circulating reticulocytes is immediately increased after strenuous exercise (Schmidt et al., 1988). Jelkman (2003) suggested that the reduced renal blood flow is only a minor factor in Epo production, but stress hormones such as cortisol and catecholamines may be more important in the regulation of erythrocyte production.
To improve their sea level endurance performance, some endurance athletes live at moderate altitude (2000-3000 m above sea level) and train at low altitude to increase [Hb] due to hypoxia induced stimulation of erythropoiesis (Levine and Stray-Gundersen, 1997; Wilber et al., 2007). Unscrupulous athletes, coaches, and practitioners artificially increase the O_2 transport capacity of the blood and thus exercise performance by using rhEpo and blood transfusions of red blood cells.

3.3.4.1. Impact of recombinant human erythropoietin

In 1987, rhEpo became commercially available. It was developed for the treatment of anemia occurring as a consequence of ailments such as chronic renal failure, HIV-infection and cancer. Unfortunately, rhEpo is also well known as a doping agent in endurance sports, and even worse, might be a target for gene doping. Since its launch onto the market, several studies have investigated its effects on [Hb] and \( \dot{V}_{O_2}\text{max} \). In the first published study, Ekblom and Berglund (1991) reported that injecting 20-40 IU/kg body mass rhEpo over a period of 6 weeks, three times per week, induces an increment of 11.2% in [Hb], resulting in an 8.9% improvement in \( \dot{V}_{O_2}\text{max} \). The effect of injected rhEpo is even more pronounced at submaximal exercise intensities than at maximal exercise intensities (Thomsen et al., 2007). Similar results are observed when [Hb] is elevated by blood infusion (Brien and Simon, 1987; Buick et al., 1980; Ekblom et al., 1972; 1976; Robinson et al., 1966; Turner et al., 1993; Williams et al., 1981). For instance, Ekblom and co-workers (1972) demonstrated that autologous blood re-infusion of 800-1200 ml results in an increase in [Hb] of 13%, whereas \( \dot{V}_{O_2}\text{max} \) and endurance time were increased by 9% and 23% respectively. Thus, it may seem obvious that the ergogenic effect of rhEpo administration and blood infusion on the exercise capacity is mediated through the increasing [Hb].

An increment in [Hb] is associated with elevated Htc levels and thus with blood viscosity (Crowell and Smith, 1967; Vogel et al., 2003). Elevated blood viscosity may unfavorably affect the microcirculatory blood flow and O_2 delivery to the tissue (Crowell and Smith, 1967). Therefore, the peripheral resistance to blood flow within the vascular system is regulated not only by the calibre of the vessels, but also by the viscous characteristics of the blood (El-Sayed et al., 2005). Blood viscosity is linearly correlated over an Htc range of 0.2 to 0.6, beyond which the increment in whole blood viscosity becomes disproportionately higher with increasing Htc levels (Chien et al., 1966). Note that blood viscosity is globally measured, but it may vary locally within the different parts of the vascular system when blood flow is forced (Dorandy, 1979). Disruption of the normal rheological properties of the blood is considered an independent risk factor for the cardiovascular system and
might also reduce exercise performance and may be life-threatening (Jelkmann, 2003). Therefore, our transgenic mouse line termed tg6 overexpressing human Epo cDNA reached Htc values of up to 0.9 (Ruschitzka et al., 2000) and showed a dramatically reduced exercise performance (Heinicke et al., 2006; Wagner et al., 2001). This implies that there is an optimal Htc value at which the O2 carrying capacity of blood is maximized without compromising cardiac output due to the elevated blood viscosity associated with higher Htc levels (Crowell et al., 1959, Guyton and Richardson, 1961; Richardson and Guyton, 1959; Villafuerte et al., 2004). It was proposed that $\dot{V}O_2^{max}$ is limited by 70% of blood O2 transport capacity within the circulatory system; all other systems being responsible for the remaining 30% (DiPrampero and Ferretti; 1990). Thus, there may potentially exist an optimal Htc for maximal endurance performance. But no data are available for higher vertebrates.

If Htc levels sharply rise, cardiac output falls (Richardson and Guyton, 1959). Interestingly, the tg6 mice showed no changes in resting cardiac output (Vogel et al., 2003). Animals grow up normal and showed no symptoms of thromboembolism, but had a lifespan that was a third shorter than that of their wild type (wt; Wagner et al., 2001) and developed organ failures such as degenerative processes in the liver, kidney, hepatic system, and nerve and skeletal muscle fibers (Heinicke et al., 2006). However, these findings indicate that adaptive mechanisms enacted in response to excessive erythrocytosis exist. Adaptive processes in response to excessive erythrocytosis include increased plasma nitric oxide levels, elevated erythrocyte deformability and reduction of erythrocyte’s lifetime (Bogdanova et al., 2007; Ruschitzka et al., 2000; Vogel et al., 2003). Interestingly, there are also humans who cope with excessive erythrocytosis. In one case, due to an autosomal dominant erythrocytosis, a Finnish cross-country skier reached Htc levels of up to 0.68 (Juvonen et al., 1991), won several Olympic gold medals and showed no organ failures. In another case, some miners in the South American Andes - living and working at an altitude of 5960 m above sea level and exposed to cobalt - were reported to have an acute Htc level of between 0.75 and 0.91 (Jefferson et al., 2002).

To study the impact of varying Htc levels on exercise performance and the cardiovascular system, the technology to measure blood pressure as well as heart rate during submaximal and maximal exercise in mice was improved by using telemetry in the first study. In general, the transmitter implantation is associated with a high failure rate. Thus, a further aim was to improve the surgery to reduce that number. The second study investigated whether there is an Htc value which allows maximal endurance performance for whole body exer-
cise. To this end, Htc levels of wt mice were increased by graduated application of novel erythropoiesis stimulating protein (NESP). On the other hand, the Htc of our tg6 mice was decreased in steps to different levels by the application of phenylhydrazine, a compound inducing hemolysis (Lim et al., 1998). We postulate that the optimal Htc levels may not be identical for wt and tg6 mice. Thus, it was hypothesized that tg6 can adapt better to various Htc levels than wt mice can.

3.3.4.2. Altitude acclimatization

When humans are exposed to hypoxia, besides the physiological changes that occur in the muscular, respiratory, cerebral, cardiovascular and hormonal systems, and the changes in fluid and electrolyte balances, the O₂ transport capacity is particularly affected. This is perhaps the reason why one of the most frequently studied adaptations to high altitude is the increase in red blood cells.

Within two hours of acute onset of hypobaric hypoxia, circulating Epo plasma levels begin to increase significantly (Eckardt et al., 1989). The peak is reached after between 24 and 28 hours of a stay at moderate (1500-3000 m) and high (> 3000 m) altitude (Abbrecht and Littell, 1972; Gunga et al., 1994; Heinicke et al., 2003; Milledge and Cotes, 1985). After this time, plasma Epo levels decline to close to pre-exposure levels, but may remain slightly elevated when subjects keep staying at higher than normal altitudes (Heinicke et al., 2003; Mairbaurl et al., 1990; Milledge and Cotes, 1985). Mountain climbers usually undergo a gradual increase in the degree of hypoxia until they reach a high altitude destination. Therefore, increasing Epo levels are detected not only after an ascent from sea level to altitude, but also when ascending from moderate altitude to high altitude. The effect of acute exposure to hypoxia on Epo levels is maintained after 3 weeks of acclimatization, and even after life long intermittent residency at high altitude (Heinicke et al., 2003). However, despite the reduced Epo level in serum with prolonged altitude, erythropoiesis remains stimulated (Milledge and Cotes, 1985). This is indicated by elevated reticulocyte counts and decreased serum iron and ferritin (Mairbaurl et al., 1990). Note that [Hb] increases during the first 24-48 hours of exposure to altitude due to a reduction in plasma volume and that this increase is not due to the enhanced erythropoiesis. The latter is caused by an enhancement of diuresis and displacement of water from the vascular to the extravascular and intracellular spaces.

When humans are acutely exposed to high altitude, V̇O₂max is reduced as a function of the reduction of arterial O₂ saturation (SaO₂; Fulco et al., 1998; Wehrlin and Hallen, 2006).
During altitude acclimatization [Hb] continues to increase in order to make up for the loss in arterial O₂ content (CaO₂) due to decreased arterial SaO₂ (Calbet et al., 2003a; Mair-baurl, 1994). Accordingly, CaO₂ is normalized or is even higher than at sea level during acclimatization above 4100 m altitude (Calbet et al., 2003b; Ceretelli, 1976; Lundby et al., 2004), whereas \( \dot{V}O_{2\text{max}} \) remained depressed. Interestingly, \( \dot{V}O_{2\text{max}} \) still does not increase when CaO₂ is elevated by NESP injection after acute hypoxia at 12.6% O₂ (= 4100 m above sea level; Lundby and Damsgaard, 2006a). NESP has been developed as an Epo-analogue with prolonged survival in the circulation (MacDougall, 2000) and thus, it has a longer half-life than rhEPO (NESP: 24-26h, rhEpo: 4-8h; Jelkmann, 2002).

However, there is a discrepancy in the ergogenic effect of increasing [Hb] observed at sea level compared to that observed at high altitude and this suggests that an altitude threshold exists over which increasing [Hb] has no advantageous effect on endurance performance, but below this threshold increasing [Hb] improves endurance performance. However, Calbet and co-workers (2002) reported that during maximal exercise on a cycle ergometer test after 9 weeks at 5260 m, systemic O₂ delivery was 10% lower than during maximal exercise at sea level, due to a reduction in peak cardiac output during exercise in hypoxia. Although the CaO₂ was almost normalized by the increasing [Hb] to pre-altitude values, \( \dot{V}O_{2\text{max}} \) was only partly improved and remained 30% below the values observed at normoxia. This implies that part of the systemic O₂ delivery gained with acclimatization is not made available to the exercising muscle (Calbet et al., 2003b).

While it seems clear that \( \dot{V}O_{2\text{max}} \) does not improve during altitude acclimatization above 4100 m, the data below are controversial. On one hand, five studies reported no change or a slight increment in endurance after 14 and 20 days training and living at 2300 m respectively (Adams et al., 1975; Daniels and Oldridge, 1970; Faulkner et al., 1967; 1968; Pugh, 1967). On the other hand, \( \dot{V}O_{2\text{max}} \) and endurance performance increase after 19 days of acclimatization to 2300 m and 21 days to 1822 m respectively (Jensen et al., 1993; Saltin 1967). The difficulties of these studies can mainly be explained by different training regimes, different physiological organic systems in the human body reacting to training at altitude at different times, the different degree of reaction of various systems and the varying overall training status of the studied athlete. Levine and Stray-Gundersen (2005) stated the success of altitude training depends on two factors: 1.) live high enough to initiate and maintain erythropoiesis to improve blood O₂ transport capacity and thus \( \dot{V}O_{2\text{max}} \), and 2.) train low enough to avoid intensified training stimulus, which results in an opposite effect – such as reduced speeds and reduced power output. Thus, athletes should live in hypoxia,
but perform their training at sea level conditions in order to perform well at sea level. It was reported that there are two groups (Levine and Stray-Gundersen, 2005). One group, termed responders, displayed a larger increase in plasma Epo concentrations at altitude compared to the concentrations in the second group of “non-responders”. The higher Epo concentrations found in responders led to increased total red blood cell volume and $\dot{V}O_{2\text{max}}$ values at sea level in contrast to the data from the non responders. The variation of increase in Epo concentrations could be explained by the genetic differences of individuals (Chapman et al., 1998).

The third study of this thesis investigates the impact of acclimatization to moderate altitude on exercise performance. To exclude detraining, elite athletes had to train according to the “live high-train low” procedure (Levine and Stray-Gundersen, 2005). The outcome of this study is particularly interesting for athletes who plan to participate in a competition at around this altitude in order to be optimally prepared for competition day.

3.4. Aim of the project

The aims of the present work were:

**In mice**
- to establish and improve the telemetry technique during exercise as well as the transmitter implantation to reduce the failure quota of the animals dramatically.
- to investigate the Htc level at which mice reach maximal exercise performance by titrating Htc levels of wt and tg6 mice.
- to examine the hypothesis that mice with chronically elevated Htc adapt better to the excessive erythrocytosis compared to acutely NESP-injected animals.

**In humans**
- to test the hypothesis that $\dot{V}O_{2\text{max}}$ and endurance performance increase after acclimatization to an altitude of 2360 m.
4. Study 1: Optimizing transmitter implantation and postoperative care to improve telemetric signal in exercising mice

4.1. Abstract

Genetically modified laboratory mice are increasingly used to study cardiovascular physiology and diseases. The measurement of blood pressure in the free roaming, unanesthetized and unstressed mouse is most reliably and accurately performed with telemetry. However, implantation of telemetric transmitters can cause serious postoperative complications and death, in particular if animals with genetically induced abnormalities undergo such major surgery. Moreover, data recording can be hampered if the measurements have to be carried out in an exercising rodent.

Here we show an optimized telemetric transmitter implantation technique (fixation of the transmitter body on the back of the animal with stainless steel wires) for measuring arterial blood pressure during maximal exercise on a treadmill. This technique is used on transgenic mice that constitutively overexpress erythropoietin (Epo) resulting in hematocrit up to 0.9 and on the corresponding wildtype mice. In addition, we have established a regime for postoperative intensive care and analgesia: warmth, subcutaneous fluid therapy (600 μl) and analgesics (flunixin 5 mg/kg bodyweight) twice per day, and offering high energy liquid in a drinking bottle. The postoperative care was performed for 14 days and led to substantially improved morbidity and mortality. The refined postoperative care and surgical technique were particularly successful in our genetically modified mice with severely compromised physiological capacities.

4.2. Introduction

The mouse has become the most commonly used animal model to study aspects of cardiovascular physiology important for drug development, safety, pharmacology and basic research goals (Kramer and Kinter, 2003). Thus, the ability to record cardiovascular parameters in mice has become an important tool for understanding the response of the cardiovascular system in various experimental approaches (Kurtz et al., 2005). Under certain circumstances, it is necessary to investigate cardiovascular performance under different challenging body conditions. Treadmill exercise tests are a commonly used clinical approach in human beings to induce cardiovascular stress in order to detect cardiovascular abnormalities which may not be observed at rest (Sullivan and Hawthorne, 1995). Simultaneously,
metabolic parameters usually determined by indirect calorimetry are often measured during exercise (Ba et al., 2008). In contrast to the situation in humans, cardiovascular function and metabolic parameters have been very rarely measured directly in rodents during submaximal and maximal exercise.

Radiotelemetry is the unique approach to measure cardiovascular parameters in unanesthetized, freely moving small rodents using this method, physiological parameters are efficiently recorded and the results are reliable and objective compared to the results obtained using previous measuring techniques described in the literature (Butz and Davisson, 2001; Clement et al., 1989; Feng et al., 2008; Kramer and Kinter, 2003; Kubota et al., 2006; Whitesall et al., 2004). However, the disadvantages are the high costs of the equipment and the need for experience in the microsurgical technique for implantation of the transmitters. Usually, the pressure-sensing catheter tip is implanted in the thoracic aorta via the left carotid artery and the transmitter body is subcutaneously placed along the right flank (Butz and Davisson, 2001). Thus, animals have to carry the transmitter’s weight unilaterally. This is particularly uncomfortable during exercise, resulting in the abandonment of the exercise test. Moreover, only weak telemetric signals or even no signals at all are obtained during treadmill exercise due to the long distance between the transmitter (located at the flank of the body of the mouse) and the receiver plate (placed over the treadmill). However, in spite of the advancement of the surgical technique over the last years, there is still a high morbidity and mortality if telemetric transmitters are implanted in mice. From their specific phenotypical characteristics, they often react by showing a severe impairment of their general condition and symptoms of suffering as a result of the trauma of implantation (Brown et al., 2006; Chen et al., 2005). Thus, even if the surgical procedure appears to be successful, a quick recovery from anesthesia and intensive postoperative medical support are necessary to prevent severe physiological aberrations and possible high death rates after transmitter implantation in genetically modified mice.

Here we show an alternative approach that places the transmitter in the midline of the mouse’s back. The aim is to minimize the negative impact of the transmitter’s weight on the running performance while obtaining telemetric signals in a constant and reliable manner during maximum exercise. Furthermore, the positive effects of the postoperative analgesia and intensive care regimen on the survival rate was demonstrated in our transgenic mouse line termed tg6 that constitutively overexpresses human erythropoietin (Epo) cDNA resulting in hematocrit levels of up to 0.9, and in the corresponding wild type (wt) mice.
4.3. Methods

Mouse model
The tg6 mice were generated by pronuclear microinjection of the full-length human Epo cDNA driven by the human platelet-derived growth factor (PDGF) B-chain promoter as described previously (Rutschitzka et al., 2000). The resulting tg6 mouse line B6D2-TgN(PDGFBEPO)321Zbz showed increased Epo levels in plasma and brain (Soliz et al, 2007; Wiessner et al., 2001). Breeding was performed by mating hemizygous males to wt C57BL/6 females. As expected, one half of the offspring was hemizygous for the transgene while the other half was wt and served as controls.

Animals and housing conditions
Male animals were used at the age of 30.9 ± 5.3 days. Mice were kept in standard rodent cages with food and water supplied ad libitum in 12:12 hour light-dark cycle. The experimental protocols were approved by the Kantonges Veterinärabt Zurich and were performed in accordance with the Swiss animal protection laws and institutional guidelines.

Side effects of carotid artery occlusion
To rule out that the occlusion of the carotid artery would induce ischemia in the brain or even stroke, seven age matched (wt: n = 5, tg6: n = 2) mice underwent a preliminary experiment. Anesthesia and surgical conditions as aseptic precautions, handling animals under laminar flow and use of sterile instruments were set according to the procedures for transmitter implantation (details see below). After removal of hairs and disinfection of the anterior neck region, the skin was incised, connective tissues and muscles were prepared and the left common carotid artery was exposed. The artery was ligated with two silk sutures (PERMA-Handseide 6-0, Ethicon, Norderstedt, Germany), a distance of 5mm apart from each other (similar to the ligations applied for the catheter fixation). Connective tissues and muscle layers were closed with resorbable sutures (VICRYL 6-0, Ethicon, Norderstedt, Germany) and animals were allowed to recover on a warmed mat (38°C). Animals were screened daily for symptoms of cerebral ischemia and stroke by use of a published neurological deficit score system (Huang et al., 1994).

Mice were sacrificed at different time points after occlusion of the left common carotid artery: three mice (wt: n = 2, tg6: n = 1) at 36 hours, 2 mice (wt: n = 1, tg6: n = 1) at 72 hours and 2 mice (wt: n = 2) at 7 days. Brains were isolated, fixed in 4% neutral buffered formalin and processed routinely for neurohistological examination (brains were cut sys-
systematically in layers to take sections at certain distances). Tissue sections (2 μm) were stained with hematoxylin and eosin and evaluated by neuropathologists, who were blinded to the treatment protocol.

**Surgical procedures**

The implantations were carried out under aseptic conditions in a laminar flow hood using sterile equipment. Inhalation anesthesia was initiated with 7-8% and maintained with 3.5-4% sevoflurane (Sevorane®, Abbot, Cham, Switzerland) in pure oxygen (O₂). After removal of hairs and disinfection of the anterior neck region, a longitudinal skin incision of 1 cm was performed, the connective tissues and muscles were prepared and the left common carotid artery (Arteria carotis communis sinistra) was exposed. The artery was ligated with a silk suture (PERMA-Handseide 6-0, Ethicon, Norderstedt, Germany) caudal to the bifurcation in the internal and external branches. A second suture was placed around the artery at a distance of 4-6 mm below the bifurcation and the blood flow was stopped by retracting the suture. A third suture was loosely placed around the artery between the other two. Then, a hole was cut in the artery using fine bladed scissors and the catheter of the TA11PA-C10 transmitter (DataSciences International, St. Paul, MN, USA) was inserted into the vessel, while the second suture was opened to allow the tip of the catheter to be introduced into the thoracic aorta. The catheter was then fixed with the sutures in the artery. The muscle layers and connective tissues were restored by resorbable sutures (VICRYL 6-0, Ethicon, Norderstedt, Germany). By blunt dissection with an atraumatic scissor a pocket underneath the skin was prepared, which reached from the right side of the skin incision to the area between the shoulders and the midline of the thorax on the back of the animal. The transmitter body was put between the skin and the subdermal connective tissue at the right edge of the skin incision and was advanced in a dorsal direction until it was located on the back of the mouse. The transmitter body was fixed there by 3 to 4 loops of surgical stainless steel sutures (3-0 2xTS+FS; Ethicon, Norderstedt, Germany), which were laid from one side to the other through the skin in the subdermal connective tissues underneath the transmitter body. One to two loops of stainless steel wire were placed behind the transmitter body to prevent its dislocation to the lower back. Finally, the skin incision in the neck was closed with resorbable sutures and the animals were allowed to recover for 1-2 hours on a heated, water bath surface of the operating table (38°C).
**Analgesia and postoperative care regimens**

As analgesics either buprenorphine (Temgesic®, Essex Chemie AG, Lucerne, Switzerland) was used in a dosage of 0.1 mg/kg body weight or flunixine (Biokema Flunixine®, Biokema SA, Crissier-Lausanne, Switzerland) was applied at 5 mg/kg body weight. Analgesics were administered subcutaneously twice per day (i.e. every 12 hours) for 7 days; the first injection of analgesics was performed during anesthesia for transmitter implantation. To support fluid homeostasis during surgery, 1 ml of saline at a temperature of 36°C was injected intraperitoneally after inducing anesthesia.

**Three different postoperative care regimens were compared**

In the first group, mice were treated with buprenorphine (wt: n = 2, tg6: n = 2); in the second group flunixine was used as analgesic (wt: n = 1, tg6: n = 4).

A third group received flunixine as an analgesic for 7 days and an additional fluid therapy of 300 μl glucose (5%) and 300 μl saline (0.9%), injected subcutaneously twice per day for 14 days (wt: n = 47, tg6: n = 48). Before injection, the mice were weighed. All analgesic agents including glucose liquid and saline were heated up to body temperature before injection. In the third group, all animals’ cages were kept on a heating mat during the whole postoperative period of 2 weeks. In addition, only the third group had free access to glucose (15%), offered in a second water bottle, and to high-energy, wet food (Solid Drink®-Energy, Triple A Trading, Tiel, Netherlands). The high-energy food and glucose-containing water bottles were given in the animals’ cages from 2 to 3 days before surgery until 2 weeks afterwards. O₂ was introduced via a tube into the cages (Fig. 4.1). After this period, the implanted animals were transferred to a non-implanted ovariectomized Crl:CD (ICR) female.

**Fig. 4.1.** Cages kept on the heating pad and perfused with poor oxygen via red as well as white tubings.
Telemetric signal verification during maximal exercise

The telemetric signal was measured during maximal exercise using a Simplex II metabolic rodent treadmill, fitted with Oxymax gas analyzer (Columbus Instruments, Columbus, OH, USA). For the maximal incremental exercise test, mice were placed in the exercise chamber and allowed to equilibrate for 30 min. Treadmill activity was initiated at 2.5 m/min and 0° inclination for 10 min and then, increased by 2.5 m/min and 2.5° every 3 min thereafter until exhaustion. Mice were gently encouraged to run for as long as possible by the use of a mild electric grid at the end of the treadmill (0.2 mA, pulse 200 ms, 1 Hz). Exhaustion was defined as the inability to continue regular treadmill running despite a repeated electric stimulus to the mice.

Statistics

All data were analyzed using StatView software (Version 4.57, Abacus Concepts, Berkeley, California, USA). Results are expressed as means ± standard deviation (SD).

4.4. Results

All animals scored 0 (i.e. showed no behavioral signs of neurological deficits) after ligation of the left common carotid artery. Systematic histological examination of brains confirmed no abnormalities that would hint at ischemia or even infarction of the brain. A total number of 50 wt and 54 tg6 mice were used with a body weight between 18g and 27g (mean: wt = 21.6 ± 1.6g; tg6 = 22.2 ± 2.1g) on implantation day (Fig. 4.2). Mice were treated either with buprenorphine (first group) or flunixine (second group). In addition to flunixine, third group received a warmth fluid therapy and had free access to high energy liquid. No animal from the first (wt: n = 2; tg6: n = 2) or second (wt: n = 1; tg6: n = 4) groups reached the final time point between 7 and 13 weeks, as the mice were euthanized due to complications because they exhibited hypothermia, apathy, exsiccosis or an obviously moribund state (Fig. 4.3 a, b, c). In contrast to animals of the first and second group, no animal of the third group (wt: n = 47 wt; tg6: n = 48) showed such complications. But, three wt mice were euthanized between 23 and 34 days after implantation as these animals were fighting with their female companions. Four tg6 mice showed hematoma in the right neck and shoulder area at 1-2 hours after surgery. Three of these animals could be rescued by re-opening the wound and removing the blood clots, while additional saline (1 ml) was injected intraperitoneally. Despite removing the haematoma, one of these animals died 6 days after the implantation from repeated bleeding from the implantation site. Two tg6 mice were euthanized 23 and 41 days after the implantation because the telemetric signals
hinted at signs of blood clot formation at the tip of the catheter. This was confirmed during necropsy. The thrombus formation was supposed to be a consequence of the repeated use of the transmitters. Despite these limitations, the methodology was rather successful. Fig. 4.4 shows the relationship between establishing and refining the postoperative care regimens and the survival time. The increasing experience in postoperative care was associated with survival times of up to 100 days. After that time, survival time remained unaltered.

Fig. 4.2. Correlation between weight at implantation and survival time. Each value represents a single animal.

In all animals, the transmitter body was fixed in the midline of the mouse’s back. In two animals from the third group, the transmitter body had to be re-fixed one day after the implantation because it had turned and moved to the upper neck region, where it could hamper the movement of the mouse’s head. In general, the mice showed no signs of reduced physical activity or restricted head movement. In contrast to the commonly used fixation technique of the transmitter body, the modified fixation method improved the telemetric signal, as the distance between the telemetric receiver plate and the transmitter body was shortened (Fig 4.5).
Fig. 4.3. Correlation between final body weight and survival time of (a) all animals, (b) wt mice, and (c) tg6 mice. Each value represents a single animal.
Fig. 4.4. Correlation between the survival time and the progression in the refinement of the postoperative care regime. Each value represents a single animal.

Fig 4.5. Enclosed treadmill and telemetry receiver plate
4.5. Discussion

The approach of inducing a genetically modified mouse model of compromised phenotype to perform maximum exercise in order to obtain real-time blood pressure data was realized by elaborating refined methods for telemetric transmitter implantation and postoperative intensive care.

When telemetric transmitters for measuring blood pressure in mice first became available, a technique of implantation was developed, in which the catheter was implanted in the abdominal aorta and the transmitter body was located in the abdominal cavity (Kramer et al., 2000; Mills et al., 2000; Van Vliet et al., 2000). This implantation method was of limited success, because it induced thrombosis and embolism at high rates leading to the death of more than half of the mice implanted within 2 after days of surgery. Therefore, implantation of the catheter in the thoracic aorta arch via the common carotid artery was soon preferred. Because of the limited length of the catheter, the transmitter body must be placed under the skin of the back, from where it moved in most cases to the right body wall of the mouse (Butz and Davisson, 2001; Carlson and Wyss, 2000). Thus, fixation of the transmitter body with sutures on the muscles of the animals’ backs was proposed, but induced difficulties and additional injury, because a second skin incision at the shoulder region was necessary in addition to the one in the neck (that was used for placement of the catheter). Thus, it was generally preferred that the transmitter body was introduced via the wound to the right flank, without any further fixation. This technique, in which the transmitter body hung at the lateral body wall of the mouse, seemed feasible for recordings at rest and during short-time exercise, e.g. for 15 minutes of treadmill running (Davis et al., 2003). However, we found in a previous pilot experiment (not shown), that transmitter signals were weak, undetectable or disturbed by artifacts during incremental exercise to exhaustion on the treadmill. Moreover, the lateral placement of the transmitter body, which lay in front of the right hind leg, seemed to hamper movement and consequently the exercise performance of the animal. Thus, we put the transmitter body through the wound in the neck under the skin of the back approximately between the shoulders. The stainless wire loops between the transmitter and the connective tissue fixed it in the midline over the axis. One or two loops behind the transmitter body prevented it from moving to the lower back and meant that the catheter tip could be withdrawn from the aortic arch. The drawbacks of this method were seen only in the genetically modified mice, in which the tissue damage from suturing with the wires or preparing the pocket for the transmitter injured subcutaneous vessels, which led to prolonged bleeding due to the impaired blood clotting properties of
these transgenic mice. As a consequence, prominent haematoma were exhibited in the area of the right shoulder and the neck 1 to 2 hours after surgery had been completed. Removing the haematoma by re-opening the wound in the neck and using an intraperitoneal injection of additional saline could rescue three of these mice, while one died at six days following implantation from repeated bleeding and hypovolemia. Apart from this technical aspect, three wt mice were euthanized before reaching the final time point because of serious injuries after fighting with their female cage mates. In these cases, there was no direct relationship with the new transmitter body implantation technique. Taken together, the fixation of the transmitter in the back of the mouse allowed us to measure reliable signals at any time point during maximum exercise.

The use of genetically modified mice with phenotypes that hint at deficiencies in physiological and bodily adaption capacities is increasing. Such specific disabilities in the tg6 mice were particularly impairing the outcome of our surgical efforts at the beginning of the study. This observation is supported by the finding that the final body weights of wt mice from the first and second groups were not different from those from the third group. In contrast, the genetically modified mice showed clear symptoms of exsiccosis, hypothermia, and energy deficits, which led to severely depressed general condition and overall appearance, in particular at 2-9 days following surgery. From this, we took supportive postoperative means to improve the condition of animals, which led to a high survival rate. We could not find a relationship between body weight and survival rate, which was suggested by others (Johnston et al., 2007). Also, we suppose that administering of warmth for a prolonged period of 7 days after implantation was a beneficial intervention, because this would save energy for the animal, as has been proposed by others (Van Vliet et al., 2006). In addition, we consider that the injection of warmed fluid at 12 hour intervals for seven days was a key intervention supporting survival. Indeed, this intervention implies more stress for the animals but the positive effect of this therapy appears to outbalance. Finally, we suggest to provide energy in the form of glucose 15% as the animals consumed this liquid in high amounts postoperatively. With these methods, we could overcome the problems which the phenotype of tg6 mice presented and which are most probably the reason for the poor survival rate at the beginning of our study. The postoperative intensive care and the analgesic regimens described in this study may be useful for others who are confronted with similar difficulties when implanting probes in genetically modified mice.
**Chronic side effects of telemetric transmitter implantation**

The effect of the occlusion of one carotid artery (caused by inserting the catheter of a telemetric transmitter) on arterial blood pressure in general was described in a study of Carlson and Wyss in 2000. With simultaneous measurements in the femoral artery, the authors found a slight elevation of the arterial blood pressure, which returned to baseline within 30 seconds and thus can be estimated as an acute reaction that has no influence on the long-term, chronic measurements after the recovery phase from transmitter implantation (Carlson and Wyss, 2000). However, it is known that mice show different blood flow and vessel conformation depending on the strain (Van Vliet et al., 2006). To rule out that ligation of the left common carotid artery induced any ischemia or even infarction in the brain, in a preliminary experiment this vessel was occluded in age matched wt and tg6 mice. In these mice, no symptoms of neurological deficits were detected. Systematic histological examination by experts in neuropathology showed no aberrations in the brain at various time points due to left common carotid artery occlusion, confirming no detectable influence of this manipulation on the experimental outcome in wt C57BL/6 and tg6 mice.

The present findings indicate that the placement of the transmitter body between the shoulders of the mice is an important feature to improve the detection and recording of the telemetric signals, allowing monitor the cardiovascular function in the exercising rodent. However, the difficulties of surgical procedure remain challenging. The small size and delicate nature of the arteries of the mouse require excellent hand-eye coordination and steady hands in order to catheterize the vessel successfully. Intensive postoperative care and analgesia improves the survival and body condition after implantation of probes, which is particularly important in genetically modified mice with impaired bodily capacities.
5. Study 2: Optimal hematocrit for maximal exercise performance in erythropoietin-treated mice

5.1. Abstract
This study was performed to investigate the impact of varying hematocrit (Htc) levels on endurance performance and the cardiovascular system using the telemetry technique. Therefore, two strategies were combined. Htc levels of wild type (wt) mice were acutely elevated by applying novel erythropoiesis stimulating protein (NESP; wtNESP). On the other hand, Htc levels of our transgenic mice line (tg6) that reached Htc levels of up to 0.9 due to constitutive overexpression of erythropoietin (Epo) were reduced by the hemolysis-inducing compound phenylhydrazine (PHZ; tg6PHZ). Highest maximal oxygen (O₂) uptake (\(\dot{V}O_{2\text{max}}\)) and best time to exhaustion were reached at Htc values of 0.58 and 0.57 for wtNESP, and 0.68 and 0.66 for tg6PHZ, respectively. Interestingly, the closest correlation between \(\dot{V}O_{2\text{max}}\) and whole body hemoglobin was only found in an Htc range from 0.4 to 0.55 in wtNESP. Maximal stroke volume was reached at Htc values of 0.58 for wtNESP and 0.68 for tgPHZ, whereas maximal heart rate was unaffected at varying Htc levels. Blood viscosity correlated with \(\dot{V}O_{2\text{max}}\). In conclusion, tg6PHZ adapted better to varying Htc values than wtNESP did. Furthermore, the close relationship between \(\dot{V}O_{2\text{max}}\) and whole blood hemoglobin is only valid in the physiologically occurring Htc range and when Htc levels were increased by NESP-injection.

5.2. Introduction
The rate of maximal oxygen (O₂) uptake (\(\dot{V}O_{2\text{max}}\)) is mainly limited by the O₂ delivery to the skeletal muscle (Di Prampero and Ferreti, 1990; Turner et al., 1993). Accordingly, when hemoglobin concentration [Hb] is increased by blood reinfusion or recombinant human erythropoietin (rhEpo) administration, \(\dot{V}O_{2\text{max}}\) increases (Ekblom and Berglund, 1991; Robertson et al. 1988). In line with this, \(\dot{V}O_{2\text{max}}\) is impaired, when [Hb] is acutely reduced isovolemically (Lundby et al., 2008b, Woodson et al., 1978).

Although the role of [Hb] as an O₂ transport carrier in circulating blood systems seems to be clear, its impact on the endurance performance over a wide hematocrit (Htc) range is still poorly understood. Increasing [Hb] results in elevated Htc levels that lead to an exponential rise in blood viscosity and are associated with higher peripheral vascular resistance in the tissue. Acutely increasing Htc levels are inversely related to the cardiac output (Crowell and Smith, 1967; Stone et al., 1968; Richardson and Guyton, 1959), which may theo-
oretically reduce $\dot{V}O_{2\text{max}}$ (Connes et al., 2006). Surprisingly, cardiac output remains unchanged when Htc levels are chronically elevated (Vogel, 2003; Wagner et al., 2001). This was observed in our transgenic mouse line termed tg6 that due to constitutively overexpression of human erythropoietin (Epo) cDNA reached Htc values of 0.8 to 0.9, nevertheless had a reduced exercise performance (Heinicke et al., 2006; Wagner et al., 2001). Enhanced erythropoiesis was also found in endurance athletes that due to a mutation in Epo receptor reached Htc levels of up to 0.68 (Juvonen et al., 1991). This endurance athlete won several Olympic gold medals and showed no signs of obvious organ failures. These findings indicate that some adaptive mechanisms to chronic excessive erythrocytosis exist.

However, some authors suggest that there is an optimal Htc value for maximal blood $O_2$ transport capacity to the tissue due to counteracting the effect of increased blood $O_2$ binding activity and viscosity (Crowell and Smith, 1968; Gaehhtgens et al., 1979; Guyton and Richardson; 1961; Villafuerte et al., 2003).

In higher vertebrates, Gaehhtgens and co-workers (1979) investigated the effect of varying Htc levels in isolated canine gastrocnemius muscle during isotonic rhythmic exercise. A plateau of maximal $\dot{V}O_2$ and contractile power in this ex-vivo set-up was found at Htc levels of between 0.4 and 0.7 with a slight tendency between 0.5 and 0.7. Below 0.4 and above 0.7, both parameters decreased. The problem of this investigation is that optimal Htc values may vary under different circumstances due to non-newtonian behavior of the blood. Factors affecting this variation include the species, the organs involved, and whether the organism is resting or exercising (Connes et al., 2004, Gaehhtgens et al., 1979; Kusunoki et al., 1981; Lee et al., 1994; Tu et al., 1997; Villafuerte et al., 2003). Thus, these results do not necessarily reflect the situation in exercising mammals and humans.

The present study tested the hypothesis if there is an optimal Htc value that allows maximal systemic endurance performance. These levels might not be identical in subjects having an acutely or chronically increased Htc. Thus, we hypothesize that subjects suffering from excessive erythrocytosis can adapt better to varying Htc levels than subjects having acutely increased Htc. For this purpose, wild typ (wt) mice were injected or not with the novel erythropoiesis stimulating protein (NESP). On the other hand, tg6 mice were treated or not with the hemolysis-inducing compound phenylhydrazine (PHZ). At the end of a period of 4 and 3 weeks, respectively, metabolic and cardiovascular measurements were performed at rest and during endurance performance, while whole blood analysis including rheology was carried out at rest.
5.3. Materials and Methods

Mouse models

The tg6 mice line was generated as described previously (Rutschizka et al., 2000). Compared to wt control, the tg6 mouse line had a 10 to 12-fold increase in plasma Epo-levels, resulting in Htc levels of up to 0.9 (Bogdanova et al. 2007; Rutschizka et al., 2000; Vogel et al., 2003). About half of the offspring was hemizygous for the transgene and was used for the hemolysis-inducing experiments, while the other half was used as wt for the hemocentration experiments. Mice were 12 weeks old during the first exercise test (Tab. 5.1). In total, 41 wt- and 40 tg6-mice were investigated. No weight loss occurred during the study period. Mice were kept in standard rodent cages (T3) with food and water supplied ad libitum in 12:12-hour light-dark cycle. The experimental protocols were approved by the Kantonales Veterinäramt Zürich and were performed in accordance with the Swiss animal protection laws and institutional guidelines.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>1</th>
<th>2</th>
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<th>6</th>
<th>7</th>
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<th>9</th>
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<th>12</th>
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<tbody>
<tr>
<td>wt/wtNESP</td>
<td>I</td>
<td>R</td>
<td></td>
<td></td>
<td>C</td>
<td>N</td>
<td></td>
<td>C</td>
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<tr>
<td>tg6/tg6PHZ</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td></td>
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Fig. 5.1. Timeline of animal age for the experimental set-up illustrated. S: Splenectomy; I: Implantation of telemetric blood pressure transmitter; R: Postoperative recovery; N: NESP injection and blood sampling; C: NESP injection; P: Phenylhydrazine injection and blood sampling; E: Phenylhydrazine injection; A: Treadmill adaptation; V: Incremental exercise test; T: Constant workload test; D: Terminal measurements.

Experimental design

Fig. 5.1 shows the experimental design. At an age of 3 weeks, only tg6 mice were splenectomised to keep Htc levels low, since extramedullary erythropoiesis mainly occurs in the spleen (Vogel et al., 2003). One week later, telemetric blood pressure transmitters were implanted in 20 wt and 19 tg6 mice, which were 4 weeks old. In the remaining animals, dummy transmitters were implanted (wt: n = 21; tg6: n = 21). Adjustments of the Htc lev-
els were started in 8 and 9 week old animals respectively. At an age of 12 weeks, the main experiments were conducted including incremental as well as constant workload exercise tests (see below) followed by measurements of the arterial O₂ saturation (SaO₂), [Hb], Htc, blood viscosity, plasma and blood volume. To exclude the impact of circadian rhythm all measurements were performed at the same time of day.

**Htc adjustments**

**wt + wtNESP**

NESP (Aranesp, Darbepoietin Alpha, Amgen Europe B.V., Breda, Netherlands) of 3.125 μg/kg to 12.5 μg/kg (wtNESP) or saline 0.9% was subcutaneously (s.c.) injected twice a week to increase and maintain the Htc levels. To this end, the animals were anaesthetized with 7-8% sevoflurane (Secorane™, Abbot, Cham, Switzerland) in pure O₂ to avoid un-controlled moving of the blood pressure catheter (Arras et al., 2001). After the injections blood sample (10 μl) was taken from the tail vein.

**tg6 + tg6PHZ**

Htc was adjusted to a range between 0.3 and 0.9 by s.c. administration of freshly prepared PHZ that causes chemical hemolysis (Lim et al., 1998; Vannucchi et al., 2001). To this end, PHZ hydrochloride (Sigma, P6926, Switzerland) was prepared as previously described (Lim et al., 1998). Animals received two PHZ injections (0.125 to 1.2 mg/10g body weight; tgPHZ) or saline 0.9% (tg6) spaced 2 days after the first injection to decrease the Htc. To maintain Htc levels PHZ was injected at a concentration between 0.065 and 0.5 mg/10g body weight every third day. Mice were anaesthetized and blood samples were taken after as described above.

**Surgical procedures: Splenectomy (tg6 mice only) and implantation of telemetric transmitter.**

Inhalation anesthesia was induced as described above. Anesthesia was maintained with 3.5-4% sevoflurane (Arras et al., 2001). Preliminary experiments showed that decreased Htc levels in our tg6 mice recovered within days after the PHZ injection. Compared to wt, tg6 mice showed that massive extramedullary erythropoiesis occurred in the spleen (Gassmann et al. 2008; Vogel et al, 2003). To maintain constant Htc levels, the spleens of 3 week old tg6 males were removed by left side-abdominal laparatomy as described previously (Vogel et al, 2003). One week later, blood pressure sensors were implanted in tg6 and wt mice of the same age. After shaving and disinfecting the neck, the left common carotid artery was isolated. The transmitter’s catheter was inserted into the artery and
pushed forward until the tip was just inside the thoracic aorta. The transmitter body was fixed under the skin. Splenectomy and blood pressure transmitter implantation were carried out under aseptic conditions. Mice were allowed to recover for two weeks. Measurements were performed using a TA11PA-C10 transmitter (DataSciences International, St. Paul, MN, USA). Data were generated by the Dataquest A.R.T 3.0 software (DataSciences International, St. Paul, MN, USA).

**Measurements**

**Exercise tests**

The exercise tests were performed on an Instrument Simplex II metabolic rodent treadmill (Columbus Instruments, Columbus, OH, USA) connected to an Oxymax gas analyzer (Columbus Instruments, Columbus, OH, USA). This system enables the measurement of $\text{VO}_2$ and carbon dioxide production ($\text{VCO}_2$), thus indirect calorimetry. Respiratory exchange ratio (RER) was calculated as $\text{VCO}_2/\text{VO}_2$. Before performing each exercise test, the gas analyzer was calibrated with a high precision gas mixture. Mice were gently encouraged to run on the belt until exhaustion with the use of a mild electric shock from the shock grid at the end of the treadmill (0.2 mA, pulse 200 ms, 1 Hz).

To determine $\text{VO}_{2\text{max}}$, systolic blood pressure, mean arterial blood pressure and heart rate were telemetrically monitored at rest and during exercise. Cardiac output depends on the stroke volume and heart rate. Unfortunately, there is no device available to measure the cardiac output telemetrically. The advantage of an implantable transmitter is the ability to monitor cardiovascular parameters in conscious freely moving animals directly. But stroke volume has to be assessed indirectly using $\text{O}_2$ pulse, which correlates closely with the stroke volume during effort, at least in healthy humans (Bhambhani, 1995; Crisafulli et al., 2007). $\text{O}_2$ pulse was calculated from division of $\text{VO}_{2\text{max}}$ by heart rate. As an indicator of myocardial $\text{VO}_2$ rate pressure product was calculated from the multiplication of heart rate and systolic blood pressure.

10 μl blood samples were taken three hours before initiation of the exercise test from the tail vein for the Htc determination. Mice were placed on individual treadmill lines. After acclimatization for at least one hour, basal heart rate, blood pressure, $\text{VO}_2$ and $\text{VCO}_2$ were monitored over the last five minutes of this period. Mice began running at 2.5 m/min and 0° inclination for 10 min. The intensity was then increased by 2.5 m/min and 2.5° every 3 min thereafter until exhaustion. Exhaustion was defined as the inability to continue regular treadmill running despite the repeated stimulus to the mice. $\text{VO}_{2\text{max}}$ was achieved when
\( \dot{V}O_2 \) did not increase in spite of an increase in work load. For reported values at maximal exercise, 1-min averages of blood pressure and heart rate were taken during last minute of exercise, while for \( \dot{V}O_{2\text{max}} \) the highest 1-min interval was considered. From the maximal value rate, pressure product and O\(_2\) pulse were calculated. After 24 hours at rest, all mice performed a constant workload exercise test to exhaustion. The workload was set to 80% of the maximal attained workload of the incremental exercise set. Before performing the time to exhaustion test, blood samples were taken at rest and the animal warmed up for 10 min at 20% followed by an additional 10 min at 40% of the maximal attained power output of the \( \dot{V}O_{2\text{max}} \)-test.

**Terminal measurements**

The day after performing the constant workload test, mice were anesthetized with a s.c. injection of a mixture of 100 mg/kg ketamine (Ketasol-100\(^\text{TM}\), Dr. Graub, Bern, Switzerland), 20 mg/kg xylazine (Rompun\(^\text{TM}\), Bayer, Leverkusen, Germany) and 3 mg/kg acepromazine (Sedalin\(^\text{TM}\), Chassot, Belp, Bern, Switzerland). Catheters were introduced into the left femoral artery and vein. Arterial blood was collected in a heparinised capillary 35 min after the injection of the anesthesia and the arterial acid-base status and SaO\(_2\) was immediately measured. Plasma volume and whole blood volume were measured by the injection of Evans blue directly after blood sampling for the arterial acid-base status and SaO\(_2\) determination. Twenty minutes later, animals were bled to death to measure blood viscosity, blood volume, plasma volume, Htc, whole body haemoglobin and [Hb].

**Blood analysis**

Htc was measured in duplicate of heparinized blood using micro centrifuge (Autokrit II, Pharmap, Geneva, Switzerland). [Hb] were determined with the automatic blood analyzer Abbott Cell Dyn 3500 (Abbott Diagnostic Division CA, USA). Whole body hemoglobin was calculated from the [Hb] and blood volume. SaO\(_2\) was evaluated by a gas analyzer (AHVL Compact 3, AHL List, Graz, Austria).

Quantification of whole blood volume has been previously described (Vogel et al., 2003). Briefly, 10 \( \mu \)l Evans blue solution (1% in saline) was injected into a femoral vein catheter. Twenty minutes after the injection, 10 \( \mu \)l samples of blood were drawn into heparinised capillaries. Absorbance of the dye in the plasma volume was read at 620 nm with a Nano-Drop spectrometer (NanoDrop products, Wilmington, USA). Evans blue concentrations were derived from a calibration curve and used to calculate plasma volume. Blood volume was calculated from plasma volume and Htc.
Blood viscosity was measured in heparinised blood samples with a rotation viscoimeter DV-II+PRO (BROOKFIELD, Brookfield Engineering Laboratories, Middleboro, MA, USA) using Rheocalc software (BROOKFIELD, Brookfield Engineering Laboratories, Middleboro, MA, USA) as previously described (Vogel et al., 2003). But, due to the non-newton fluid characteristics of blood, only blood temperature at 37ºC and shear rates of 450 s⁻¹ were compared.

Statistics

All data were analyzed using StatView software (Version 4.57, Abacus Concepts, Berkeley, California, USA). The relationship between the two parameters was analyzed with linear or polynomial regression. Significances were performed by a one-way analysis of variance (ANOVA). Results are expressed as mean ± standard deviation (SD). Statistical difference was set at P < 0.05.

5.4. Results

Male wt and tg6 mice were about 8 and 9 weeks old, respectively, at the beginning of the corresponding injections and showed differences in Htc levels (Tab. 5.1). While wt males had an Htc of 0.46 ± 0.03, the Epo overexpressing transgenic tg6 males developed excessive erythrocytosis, and showed Htc values of 0.78 ± 0.06. No differences in resting mean arterial blood pressure, heart rate, \( \dot{V}O_2 \) or RER were observed between wt and tg6 at the beginning of the incremental exercise test.

<table>
<thead>
<tr>
<th>Type</th>
<th>Weight [g]</th>
<th>Age [Days]</th>
<th>Htc [ml kg⁻¹min⁻¹]</th>
<th>( \dot{V}O_2 ) [ml kg⁻¹min⁻¹]</th>
<th>RER</th>
<th>Mean arterial blood pressure [mmHg]</th>
<th>Heart rate [beats·min⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt/wtNESP</td>
<td>25.0 ± 1.5</td>
<td>84.1 ± 5.2</td>
<td>0.46 ± 0.03</td>
<td>48.1 ± 2.7</td>
<td>0.81 ± 0.01</td>
<td>108.3 ± 6.7</td>
<td>513.8 ± 38.8</td>
</tr>
<tr>
<td>tg6/tg6PHZ</td>
<td>24.9 ± 1.6</td>
<td>86.9 ± 4.0</td>
<td>0.78 ± 0.06</td>
<td>50.1 ± 3.5</td>
<td>0.82 ± 0.01</td>
<td>103.4 ± 14.7</td>
<td>525.6 ± 55.0</td>
</tr>
</tbody>
</table>

Tab 5.1. Weight, age, and cardiac and metabolic parameters at baseline before performing an incremental exercise test. Note that the body weight was measured prior to hematocrit (Htc) manipulation. Values represent means ± SD. \( \dot{V}O_2 \): \( O_2 \) uptake; RER: Respiratory exchange ratio.
The impact of Htc manipulation

The results of wt/wtNESP and tg6/tg6PHZ after about 4 and 3 weeks of treatment, respectively, are depicted in Fig. 5.2. It shows [Hb], SaO₂, plasma and blood volume in relation to the Htc value for each individual mouse used in this study. Note that the blood samples analyzed were taken from anaesthetized animals prior to euthanizing them.

Fig. 5.2.1. Relationship between hematocrit (Htc) and (a) hemoglobin concentration ([Hb]) as well as (b) arterial oxygen saturation (SaO₂) during terminal determination in wtNESP and tg6PHZ mice. Values represent individual values. Regression plot of wt/wtNESP: ⋯⋯ and tg6/tg6PHZ: ——.
As shown in Fig. 5.2.1a, there was a linear increase in [Hb] as Htc values increased in all wt and tg6 mice used. While SaO2 (Fig. 5.2.1b) and plasma volume (Fig. 5.2.2a) did not significantly change in either treated or untreated wt or tg6 mice, elevated Htc levels were paralleled by dramatically increased blood volumes (Fig. 5.2.2b). As reflected by a calculated (degree two) polynomial equation, alterations at lower Htc values had a lower impact
on blood volume changes than was the case at higher Htc levels. Indeed, the increment of blood volume at Htc levels from 0.4 to 0.5 was about 19 ml/kg for wtNESP and 10 ml/kg for tgPHZ, while the increment between 0.6 and 0.7 was about 37 and 50 ml/kg, respectively.

**Optimal Htc for maximal endurance performance**

Endurance performance consists of the product of the subject’s $\dot{V}O_{2\text{max}}$ and exercise duration at a certain percentage of $\dot{V}O_{2\text{max}}$ that the subject can undertake until exhaustion (Bassett and Howley, 2000). Thus, to investigate the impact of varying Htc levels on endurance performance, individual data of $\dot{V}O_{2\text{max}}$ (Fig. 5.3.1) and time to exhaustion (Fig. 5.3.2) were plotted against Htc.

\[ y = -252.78x^2 + 292.75x + 59.812 \]
\[ R^2 = 0.7319; P < 0.0001 \]

\[ y = -337.58x^2 + 461.8x - 14.515 \]
\[ R^2 = 0.7771; P < 0.0001 \]

Fig. 5.3.1. Relationship between hematocrit (Htc) and maximal oxygen uptake ($\dot{V}O_{2\text{max}}$) in wtNESP and tg6PHZ mice. Values represent individual values. Regression plot of wt/wtNESP: ······· and tg6/tg6PHZ: —. 
Fig. 5.3.2. Relationship between hematocrit (Htc) and time to exhaustion in wtNESP and tg6PHZ mice. Values represent individual values. Regression plot of wt/wtNESP: ⋯⋯⋯⋯⋯⋯ and tg6/tg6PHZ: ⋅⋅⋯⋯⋯⋯.

\[ y = -575.87x^2 + 764.75x - 170.32 \]
\[ R^2 = 0.7719; P < 0.0001 \]

\[ y = -1225.5x^2 + 1389x - 298.72 \]
\[ R^2 = 0.7691; P < 0.0001 \]

\[ V_\text{O}_{2\text{max}} \] and time to exhaustion in both mouse lines showed a well shaped function and were more affected by Htc alterations at lower and higher Htc levels. Furthermore, time to exhaustion was more sensitive to Htc alterations than \( V_\text{O}_{2\text{max}} \). Mathematical calculations showed that best \( V_\text{O}_{2\text{max}} \) and time to exhaustion were reached at Htc values of 0.58 and 0.57 for wtNESP, respectively, and 0.68 and 0.66 for tg6PHZ-- mice, respectively.

**Effect of varying blood volume, whole body hemoglobin and viscosity on endurance performance**

Besides Htc, blood volume and whole body hemoglobin play an important role in cardiovascular performance (Åstrand, 1952, 1977; Ekblom and Hermansen, 1968; Kanstrup and Ekblom, 1984). To determine the impact of the blood volume and whole body hemoglobin which is required to reach maximal endurance performance, both parameters were corre-
lated with $\dot{V}O_{2\text{max}}$. As shown in Fig. 5.4a, b, $\dot{V}O_{2\text{max}}$ of wtNESP and tg6PHZ expressed as a function of blood volume or whole body hemoglobin behaved as a polynomial second degree equation. The graphs illustrate that about twice as much blood volume or whole body hemoglobin was necessary to reach maximal $\dot{V}O_{2\text{max}}$ in tgPHZ compared to the levels required in wtNESP. When comparing all blood parameters to $\dot{V}O_{2\text{max}}$, the best correlation was found with various Htc levels. This was not the case, however, with an Htc range of 0.4 to 0.55, in which whole haemoglobin correlated most closely with $\dot{V}O_{2\text{max}}$ in wtNESP mice ($R^2 = 0.513; P < 0.001$).

![Graph](image)

**Fig. 5.4.** Relationship between maximal oxygen uptake ($\dot{V}O_{2\text{max}}$) and (a) whole blood volume, and (b) whole body hemoglobin in wtNESP and tg6PHZ mice, respectively. Values represent individual values. Regression plot of wt/wtNESP: ⋯⋯⋯⋯⋯⋯ and tg6/tg6PHZ: ⋯⋯⋯⋯⋯⋯.
Polyzythemic condition impairs the blood flow due to Htc-dependent increasing blood viscosity and thus, less $O_2$ is transported to the tissue (Crowell et al., 1959) which may result in reduced exercise performance. We observed a correlation between blood viscosity and $\dot{VO}_{2\text{max}}$ (Fig. 5.5). Compared with wtNESP, Epo-overexpressing tg6 mice reached maximal $\dot{VO}_{2\text{max}}$ at a higher blood viscosity. Furthermore, blood viscosity versus Htc of wt and tg6 mice was in line with previously published data (Vogel et al., 2003).

![Graph](Viscosity.png)

Fig. 5.5. Relationship between maximal oxygen uptake ($\dot{VO}_{2\text{max}}$) and blood viscosity in wtNESP and tg6PHZ mice. Values represent individual values. Regression plot of wt/wtNESP: \ldots and tg6/tg6PHZ: \ldots.

**Increasing mean arterial blood pressure, constant heart rate and altered stroke volume, with rising Htc levels at $\dot{VO}_{2\text{max}}$.**

As the heart is the main generator of systemic blood circulation, mean arterial blood pressure, heart rate and stroke volume were investigated. Mean arterial blood pressure rose with increasing Htc levels in wtNESP and tg6PHZ (Fig. 5.6a). Overall, wtNESP reached higher mean arterial blood pressure values compared to those in tg6PHZ. Heart rate did not alter with increasing Htc levels and also did not alter from group to group (Fig. 5.6b).

Previous studies found a correlation between the stroke volume and $O_2$ pulse during exercise in healthy humans (Bhambhani, 1995; Crisafulli et al., 2007). Fig. 5.6c shows $O_2$ pulse as a function of Htc at $\dot{VO}_{2\text{max}}$. $O_2$ pulse of wtNESP and tg6PHZ rises slightly with increasing Htc levels and reaches a calculated maximum at Htc values of 0.58 for wt and
Fig. 5.6. Relationship between hematocrit (Htc) and (a) mean arterial blood pressure, (b) heart rate and (c) oxygen (O₂) pulse at maximal oxygen uptake (VO₂max) in wtNESP and tg6PHZ mice. Values represent individual values. Regression plot of wt/wtNESP: ⋮⋮⋮ and tg6/tg6PHZ: ---.
0.68 for tg6, and decreases with higher values. Fitted curves display polynomial second degree characteristics. In comparison to wtNESP, tg6PHZ showed a greater impact of varying Htc levels on O2 pulse.

**Evidence of increasing myocardial \( \dot{V}O_2 \) with rising Htc levels at \( \dot{V}O_{2\text{max}} \)**

To study the impact of the myocardial \( \dot{V}O_2 \) on \( \dot{V}O_{2\text{max}} \), rate pressure product was correlated with Htc levels. Myocardial \( \dot{V}O_2 \) increased with increasing Htc values in both groups (Fig. 5.7). Both graphs showed a similar slope, but wtNESP mice had higher rate pressure product values at corresponding Htc levels than tg6PHZ did, indicating that the heart of wtNESP had a higher myocardial O2 supply requirement.

![Graph showing rate pressure product vs. Htc for wtNESP and tg6PHZ mice](image)

**Fig. 5.7.** Relationship between hematocrit (Htc) and rate pressure product in wtNESP and tg6PHZ mice. Values represent individual values. Regression plot of wt/wtNESP: \( \cdots \) and tg6/tg6PHZ: \( \cdots \).

### 5.5. Discussion

This is the first report demonstrating optimal Htc values for maximal systemic endurance performance in mice. Interestingly, acutely increasing Htc levels resulted in lower optimal Htc values than those measured when Htc values were chronically increased. In addition, the data showed that (1) the optimal Htc levels for maximal stroke volume and maximal systemic exercise performance were similar but mouse line dependent; (2) mean arterial blood pressure and rate pressure product increased with increasing Htc levels, whereas
heart rate remained unaffected; and (3) blood volume was dramatically elevated at higher Htc levels.

Some terrestrial vertebrate species, such as dogs and horses respond to strenuous exercise by splenic contraction due to their very high O₂ demand for behaviour and survival (Dane et al., 2006; Fedde and Wood, 1993). Splenic contraction releases extra erythrocytes into the circulation to increase the blood O₂-carrying capacity. As a consequence, Htc is elevated from approximately 0.4 at rest to approximately 0.6 at exercise (Wagner et al., 1996; Wu et al., 1996), resulting in improved exercise performance. These parameters return quickly to resting values when the animals stop exercising to avoid constant overload of the cardiovascular system (Wagner et al., 1995; Wu et al., 1996). Considering our findings, it is tempting to speculate that these species elevate temporary their Htc levels close to the optimal value Htc for maximal systemic endurance performance to enhance their exercise performance.

In the present study, \(\dot{V}O_2\max\) and time to exhaustion initially rose with increasing Htc and reached a maximum value. After this point, both parameters began to decrease. Maximal \(\dot{V}O_2\max\) and time to exhaustion values were found at Htc levels of 0.58 and 0.57 for wtNESP, and 0.68 and 0.66 for tg6PHZ, respectively. These findings are in agreement with the optimal Htc hypothesis (Crowell et al., 1959; Gaetgengs et al., 1979; Guyton and Richardson; 1961; Villafuerte et al., 2003). Buick et al. speculated (1980) that optimal systemic Htc is in excess of the commonly observed 0.45. These calculations were based on the condition existing in the circulatory system at rest, since exercise induces changes in vessel diameters, blood flow, internal temperature and blood distribution. However, there was no experimental proof. Gaethgens and co-workers (1979) showed in isolated dog muscle that during rhythmic isotonic exercise, various Htc levels lead to a plateau of maximal \(\dot{V}O_2\) and contractile power, but with a slight tendency toward maximal values in an Htc range between 0.5 and 0.7. Indeed, the result may not be transferable to systemic exercise, since each organ may have its own individual optimal Htc (Gaethgens et al., 1979; Lee et al., 1994; Tu et al., 1997). Therefore, the systemic Htc value has to be interpreted as an average value of all organ specific optimal Htc values to provide the whole organism with adequate O₂. During exercise, O₂ supply is disturbed because the impact of the skeletal muscle is increasing and thus, a shift from the optimal physiological to the optimal Htc value for best endurance performance may be observed. Endurance athletes make use of this knowledge by the combination of living at moderate altitude and training at low altitude (termed “live high-train low”) to improve their performance at sea level and moderate
altitude due to increased [Hb] (Levine and Stray-Gundersen, 1997; Schuler et al., 2007). Particularly unscrupulous athletes artificially increase their circulating red blood cell number by misusing Epo, resulting in improved endurance performance by doping abuse (Eichner, 2007; Warburton et al., 2000). This is not only unfair and criminal, but also life-threatening.

At first glance, the observed relationship between $\dot{V}O_{2\text{max}}$ and Htc in our study is in contradiction to the one observed in human studies. Several investigators have shown that there is a strong correlation between $\dot{V}O_{2\text{max}}$ and whole body hemoglobin and blood volume (Åstrand, 1952, 1977; Ekblom and Hermansen, 1968; Kanstrup and Ekblom, 1984; Heinicke et al., 2001; Warburton et al., 2000) but not Htc. It has to be mentioned that most human studies are carried out with Htc levels of up to 0.5. Interestingly, if only the animals in the present study in an Htc range of 0.4 and 0.55 were considered, we also found the closest relationship between $\dot{V}O_{2\text{max}}$ and whole body haemoglobin in wtNESP animals. Thus, the potential ergogenic effect of altered total body hemoglobin to improve endurance performance seems only to be valid in an Htc range of 0.4 to 0.55. At higher Htc levels, other factors may be more important.

The changes in the blood volume at higher Htc values are caused by the dramatically increased number of erythrocytes, since plasma volume remained unchanged. Elevated blood volume enhances end-diastolic volume (preload) which results in increased stroke volume, leading to enhancement of $\dot{V}O_{2\text{max}}$ as long as heart rate is not altered. This finding is in line with a previous study (Kanstrup and Ekblom, 1982). But, after reaching maximum at 0.57 for wtNESP and 0.68 for tg6PHZ, the blood viscosity may play a major role. Winslow and Monge (1958) showed that blood viscosity increases exponentially with Htc levels of above 0.55. High viscosity increases arterial blood pressure and diminishes venous return due to the increased peripheral resistance (Richardson and Guyton 1959; Villafuerte et al., 2004). Thus, the changes in stroke volume are not only a function of whole blood volume, but also simultaneously of blood viscosity and peripheral resistance.

The cardiac output is calculated by the multiplication of stroke volume and heart rate. Since the maximal heart rate in all our mice remained unaffected by the varying Htc level, cardiac output alterations were induced by the stroke volume. Other studies have already reported that stroke volume is a main regulator of the cardiac output in healthy human beings during maximal exercise (Ekblom and Hermansen, 1968; Grimby et al., 1966; Mitchell et al., 1958; Saltin and Stenberg, 1964).
When Htc is acutely raised, cardiac output begins to fall (Crowell and Smith, 1967; Richardson and Guyton, 1959; Robertson et al., 1988; Stone et al., 1968). Otherwise, cardiac output increases, when Htc acutely decreases (Kanstrup and Ekblom, 1982; Lundby et al., 2008b; Richardson and Guyton, 1959). Interestingly, we did not observe this phenomenon in our mice. The discrepancy in our study may be explained by the different species used and experiments performed. Most likely, the wtNESP and tgPHZ animals could compensate for the increasing blood viscosity since Htc levels stayed constant at a certain level for two weeks. This may have provided sufficient time for the cardiovascular system of both mouse lines to adapt. Since maximal stroke volume of tg6PHZ was reached at higher Htc levels compared to those for wtNESP, we conclude that tg6PHZ can adapt better to varying Htc levels. Physiological adaptations to excessive erythrocytosis are also observed in humans. In the case of sports medicine there was the case of a Finnish cross-country skier with autosomal dominant erythrocytosis resulting in Htc levels of up to 0.68. He won several Olympic gold medals (Juvonen et al., 1991). Based on results of our study, we can conclude that Htc of this athlete may be equal to the optimal Htc for maximal endurance performance of tg6PHZ.

Adaptational mechanisms to excessive erythrocytosis include peripheral vasodilatation and regulation of blood viscosity (Bogdanova et al., 2007; Ruschitzka et al., 2000; Vogel et al., 2003). Vasodilatation is induced by the enhancement of endothelial nitric oxide synthase activity, which results in peripheral vasodilatation despite concomitant increased endothelial-1 levels (Quaschning et al., 2003), whereas blood viscosity is regulated by the flexibility of the erythrocytes (Vogel et al., 2003). Of note, the regulation of blood viscosity appears to be at least as important as vasodilatation (Bogdanova et al., 2007). The simplest way to regulate blood viscosity is to increase the level of juvenile erythrocytes in the circulatory blood system, since reticulocytes have a higher deformability than erythrocytes (Shiga et al., 1990). Deformability is of crucial importance for microcirculation, in which cells have to deform to pass through capillaries but also for the enhancement of O₂-release (Stuart and Nash, 1990). In response to PHZ treatment (Lim et al., 1998), the number of erythrocytes decreases, and Epo plasma and the population of reticulocytes dramatically increase (Cherukuri et al., 2004; Criswell et al., 2000; Rothman et al., 1970). Thus, tg6PHZ may have a higher population of juvenile erythrocytes than wtNESP have. This would induce a shift to a higher optimal Htc value.

The close correlation between \( \dot{V}O_{2\text{max}} \) and blood viscosity in the present study confirms the classical explanation of the existence of critical Htc to reduce cardiac output due to the
increased viscosity and hence limit endurance performance. These findings support the
notion that $\dot{V}O_{2\text{max}}$ is primarily limited by $O_2$ delivery to the exercising muscle (Calbet et al., 2005; Ekblom et al., 1976; Krip et al., 1997).

Maximal heart rate at exhaustion was unaffected by the Htc level. This implies that sympathetic nervous activity remained unchanged at maximal exercise and thus, other factors such as heart contractility were not influenced by the NESP and PHZ treatment. Moreover, the fact that rate pressure product and blood pressures were increasing with incremental elevation of Htc levels shows that the heart did not reach its maximal work capacity at optimal Htc. This is against the “Central governor theory” as the limiting factor of $\dot{V}O_{2\text{max}}$ (Noakes, 1997, 1998). The theory proposes that the central nervous system regulates the extent of skeletal muscle recruitment avoiding myocardial ischemia and therefore, maximal cardiac output is never reached. As consequence, $\dot{V}O_{2\text{max}}$ is limited by the maximal rate of work which the heart allowed. In the present study, maximal cardiac output was decreased after reaching a maximum, whereas rate pressure product was constitutively increased with increasing Htc levels. This suggests that maximal cardiac output is established by a regulatory mechanism and not by the heart that is working at its maximum. Our result is in line with a recent published study that heart working capacity does not limit exercise performance (Brink-Elfegoun et al., 2007). It was demonstrated that two maximal combined arm and leg exercise tests performed at identical $\dot{V}O_{2\text{max}}$ and cardiac output result in different rate pressure products.

Apart from the cardiovascular limitation, we found no organ degeneration of the wtNESP and tg6PHZ mice, despite the fact that previous studies showed that excessive erythrocytosis leads to multiple organ failures (Heinicke et al., 2006). In contrast to that study, our study used younger mice.

It has been reported that Epo promotes angiogenesis by enhancing vascular endothelial growth factor (VEGF) in the issue (Alvarez Arroyo et al., 1998; Bellomo et al., 2006). Its importance in the skeletal muscle is the capillary growth. Interestingly, no difference in skeletal muscle capillary density was found between wt and tg6 (Gassmann et al., 2008) and thus, it may have no impact on the endurance performance of wtNESP and tg6PHZ.

In summary, the results of the present study confirm the optimal Htc hypothesis during systemic exercise in mice. The reason for this is that blood viscosity increases with increasing Htc levels. Furthermore, the animals with chronic excessive erythrocytosis adapted better to different Htc levels than acutely NESP-injected animals did. At nor-
moxia, the heart can tolerate higher rate pressure product at higher Htc levels. Thus, the optimal Htc values for maximal endurance performance were independent of the working capacity of the heart. $\dot{V}O_{2\text{max}}$ is mainly limited by $O_2$ delivery.

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6. Study 3: Timing the arrival at 2340 m altitude for aerobic performance

This study has been published:


6.1. Abstract

This study tested the hypothesis that \( \dot{V}O_{2\max} \) and performance increase upon altitude acclimatization at moderate altitude. Eight elite cyclists were studied at sea level, and after 1 (Day 1), 7 (Day 7), 14 (Day 14) and 21 (Day 21) days of exposure to 2340 m. Capillary blood samples were taken on these days before performing two consecutive maximal exercise trials. Acclimatization increased hemoglobin concentration ([Hb]) and arterial oxygen content (\( CaO_2 \)). On Day 1, \( \dot{V}O_{2\max} \) and time to exhaustion (at 80% of sea level maximal power output) decreased by 12.8% (P < 0.05) and 25.8% (P < 0.05), respectively, compared to the corresponding sea level values. Subsequently, these parameters increased by 3.2% (P < 0.05) and 6.0% (P < 0.05) from Day 1 to Day 7, by 4.8% (P < 0.05) and 5.7% (P < 0.05) from Day 7 to Day 14, and by 0.7% (P > 0.05) and 1.4% (P > 0.05) from Day 14 to Day 21, respectively. These data suggest that endurance athletes competing at altitudes around 2340 m should expose themselves to this altitude at least 14 days prior to competition.

6.2. Introduction

Most sporting competitions are held near sea level, where the majority of athletes also reside. A few exceptions include the 1968 Olympic games held in Mexico City at around 2300 m altitude, the 1995 road cycling championships in Bogotá at 2640 m, and the 2000 winter Olympics at 1250-2003 m near Salt Lake City. Also, in 2006, the Olympic ski races of the Turin games were held near Sestriere at altitudes between 1509 and 2800 m. Thus, although the majority of competitions are generally held near sea level, some events take place at higher altitudes. The present investigation focuses on the importance of timing the arrival at altitude in order to optimize competitive performance.

In humans \( \dot{V}O_{2\max} \) is limited by approximately 70% by oxygen (O\(_2\)) delivery, and all other systems are responsible for the remaining 30% (Di Prampero & Ferretti, 1990). Moreover, experimental data show that \( \dot{V}O_{2\max} \) is limited by maximal O\(_2\) delivery, which in turn de-
pends on maximal cardiac output and maximal O₂ extraction (Calbet et al., 2004a, 2005). Accordingly, when arterial O₂ content (CaO₂) is increased by hyperoxia (Amann et al., 2006; Knight et al., 1993; Nielsen et al., 1998), erythropoietin (Epo) administration (Ekblom and Berglund, 1991) or autologous blood infusions (Thomson et al., 1982), VO₂max increases. Conversely, when CaO₂ is acutely reduced due to moderate hypoxia, VO₂max decreases (Lundby et al., 2004). Thus, there is good evidence that a close relationship between CaO₂ and VO₂max within a given individual exists. With acclimatization to altitudes at or above 4100 m, however, CaO₂ is resumed, or values even higher than sea level values are observed without a concomitant normalization in VO₂max (Calbet et al., 2003b; Lundby et al., 2004). More recently, it was shown that VO₂max, upon acute hypoxic exposure to 4100 m, does not increase despite increases in CaO₂ that were achieved by enhanced erythropoiesis upon application of the novel erythropoiesis stimulating protein (NESP; Lundby and Damsgaard, 2006a). On the other hand, exercise performance can be increased by erythrocyte infusion at 2255 m and 3566 m (Robertson et al., 1982, 1988). Thus, it is tempting to speculate that there is an altitude threshold over which increased hemoglobin concentration ([Hb]) has no beneficial effect on VO₂max. Below this threshold it is very likely that an increased [Hb] results in an improvement in VO₂max compared to what occurs at sea level. The fact that VO₂max does not fully recover after acclimatization at high altitudes may be a consequence of a reduced peak leg blood flow (Calbet et al., 2003b; Lundby et al., 2006b).

Most altitude sporting events are conducted at considerably lower altitudes, but unfortunately data on acclimatization, VO₂max and performance at these altitudes are sparse. Prior to the Olympic games in 1968, Faulkner and co-workers (1967) found no increase in swimming performance after 14 days exposure to 2300 m and also found similar results in runners (Faulkner et al., 1968). Accordingly, Adams and colleagues (1975) found only minimal increases in 2-mile run times (statistics not reported) after 20 days of living and training at 2300 m, and similar data for VO₂max have been presented after about 4-5 weeks at a similar altitude (Daniels & Oldridge, 1970; Pugh, 1967). On the other hand, Saltin (1967) reported that if altitude related sicknesses are avoided, VO₂max and performance are increased after 19 days of exposure to 2300 m (but are still lower than sea level values), and similar results have been presented after 21 days at 1822 m (Jensen et al., 1993). Differences in the outcomes of the above mentioned studies could be related to a deficit in iron-stores prior to altitude exposure (Stray-Gundersen et al., 1992). Another explanation for VO₂max and/or performance not to increase with acclimatization could also be related to
decrement in training intensities at altitude. This potentially offsets the effects of acclimatization on O$_2$ transport. Accordingly, Levine and Stray-Gundersen (1997) suggest that “live high-train low” is the optimal approach. Indeed, in a recent review it was suggested that altitude acclimatization lasting 10 days or longer can lead to improvements in endurance performance at altitude (Fulco et al., 2000). However, in order to test this hypothesis we took the opportunity to quantify $\dot{V}O_{2\text{max}}$ and performance in eight iron supplemented elite cyclists at sea level, and after 1, 7, 14 and 21 days of exposure to 2340 m. To maintain training intensity during the altitude exposure period, all training was performed below 1100m of altitude as suggested by the “live high-train low” approach.

6.3. Methods

Subjects

Eight elite bike racers participated in the study, and their anthropometric data are presented in Tab. 6.1. They were all sea level residents with no prior exposure to high altitude for the last 6 months. During the study the subjects did not participate in competitions, and their training regimes two weeks prior, and during, the experiments are reported in Tab. 6.2. Two weeks before altitude exposure, iron (100 mg/day, Ferro Duretta, Astra Zeneca) supplementation in all athletes was initiated and was continued during the whole study period. After being given both written and oral information on the experimental protocol and procedures, the subjects gave their informed, written consent to participate. The study conformed to the guidelines laid down in the Declaration of Helsinki.

Protocol

All subjects were studied on five occasions: In Malaga, Spain, at sea level, and after 1 day (Day 1) of exposure to 2340 m altitude at Centro de Alto Rendimiento in Sierra Nevada, Spain, and again at the same location after 7 (Day 7), 14 (Day 14), and 21 (Day 21) days of altitude exposure. Thus, the experiments were conducted within 23 days. The subjects spent 16-24 hours at altitude before being tested on Day 1. All training was performed below 1100 m altitude. On training days all subjects were transported to low altitude by car (approximately 30 min) and also transported back by car (approximately 45 min) after the training session. Therefore, on a daily basis, the subjects spent approximately 19 hours at 2340 m, and the remaining time below approximately 1100 m. At the altitude facilities the subjects had the choice of a large variety of food and beverages, and they were free to consume what they preferred.
Tab. 6.1. Individual anthropometric data of the subjects at sea level. Additionally body mass on Day 1, Day 7, Day 14, Day 21. Values are means ± sd.

<table>
<thead>
<tr>
<th>Subject (No.)</th>
<th>Age (yr)</th>
<th>Height (cm)</th>
<th>Body mass (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SL</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td>174</td>
<td>62.2</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>178</td>
<td>65.2</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>173</td>
<td>62.1</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>175</td>
<td>61.2</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
<td>172</td>
<td>55.1</td>
</tr>
<tr>
<td>6</td>
<td>26</td>
<td>174</td>
<td>61.2</td>
</tr>
<tr>
<td>7</td>
<td>24</td>
<td>172</td>
<td>60.8</td>
</tr>
<tr>
<td>8</td>
<td>23</td>
<td>169</td>
<td>62.1</td>
</tr>
</tbody>
</table>

Mean ± sd 23.6 ± 1.5 173.4 ± 2.6 61.2 ± 2.8 61.3 ± 2.8 61.3 ± 2.8 61.2 ± 2.6 61.3 ± 2.7

Tab. 6.2. Average training hours per week of all subjects expressed as % of maximal heart rate (%HR) one and two weeks prior to the experiments at sea level (Sea level 1 and Sea level 2), and during week one, two, and three at altitude (Altitude 1, 2, and 3, respectively). Values are means ± sd.

<table>
<thead>
<tr>
<th>Intensity (%HR)</th>
<th>Sea level 1 (hh:mm)</th>
<th>Sea level 2 (hh:mm)</th>
<th>Altitude 1 (hh:mm)</th>
<th>Altitude 2 (hh:mm)</th>
<th>Altitude 3 (hh:mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-40</td>
<td>04:15 ± 00:38</td>
<td>03:47 ± 00:42</td>
<td>04:02 ± 00:32</td>
<td>04:48 ± 00:32</td>
<td>03:32 ± 00:32</td>
</tr>
<tr>
<td>41-60</td>
<td>06:12 ± 00:25</td>
<td>06:29 ± 00:12</td>
<td>06:48 ± 00:23</td>
<td>06:12 ± 00:42</td>
<td>06:03 ± 00:21</td>
</tr>
<tr>
<td>61-80</td>
<td>07:28 ± 00:17</td>
<td>07:02 ± 00:45</td>
<td>07:09 ± 00:51</td>
<td>07:27 ± 00:34</td>
<td>07:25 ± 00:24</td>
</tr>
<tr>
<td>81-100</td>
<td>05:24 ± 00:51</td>
<td>05:58 ± 00:25</td>
<td>05:41 ± 00:16</td>
<td>05:12 ± 00:35</td>
<td>06:11 ± 00:26</td>
</tr>
</tbody>
</table>

Mean ± sd 23:20 ± 01:03 23:46 ± 01:35 23:40 ± 00:47 23:48 ± 01:42 23:11 ± 01:14

Each subject was investigated at the same time of the day on all occasions, but some were studied in the morning; others were monitored throughout the afternoon/evening. After blood sampling, all subjects performed a maximal cycle ergometer (Ergomedic 839E, Monark, Varberg, Sweden) test. After 15 minutes of warm-up at 200 W, the workload was increased by 30 W every minute until exhaustion. After at least 6 hours of rest the subjects performed a constant workload exercise test to exhaustion. The workload (339.7 ± 15.8 W)
was the same in all conditions and calculated from 80% of the maximal attained workload at sea level, and corresponded to 92.7 ± 1, 89.8 ± 1.3, 85.1 ± 0.8, and 84.3 ± 1.6% of maximal power output on Day 1, Day 7, Day 14, and Day 21, respectively. Prior to the time to exhaustion test, subjects warmed-up for 15 minutes at 200 W.

**Measurement**

Blood samples were taken before the initiation of the first exercise test, after 30 min of supine resting. Hemoglobin concentration ([Hb]) and hematocrit (Htc) were measured in duplicate using a HemoCue Hemoglobin Photometer (HemoCue AB, Ängelholm, Sweden) and Mikro 12-24 Centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). They were measured from venous blood obtained from an antecubital vein. Arterial oxygenation (SaO₂) was determined by a Nellcor pulse oxymeter (Nellcor, Hyward, CA, U.S.A.), and the CaO₂ was calculated as CaO₂ = [Hb] x 1.34 x SaO₂. Blood samples for Epo quantification were also obtained from the antecubital vein. These samples were centrifuged for 15 minutes at 3500 rpm and -4°C. The plasma was stored at -20°C for later analysis via ELISA (R&D Systems, Minneapolis, MN, USA).

Pulmonary VO₂ and CO₂ production (VCO₂) were continuously measured (Quark b², Cosmed Srl., Rome, Italy). Before each test, ambient conditions were measured, and then the gas analyzer and the flowmeter were calibrated with high precision gases. During sub-maximal and maximal exercise the VO₂ were recorded as averages of 15 second intervals. Gross mechanical efficiency (%) was determined from the ratio of power output (kJ·min⁻¹) to energy expended (kJ·min⁻¹), as calculated from VO₂ and the respiratory exchange ratio (RER).

**Statistics**

All data were analyzed using StatView software (Version 4.57, Abacus Concepts, Berkeley, CA, USA). Statistical comparisons were performed by one-way ANOVA for repeated measurements. The Fisher’s PLSD post hoc test was used to assess differences between values at sea level and those from the different days at altitude, and from the previous measuring day during acclimatization (e.g. Day 7 to Day 1, Day 14 to Day 7 and Day 21 to Day 14). Correlations between the parameters were computed using linear regression. Statistical differences in ANOVA, post hoc test and linear regression were considered significant when P < 0.05. Results are presented as means ± standard deviation (sd).
6.4. Results

Individual anthropometric data of the subjects are given in Tab 6.1. There were no statistically significant differences in body weights observed at sea level, and on Day 1, Day 7, Day 14, and Day 21.

Hematological parameters

Plasma Epo levels peaked on Day 1 at altitude, and remained slightly, but not statistically significantly, elevated during the acclimatization period (Fig. 6.1 a). [Hb] and Htc increased during the period from Day 1 until the end of the altitude exposure period by 15.1% and 13.4%, respectively (Fig. 6.1b, c). Compared to its value at sea level, the amount of CaO₂ decreased by 8.7% on Day 1, but increased by 15.6% after 21 days of altitude exposure (Fig. 6.1d).

Fig. 6.1. Individual and mean resting values for (a) erythropoietin (Epo), (b) hemoglobin concentration ([Hb]), (c) hematocrit (Htc), and (d) arterial O₂ content (CaO₂) at sea level (SL) and on Day 1 through 21 (Day 1, Day 7, Day 14, Day 21, respectively) of acclimatization. a P < 0.05 compared to SL; b P < 0.05 compared to, e.g. Day 7 to Day 1, Day 14 to Day 7 and Day 21 to Day 14.
**Exercise performance parameters**

Acute hypoxic exposure decreased maximal power output by 13.8% resulting in an accordingly lower \( \dot{V}O_{2\text{max}} \) of 12.8% (Fig. 6.2a, b). With acute hypoxic exposure, time to exhaustion declined by 25.8% (Fig. 6.2c).

![Graphs showing exercise performance parameters](image)

Fig. 6.2. Individual and mean values for (a) maximal power output, (b) maximal oxygen uptake (\( \dot{V}O_{2\text{max}} \)), and (c) time to exhaustion at sea level (SL) and on Day 1 through 21 (Day 1, Day 7, Day 14, Day 21) of acclimatization. \( ^{a}P < 0.05 \) compared to SL; \( ^{b}P < 0.05 \) compared to, e.g. Day 7 to Day 1, Day 14 to Day 7 and Day 21 to Day 14.

![Graphs showing exercise performance parameters](image)

Acclimatization produced a marked improvement in all exercise performance parameters. At the end of the 3 week altitude period, maximal power output, \( \dot{V}O_{2\text{max}} \) and time to exhaustion had increased by 10.0%, 8.9% and 13.6% when compared to the levels on Day 1. The highest increase was observed between Day 7 and Day 14, and the lowest between Day 14 and Day 21 (non-significant change). There was a correlation between \( \dot{V}O_{2\text{max}} \) and time to exhaustion (Fig. 6.3). Moreover, the changes in [Hb] were correlated with the changes in \( \dot{V}O_{2\text{max}} \) and time to exhaustion (Fig. 6.4a, b).
Fig. 6.3. Correlation between maximal oxygen uptake ($\dot{V}O_{2\text{max}}$) and time to exhaustion. Values are means. SL: sea level.

Fig. 6.4. Correlation between the percentage of changes in (a) hemoglobin concentration [Hb] and maximal oxygen uptake ($\dot{V}O_{2\text{max}}$), and (b) between [Hb] and time to exhaustion of, e.g., sea level (SL) to Day 1, Days 1-7, Days 7-14, Days 14-21. Values are means.
Gross mechanical efficiency (%) remained constant at all time points for a given workload, and was on average 25.3 ± 0.9 at sea level, and 25.2 ± 1.0 (Day 1), 25.3 ± 1.2 (Day 7), 24.8 ± 1 (Day 14), and 25.1 ± 0.7 (Day 21) at altitude.

**6.5. Discussion**

The main finding of the present study is that with acclimatization to 2340 m (i) \( \dot{V}O_{2\text{max}} \) and time to exhaustion improve over time and (ii) this improvement occurs within the first 14 days of altitude exposure. Our data provides convincing evidence that athletes who plan to compete in endurance events at this altitude should expose themselves to the altitude of competition for at least 14 days prior to the competition.

In the present study an improvement in all measured exercise performance parameters was found during acclimatization to moderate altitude. In all athletes, the main improvement in performance was observed within the first 14 days of altitude exposure. In the following week, however, five of eight athletes improved \( \dot{V}O_{2\text{max}} \) even further, whereas eight continued to improve time to exhaustion slightly. The observation that \( \dot{V}O_{2\text{max}} \) increases with prolonged altitude acclimatization, is in agreement with data obtained by Jensen and co (1993). That study reported that three weeks of acclimatization to 1822 m is accompanied by an increase in \( \dot{V}O_{2\text{max}} \) and 6-min work capacity during rowing. Our results and those of Jensen et al. (1993), however, are not in agreement with the results of other studies determining acclimatization and performance at 2300 m (Adams et al. 1975; Daniels & Oldridge, 1970; Faulkner et al., 1967, 1968; Pugh, 1967). The data from the latter studies can be divided into two groups: Either \( \dot{V}O_{2\text{max}} \) and/or performance remained constant during acclimatization, or were slightly increased during the stay at altitude. For instance, Faulkner and co-workers (1968) found no change in \( \dot{V}O_{2\text{max}} \) and time trial performance after three weeks of acclimatization to 2300 m altitude. The main difference between the present study and those performed in the past is that our athletes performed all training at altitudes lower than 1100 m. As suggested by the “live high-train low” concept, this approach minimizes the loss of training intensity which would normally be associated with traditional high altitude training regimes (Levine and Stray-Gundersen, 1997). Thus, whereas subjects in some of the previous studies may have experienced reduced training intensities during the altitude exposures, this was not the case in the present study. The study by Jensen et al. (1993) was conducted at an altitude where the decrement in \( \dot{V}O_{2\text{max}} \), and therefore also training intensities, was less than in the remaining studies. Therefore, it is tempting to speculate that these subjects were able to keep training intensities closer to
their normal sea level values, and therefore also minimized the loss of training stimulus, while still achieving the effects of hypoxia on, for example, hemoglobin mass. Another factor that may explain the differences in results is that we supplemented all subjects with 100 mg of daily iron in order to facilitate hemoglobin production. None of the previous studies states that iron was supplemented. Yet another explanation for the different results may be the presence of altitude associated diseases. From studies performed in preparation for the 1968 Olympic Games, Saltin (1968) reported that 19 days of acclimatization to approximately 2300 m led to increments in \( \dot{V}O_{2\text{max}} \) in the athletes who were not affected by illness, whereas the infected athletes did not improve performance. In the case of Mexico, the illness was most likely associated with infections obtained from polluted drinking water and not with acute mountain sickness.

The altitude associated increase (%) in [Hb] correlated well with the increment (%) in \( \dot{V}O_{2\text{max}} \) \( (R^2 = 0.948; P < 0.05) \) and time to exhaustion \( (R^2 = 0.971; P < 0.05) \), and thus, seems to be a reasonable candidate for the increase in performance (Fig. 6.4a, b). This is in agreement with Robertson et al. (1982, 1988), who showed that erythrocyte infusion results in an improvement in exercise performance at altitudes of 2255 m and 3566 m. In the present study the increase in [Hb] was somewhat greater than in previous studies conducted with athletes at similar altitudes. This could be related to the relatively low [Hb] observed at sea level compared to that observed in other studies (Kime et al. 2003; Veicsteinas et al., 1984). The difference may be explained by different initial conditions and different training regimes. The relatively low [Hb] could make the subjects in the present study more sensitive to iron supplementation. Unfortunately, we can not access the relative contribution of iron supplementation or acclimatization to the total improvements in [Hb]. It has to be mentioned, however, that only athletes with iron deficiency anemia may have a beneficial effect on performance from iron supplementation (Zoller and Vogel, 2004). Note that in this study the subjects were supplemented daily with iron (100 mg) starting two weeks prior to, and during, the study period in order to minimize the possible influence of iron deficiency on \( \dot{V}O_{2\text{max}} \), performance and [Hb].

Although Epo increased rapidly, it is unlikely that the early increase in CaO2 was due to increases in haemoglobin synthesis, since it is well known that plasma volume decreases almost immediately with exposure to high altitude (Gunga et al., 1994), and that it remains depressed for several weeks (Alexander et al., 1967; Pugh, 1964; Reynafarje et al., 1959). Thus, the increase in \( \dot{V}O_{2\text{max}} \) and performance could also reflect a time dependent recovery in blood volume. However, plasma volume expansion does not affect \( \dot{V}O_{2\text{max}} \) after 9 weeks
of acclimatization to high altitude (Calbet et al., 2004b). Unfortunately, we did not have the opportunity to measure blood volume in the present study.

In previous “sleep/live high-train low studies”, performed to investigate subsequent performance at sea level, an increase in muscle buffer capacity and ergometer cycling economy (Gore et al., 2001) and running economy (Saunders et al., 2004) have been reported. Others, however, have not observed changes in running economy (Levine and Stray-Gundersen, 1997, 2005). Thus, this issue remains a topic of great debate (Levine and Stray-Gundersen, 2005). In a recent study including over 100 subjects from different studies and altitudes ranging from 2500 to 5260 m, however, it was concluded that low to high altitudes do not influence exercise economy (Lundby et al., 2007). Whether the differences in results stem from methodological issues such as cycling vs. running and net vs. gross economy still remains to be clarified.

In the present study we did not quantify muscle buffer capacity, but found no differences in ergometer cycling economy. Thus, at least in the present investigation, performance increments were unrelated to changes in economy. Of note is that previous studies evaluated economy at sea level after termination of the altitude training regimes, whereas we completed the trials at altitude.

It should be noted that all time to exhaustion experiments were conducted at the same absolute exercise intensity, and that this elicited different relative exercise intensities, i.e. 80% at sea level, and values ranging from 92.2% on Day 1 at altitude to 84.3% on Day 21. Therefore, the improvements in time to exhaustion could be related to decreases in relative exercise intensities alone, but unfortunately, we did not have the opportunity to conduct a second time to exhaustion test in all conditions in which the workload was matched to elicit a relative intensity of 80% as at sea level.

**6.6. Perspectives**

The results of this study show that the “live high-train low” approach can be recommended to improve \( \dot{V}O_{2\text{max}} \) and performance at moderate altitude. Accordingly, athletes who plan to compete at 2340 m altitude are advised to expose themselves to this altitude for at least 14 days prior to the competition. Although elite cyclists were investigated in this study, it also seems likely that endurance athletes from other sporting disciplines may respond similarly. Based on the present study and previous work, it has become evident that there is an altitude threshold around 3300-3500 m below which an increase in [Hb] has a beneficial
effect on $\dot{V}O_{2\text{max}}$, whereas this advantage disappears at higher altitudes. One reason that $\dot{V}O_{2\text{max}}$ does not increase with the elevation of [Hb] at higher altitudes is probably that there is a reduction in peak leg blood flow in chronic hypoxia (Calbet et al., 2003b; Lundby et al., 2007). This implies that the extra O$_2$ carrying capacity gained with the acclimatization-elicited elevation in [Hb] is not made fully made available to the exercising muscles, but deviated to secure oxygenation of other vascular beds. This hypothesis, however, needs to be tested experimentally.
7. General discussion and conclusions

This thesis investigated the impact of erythropoietin (Epo) on endurance performance under normoxic condition and upon acclimatization to moderate altitude in mice and humans. To be able to study metabolic and cardiovascular parameters in exercising mice, telemetry and indirect calorimetry during exercise were combined and improved in first study. In a newly developed injury regime, the transmitter body was subcutaneously placed in the midline of mice’s back. This is more comfortable for the animals during submaximal as well as maximal exercise and improves blood pressure signal. Furthermore, the success of the implantation depends on the choice of the postoperative regime. Note that this might also be relevant for other injuries. This new method was used in the second study to investigate the effect of varying hematocrit (Htc) levels on exercise performance and the cardiovascular system. To this end, Htc levels of wild type (wt) were acutely elevated by novel erythropoiesis stimulating protein (NESP) administration. On the other hand, Htc levels of our transgenic mice line termed tg6 that reach Htc levels of up to 0.9 due to the overexpression of Epo were reduced by the hemolysis-inducing compound phenylhydrazine (PHZ). Maximal oxygen (O₂) uptake (\( \dot{V}_O_{2,max} \)) and time to exhaustion increased with increasing Htc levels to a peak at 0.58 and 0.57 for wtNESP mice, and 0.68 and 0.66 for tg6PHZ, respectively. Increasing blood viscosity seems to be a reasonable candidate to limit exercise performance.

The third study investigated the effect of altitude acclimatization on exercise performance and hemoglobin concentration ([Hb]). To this end, elite endurance athletes were exposed during a period of 21 days to 2360 m altitude. \( \dot{V}_O_{2,max} \) and time to exhaustion increased over the experimental period, and the main increment occurred within the first 14 days of altitude acclimatization. The altitude-induced elevation in [Hb] seems to be responsible for the improvement in exercise performance.

Overall, the findings of the present work suggest that the main effect of Epo on exercise performance is aimed at improving O₂ carrying capacity by increasing [Hb]. Paradoxically, the associated increased blood viscosity that accompanies elevated Htc levels impairs O₂ delivery and thus, exercise performance. Interestingly, the effects were more pronounced at submaximal intensities than at \( \dot{V}_O_{2,max} \). This is in line with the observation by Thomsen and co-workers (2007) showing that recombinant human Epo (rhEpo) treatment in healthy humans over 14 days increases \( \dot{V}_O_{2,max} \) by approximately 12% and prolongs submaximal exercise by approximately 54%. This implies that other factors may also play a role.
number of data have been gathered on the impact of Epo under pathological condition in order to study its non-hematopoietic functions. Systemically administered recombinant human erythropoietin (rhEpo) crosses the blood-brain barrier and has neuroprotective effects on the central nervous system (Brines et al., 2000). Furthermore, Epo and Epo receptors are also expressed in the brain (Jelkmann, 2005). Elevated Epo-levels in plasma and brain enhance the ventilatory response to severe acute hypoxia as well as the acclimatization to chronic hypoxic exposure (Soliz et al., 2005). Moreover, it was shown in healthy humans that rhEpo administration positively affects the self reported mood, physical conditioning and strength scores (Miskowiak et al., 2008; Ninot et al., 2006). All of them could have an impact on exercise performance of our subjects.

The question arises as to whether the optimal Htc for best aerobic exercise performance alters upon exposure to altitude. It is likely that Htc level is optimized because of its influences on blood O₂ transport capacity. During altitude acclimatization, [Hb] increases and may lead to an arterial O₂ content (CaO₂) that is higher than that at sea level. However, the acclimatization process is multifactorial. Thus, it is difficult to define as whether the optimal Htc for best aerobic exercise performance is altered at altitude. For instance, more than controversial is the effect of hypoxia on the degree of capillarisation (Lundy et al., 2004; Hoppeler et al., 2008). Increased muscle capillarisation might reduce peripheral resistance in the exercising skeletal muscle resulting in a shift of the optimal Htc to a higher Htc value. Therefore, more studies are required to unravel this issue.

Elevated blood viscosity may not only limit exercise performance, but also is potentially dangerous for the health of the athletes. The main risk occurs with Htc above 0.55 (Jelkmann, 2003). Risks include heart failure, myocardial infarction, peripheral thromboembolic events and pulmonary embolism. During competition, athletes are at special risk, because blood viscosity may further increase due to sweating and the loss of body fluid. Thus, sport associations have introduced Htc thresholds, above which athletes are not allowed to compete to stop them from damaging their health. These limiting values are different depending on the association and are not scientifically proven. More investigation to study the morphological and functional consequences of varying Htc levels would support scientific Htc limitation to protect the athlete’s lives. Moreover, scientific evidence would demonstrate the life-threatening consequences of long-term rhEpo-abuse. This is necessary to sensitize athletes, trainers, administrators and also, importantly, spectators. It is becoming even more important, as anti-doping agencies will be confronted with a new technology – gene doping. Genetically manipulated athletes will be difficult to identify. Thus,
more scientific proofs about the health risks of long-term Epo abuse will probably hinder many athletes from abusing their bodies in this way.

On the other hand, rhEpo and its derivate are used for the treatment of anemia such as chronic renal failure, HIV-infection or cancer. Thereby, the treatment is depending on severity of the illness and the ability for regeneration of the injured organs. Thus, novel scientific knowledges of Epo’s positive and negative side effects to human health are also important for those patients suffering from anemic diseases.

Finally, Epo has a future in clinical medicine for a number of therapies in areas including neuroscience and cardiovascular diseases because it is a safe drug. Unfortunately, it is also misused by scrupulous athletes. Indeed, many publications and reviews are available on this topic. In spite of the efforts in the last years, there is still much work to do in order to understand the multiple functions of Epo.
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9. List of abbreviations

Ca\textsubscript{O2} arterial oxygen content
Epo erythropoietin
Hif hypoxia-inducible factor
Hif-\textalpha{} \(\alpha\) subunit of hypoxia-inducible factor
Hif-1 hypoxia inducible factor-1
Hif-1\textalpha{} \(\alpha\) subunit of hypoxia-inducible factor-1
Hif-1\textbeta{} \(\beta\) subunit of hypoxia-inducible factor-1
Hif-2\alpha{} \(\alpha\) subunit of hypoxia-inducible factor-2
Hif-3\alpha{} \(\alpha\) subunit of hypoxia-inducible factor-3
[Hb] hemoglobin concentration
Htc hematocrit
O\textsubscript{2} oxygen
NESP novel erythropoiesis stimulating protein
PHZ phenylhydrazine
pO\textsubscript{2} oxygen partial pressure
RER respiratory exchange ratio
rhEpo recombinant human erythropoietin
Sa\textsubscript{O2} arterial oxygen saturation
SD standard deviation
tg6 transgenic mouse line overexpressing human erythropoietin cDNA
tg6PHZ transgenic mouse line overexpressing human erythropoietin cDNA, treated with phenylhydrazine
wt wild type mouse
wtNESP wild type mouse treated with novel erythropoiesis stimulating protein
VEGF vascular endothelial growth factor
\textDot{\textVCO}_2 carbon dioxide production
\textDot{\textVO}_2 oxygen uptake
\textDot{\textVO}_\text{2max} maximal oxygen uptake
10. Curriculum Vitae

Name: Beat Schuler
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Education:

1979-1986 Primary school, Siebnen
1986-1989 Secondary school, Siebnen
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11. Publications

**Scientific Publications**


**Proceedings and Abstracts**


**Schuler B, Thomsen JJ, Gassmann M, Lundby C.** Time course of improvement in endurance performance during altitude acclimatization. 11th annual Congress of European College of Sport Science, 5-8 July 2006, Lausanne, Switzerland.


**Schuler B, Thomsen JJ, Gassmann M, Lundby C.** Optimal arrival for competing at moderate altitude in elite endurance athletes, 2th ZIHP Symposia, 22 September 2006, Zurich Switzerland.

**Other publications**

- Der Dopingverdacht rennt mit, 10vor10, Swiss Television SF, 15 August 2008.
- 175 Years Anniversary of the University of Zurich, University of Zurich, 17-18 April 2008.
- Zaubersaft Blut, ARTE (Association à la Télévision Européenne), 1 August, 2008.

Oral presentation

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- Doping and Gendoping: The impact of Epo and NESP on exercise performance and health, Forschungskredit, University of Zurich, Zurich, Switzerland, 2008, CHF: 94576.
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