UNIVERSITÄT FÜR BODENKULTUR WIEN

DEPARTMENT FÜR INTEGRATIVE BIOLOGIE & BIODIVERSITÄT

- INSTITUT FÜR WILDTIERBIOLOGIE UND JAGDWIRTSCHAFT -

The effect of altitude on mitochondrial fatty acid composition and mitochondrial enzyme activity in heart muscles from Mountain hares (*Lepus timidus varronis*)

Die Auswirkung der Höhenlage auf die mitochondriale Fettsäurezusammensetzung und mitochondriale Enzymaktivität im Herzmuskel des Alpenschneehasen (*Lepus timidus varronis*)

Masterarbeit

zur Erlangung des akademischen Grades

Master of Science

eingereicht von

Natacha Hell

aus
Montevideo, Uruguay

1. Betreuer: Univ. Prof. Dipl.-Biol. Dr.rer.nat. Univ.Prof. Klaus Hackländer
2. Betreuer: Dr.rer.nat. Wolfgang Gregor

Danksagung

Ich möchte mich bei meinem Betreuer Herrn Prof. Klaus Hackländer bedanken für die Vergabe des Masterarbeitsthemas und für die ausgezeichnete Betreuung während der Erstellung der Masterarbeit. Auch bei meinem Zweitbetreuer Herrn Dr. Wolfgang Gregor möchte ich mich bedanken für die Betreuung im Labor und für die vielen Anregungen und Ideen.

Vielen Dank auch an Gabi Kaiser, für die Vorbereitung der Proben und vor allem für die Hilfe und Geduld bei meinen zahlreichen Fragen.

Ich möchte Herrn Prof. Walter Arnold dafür danken, dass das Forschungsinstitut für Wildtierkunde und Ökologie (FIWI) die Arbeitsmittel für die Erstellung meiner Masterarbeit zur Verfügung gestellt hat.

Insbesondere möchte ich meine Eltern und meinen Bruder danken, da sie mich während meinen gesamten Studium aus der Ferne immer unterstützt haben, Interesse an meinem Studium und Arbeit gezeigt haben und mir den Rücken gestärkt haben, immer wenn es nötig war.

Contents

Abstract

To analyse the effect of increasing habitat altitude on mitochondrial phospholipid fatty acid composition and on mitochondrial enzyme activity, 10 hearts from mountain hares (*Lepus timidus varronis*) living at lower habitat altitude (1180-1760 m.a.s.l.) and 10 hearts from higher altitude (2700-2800 m.a.s.l.) in the Grisons (Switzerland) were compared. There were no significant differences in the activities of complex II and IV of the electron transport chain and in the mitochondrial yield. No significant impact of altitude on the relative content of saturated, monounsaturated and polyunsaturated fatty acids and on the unsaturaion index of mitochondrial phospholipids was found. However, there was an increase in the n-6/n-3 ratio at higher altitudes ($p<0,001$). This increase was caused by the decrease of the relative content of the n-3 fatty acids α-linolenic acid (C18:3, $p=0,002$), eicosapentaenoic acid (C20:5, $p=0,008$) and docosahexaenoic acid (C22:6, $p=0,032$). We suggest that the increased n-6/n-3 ratio is an adaptation to the cold environment: it might increase the activity of the Ca^{++} pump of the sarcoplasmic reticulum of the heart (SERCA), decreasing the risk of arrhythmia during low tissue temperature. In addition, it might contribute to regulate a low basal metabolic rate and decreased heart rate. This probably explains the finding that there were no changes in the mitochondrial activity observed in cold acclimatized hares.

Zusammenfassung

Zur Analyse der Auswirkungen zunehmender Lebensraumhöhe auf die Fettsäurezusammensetzung der mitochondrialen Phospholipide und auf die mitochondriale Enzymaktivität wurden 10 Herzen aus Alpenschneehasen (*Lepus timidus varronis*) aus niedrigen Lagen (1180-1760 m ü.M.) und 10 Herzen aus höheren Lagen (2700-2800 m ü.M.) aus Graubünden (Schweiz) verglichen. Es gab keine signifikanten Unterschiede in den Aktivitäten der Komplexe II und IV der Elektronentransportkette und in der Mitochondrienausbeute. Es wurden auch keine signifikanten Auswirkungen der Höhe auf den relativen Gehalt an gesättigten, einfach und mehrfach ungesättigten Fettsäuren und auf den Unsaturation Index der mitochondrialen Phospholipide gefunden. Allerdings gab es eine Erhöhung des n-6/n-3 Verhältnisses in höheren Lagen ($p<0.001$). Dieser Anstieg war durch den Rückgang im relativen Gehalt der n-3-Fettsäuren α-Linolensäure (C18:3, $p=0.002$), Timnodonsäure (C20:5, $p=0.008$) und Cervonsäure (C22:6, $p=0.032$) verursacht. Wir schlagen vor, dass das erhöhte n-6/n-3 Verhältnis eine Anpassung an die kalte Umgebung ist: es könnte die Aktivität der Ca^{2+}-Pumpen des sarkoplasmatischen Retikulums des Herzens (SERCA) erhöhen, was zu einer Verringerung der Gefahr von Herzrhythmusstörungen bei niedriger Gewebetemperatur führt. Darüber hinaus könnte es dazu beitragen, den niedrigen Grundumsatz und eine verringerte Herzfrequenz zu regulieren. Damit erklärt sich wahrscheinlich auch der Befund, dass es keine Veränderungen der Mitochondrienaktivität in kalt-akklimatisierten Alpenschneehasen gab.

Introduction

Unlike hibernating animals, which are capable to reduce body temperature (T_b) to slightly above ambient temperature (T_a) (Arnold 1988), mountain hares decrease their average core T_b only by 0.4℃ during winter (Nieminen & Mustonen 2 008). Most homeothermic animals are able to increase their metabolic heat production (thermogenesis) and thereby offset the heat loss and maintain their body temerature (Chaînier et al 2000). Indeed, in mountain hares a reduction of 18% of the basic metabolic rate (BMR) was observed (Pyörnilä et al. 1992). Furthermore, mountain hares neither gather fat reserves (Soveri & Aarnio 1983) nor have behavioral thermoregulation (Pyörnilä et al. 1992). Since our knowledge concerning metabolic adaptation of mountain hares to cold climates is poor, the question which physiological mechanisms of adaptation exist in cold acclimatized mountain hares must be analyzed in greater detail.

Leporids have developed several strategies to live in arctic and subarctic regions and to cope with the seasonal climatic changes in their habitat, including improvements in insulation and an increased metabolic heat production (Pyörnilä et al. 1992). Since Mitochondria are responsible for 90% of total oxygen consumption (Rolfe and Brown 1997), there might be a shift in mitochondrial respiration and mitochondrial enzyme-catalyzed reactions with changes in metabolic heat production and BMR due to cold acclimation.

Temperature influences the biophysical properties of membranes and thus the mitochondrial function (Staples and Brown 2008). Lipid composition can also affect membrane function such as mitochondrial electron transport (Gutiérrez et al. 2002), influencing the activities of mitochondrial membrane bound enzymes (Daum 1985, Chaînier et al. 2000, Crider & Xie 2003). Phospholipids (PL) are essential

components of the cell membranes (Zabelinskii et al. 1999) and there are many investigations about the influence of fatty acid (FA) composition of membranes on mitochondrial activity (Brookes et al. 1998, Clouet et al. 1995, Vázquez-Memije et al. 2005, Gerson et al. 2008). As PL consist mainly of FA, their properties influence directly membrane properties such as fluidity.

Since mitochondria play a central role in energy metabolism of organisms, it is very important to take them into account in issues related to cold acclimation. Cold can affect mitochondrial metabolism by reducing mitochondrial oxidative capacity, by limiting diffusion within the mitochondrial membrane or by modifying the sensitivity of activity regulating enzymes (Guderley & St-Pierre 2002). For cold acclimatized carp (Wodtke 1991a,b), cold exposed ducklings (Chaînier et al. 2000) and cold exposed guinea pigs (Kinnula et al. 1983) it has been demonstrated that there is an increased mitochondrial activity. This increase can be due to greater mitochondrial volume densities or due to a higher oxidative capacity of isolated mitochondria (Guderley & St-Pierre 2002).

To our knowledge, there are no present studies about the effects of altitude on the mitochondrial FA composition and enzyme activity.

The present study attempts to evaluate the effects of altitude on mitochondrial FA composition, mitochondirial enzyme activity and mitochondrial yield. Furthermore, the altitude-independent effects of FA composition on mitochondrial enzyme activity were analyzed.

We want to test the hypothesis that with increasing altitude, i.e. decreasing temperature, there is an increase in mitochondrial volume densities and/or an increased mitochondrial enzyme activity, to ensure activity during cold acclimation. Moreover, we want to test the hypothesis that there is a change in the FA composition of PL in order to increase tissue tolerance to cold.

Materials and Methods

In order to perform this study, 20 heart samples from alpine mountain hares (*Lepus timidus varronis*) were collected in Grisons (Switzerland). Ten of these samples came from lower altitudes (1180-1760 m.a.s.l.) and the other ten from higher altitudes (2700-2800 m.a.s.l.) of the habitat of the hares. It was assumed that the animals lived or were born at the site where they were hunted (Nodari 2006). The samples were collected in October/November 2004 (n=10) and October/November 2005 (n=10). The animals were weighed immediately after death with an accuracy of 5g (Kaiser et al. 2009) and their age was determined (subadult – adult) from the dried eye lens weight (Suchentrunk et al. 1991). The altitude was inferred from the map by the hunters with an accuracy of 10 m (Kaiser et al 2009). The hearts were frozen at -20℃ until further analysis (Kaiser et al. 2009).

To analyze the samples, 2.5 g were washed with homo buffer (pH 7.5, 10 mM EDTA, 100 mM TRIS, 100 mM KCl, 100 mM sucrose) until no more blood was present. Then, the tissues were homogenized with a Potter pestle (Mühlendorf & Lill 2001) in 25 ml of homo buffer containing ca. 50 mg BHT (butylated hydroxytoluene). 30 ml of homo buffer containing 15% BSA (bovine serum albumin) were added, followed by two centrifugation steps for 10 minutes each (800 rpm, 4℃). Finally the supernatants were frozen at -80℃ (Kaiser et al. 2009).

For the isolation of mitochondria, the supernatants were filled up to 30 ml with homo buffer containing BSA and centrifuged twice for 10 minutes (1500 rpm and 2500 rpm). The pellet from 1500 rpm was resuspended with wash buffer, homogenized with a hand-driven potter pestle and centrifuged for 10 minutes at 1500 rpm. The

pellet from 2500 rpm was also resuspended with wash buffer, homogenized with a potter pestle and centrifuged 10 minutes at 2500 rpm.

The supernatants were discarded. The pellets from 1500 rpm (“Mito 1”) and from 2500 rpm (“Mito 2”) were resuspended with wash buffer and stored at -80°C.

The samples used to determine mitochondrial enzyme activities and protein content (“Mito 2”) contained approximately 50-70% of the mitochondria (extrapolated from experiments on red deer samples). A small fraction of heavy mitochondria (“Mito 1”, 7%) was used for fatty acid analysis.

To characterize mitochondrial enzyme activites, the activities of complexes I-IV of the electron transport chain were measured according to the method of Gregor et al. (2006) using a Perkin-Elmer 550 photometer. The initial slopes of the kinetic curves (absorption as a function of time) were analysed under substrate saturation conditions in order to obtain the maximum reaction rate.

The activity of complex I (NADH-dehydrogenase) was measured at 340 nm (maximum absorption of NADH) in 20 mM TRIS buffer (pH 7.5), which contains 100 µM NADH and 50 µM of the short-chain ubiquinone homologue UQ-1. To determine the specificity of the reaction, an inhibitor test was performed: the specific activity of complex I was inhibited with 2 µg/ml rotenone.

The activity of complex II (succinate dehydrogenase) was measured at 280 nm in a 20 mM TRIS buffer (pH 7.5), containing 20 mM succinate and 50 µM UQ-1.

The activity of complex III was measured at 550 nm in a 10 mM TRIS buffer (pH 7,4). In addition, 200 µM ubiquinol (UQH_2-1) and 1 mM CN^-, which prevents the

reoxidation of cytochrome c, were required for measuring the activity of complex III. The reaction was tested for specificity with 20 µM antimycin.

The enzymatic reduction of cytochrome c (complex IV = cytochrome oxidase) was measured at 550 nm in a 30 mM sodium phosphate buffer (pH 7.4), containing 0.3 µg/ml cytochrome c, which was prereduced with sodium dithionite. To determine the specificity of the reaction, the activity of cytochrome oxidase was inhibited with 1 mM CN^-.

Each measurement was performed 5 times. For the evaluation of the results, at least 3 out of 5 measurements were used, i.e. the more stable kinetic curves were used and the outliers were discarded.

The protein content of the mitochondrial samples and of the supernatants (from which the mitochondria were pelleted) were also measured with the photometer. The absorption was assessed at 595 nm after mixing with Bradford reagent. The absorptions of three standard solutions containing 1, 3 and 5 mg/mL BSA were also measured. The measurements were repeated until two absorption values differed by less than 0,02. The protein content was calculated using a standard plot that shows the absorption as a function of the protein content of the BSA standard solutions.

The activities of the complexes I-IV were normalized to the protein content of the mitochondria.

Subsequently, the total amount of mitochondria was derived from the volumes and protein concentrations of the samples used in the mitochondrial activity assays, and normalized to the protein concentrations of the homogenates. Data on mitochondrial

activities (given in $\mu mol \cdot min^{-1} mg^{-1}$) and mitochondrial yield (proportion of mitochondrial proteins given in %) were calculated.

The method of Folch et al. (1957) was used for lipid extraction. The mitochondrial lipids were extracted by vortexing with a chloroform/methanol mixture (2:1 plus 0.22 mg/ml antioxidant BHT). Phospholipids and neutral lipids were separated by using thin layer chromatography (Kieselgel 60, F254, 0.5 mm, Merck). Hexane/diethylether/formic acid (80:20:2) was used as a mobile phase.

The different lipids were made visible under UV light and the phospholipids (PL) were isolated. For the transesterification the PL were heated for 30 minutes in a 20% boron trifluoride solution in methanol under nitrogen at 100℃ (Eder 1995). The fatty acid methyl esters (FAME) were extracted with n-hexane and analyzed by gas chromatography (Perkin Elmer Autosystem XL with autosampler and FID; Norwalk, USA) using a capillary column (HP INNOWax, 30 m x 0.25 mm x 0.25 µm; Hewlett Packard, USA) and the following parameters: injector 240℃, column 130-180℃ at 4℃/min, 180-200℃ at 3℃/min, 200-240℃ at 15℃/mi n, 240℃ for 8 min. External FAME standards (Supelco) were used for peak identification and calculation of the response factors.

The statistical analysis of the data was performed using Analysis of Covariance (ANCOVA) in SPSS 15.0 for windows. The significance level was set at $p<0.05$.

Fatty acids were combined depending on their degree of saturation (Valencak et al., 2003): saturated fatty acids (SFA: C14:0, C15:0, C16:0, C17:0, C18:0), monounsaturated fatty acids (MUFA: C16:1, C18:1) and polyunsaturated fatty acids (PUFA: C18:2n-6, C18:3n-3, C20:4n-6, C20:5n-3, C22:5n-3, C22:6n-3) (data given in

w/w%). Furthermore, the unsaturation index (UI: mean number of double bonds) and the n6 to n3 ratio were calculated.

ANCOVA was performed to determine the dependence of the relative amount of each FA, SFA, MUFA, PUFA, UI, n6/n3, mitochondrial activity and mitochondrial density on habitat altitude. Since the weight and age of the hares may also play a role, these were also included, as covariate and random factor respectively, in the model.

ANCOVA was also performed in order to determine if there is an influence of FA composition on mitochondrial activity. Each FA, SFA, MUFA, PUFA, UI and n6/n3 were set as covariates in each test together with the weight, while the altitude group was set as fixed factor and age as random factor of mitochondrial activity (dependent variable).

Results

There were no correlations of the enzymatic activity of complex II ($F_{1,13}$=0.957, p=0.346) and complex IV ($F_{1,13}$=1.721, p=0.212) with altitude. It was not possible to measure the activity of complexes I and III, probably due to protein denaturation. The mitochondrial yield also showed no significant differences in relation to altitude ($F_{1,13}$=0.149, p=0.705). Neither age nor weight had an effect on mitochondrial activity and mitochondrial yield.

The mean w/w% of SFA, MUFA and PUFA in mitochondrial phospholipids was 36.3%, 5.6% and 58.1% respectively. There were no significant changes of SFA ($F_{1,13}$=0.003, p=0.955), MUFA ($F_{1,13}$=1.107, p=0.312) and PUFA ($F_{1,13}$=0,298, p=0,594) with altitude. The unsaturation index also showed no significant differences with altitude ($F_{1,13}$=1.467, p=0.247). However, there was a significant increase in the n-6/n-3 ratio with increasing altitude ($F_{1,13}$=24.307, p=0,001) (Fig. 1D).

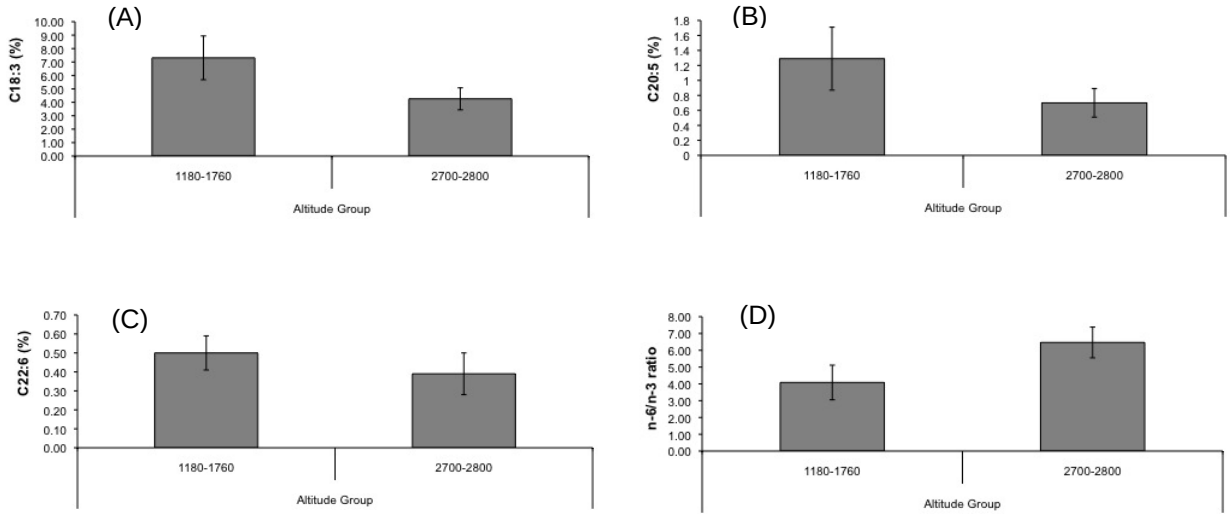

Figure 1: The effect of altitude on the content of C18:3 (A), C20:5 (B), C22:6 (C) and on the n-6/n-3 ratio (D) (Error bars show mean +/- 1 SD)

The major components of the heart mitochondrial phospholipids were stearic acid (C18:0, 24.38%) and linoleic acid (C18:2n-6, 37.93%).

Altitude affected the w/w% of some FA in the mitochondrial phospholipids: there was a significant decrease of the n-3 FA C18:3 ($F_{1,13}$=16.028, p=0,002), C20:5 ($F_{1,13}$=9,783, p=0,008) and C22:6 ($F_{1,13}$=5.786, p=0.032) of 3.05%, 0.59% and 0.11% respectively (Fig. 1). Despite an effect of age on n-6/n-3 ($F_{1,13}$=4.813, p=0.047), there was no effect of body weight or age on fatty acid composition in heart mitochondria.

The mitochondrial activity of complex IV was influenced by C20:5 ($F_{1,12}$=6.581, p=0.025), C22:5 ($F_{1,12}$=6.599, p=0.025), C22:6 ($F_{1,12}$=7.879, p=0.016) and UI ($F_{1,12}$=5.847, p=0.032) (Fig. 2). There was no effect of FA composition on the enzymatic activity of complex II.

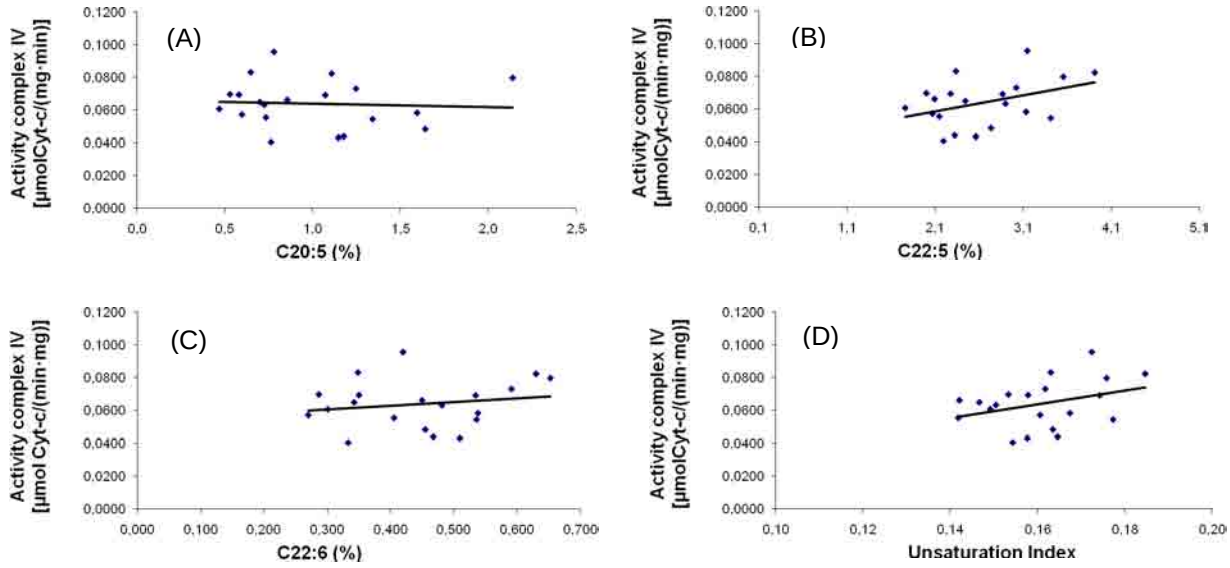

Figure 2: the effect of C20:5 (A), C22:5 (B), C22:6 (C) and the Unsaturation Index (D) on the activity of complex IV

Discussion

In mitochondria of heart muscles from mountain hares we found significant changes in the FA content of α-linolenic acid (C18:3n-3), eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3) with increasing altitude. The decrease of these three n-3 FA caused an increase in the n-6/n-3 ratio with increasing altitude. These results are in agreement with the decrease in C18:3 and C20:5 and the increased n-6/n-3 ratio in total PL of heart muscles from the same mountain hares studied by Kaiser et al. (2009). However, Kaiser et al (2009) also found changes in C16:1 content in the PL of heart muscles, which we did not detect.

Unlike other studies related to cold acclimation, no changes in mitochondrial SFA, MUFA and PUFA content were found in cold acclimatized mountain hares. Since PUFA have lower melting points, they are important in maintaining body lipids fluidity at low T_b (Altman & Dittmer 1972). In hibernating animals, such as yellow-pine chipmunk (*Tamias amoenus*) (Geiser & Kenagy 1987, Geiser et al. 1994) and golden-manteled ground squirrels (*Spermophilus lateralis*) (Frank 1992) an increased PUFA content in the diet caused a higher PUFA content in membranes which resulted in more frequent and longer torpor bouts and lower T_b during hibernation. Cold acclimation altered the FA membrane composition in ducklings: an enrichment in PUFA (especially 20:4n-6) and a higher unsaturation index in muscle mitochondria was found (Chaînier et al. 2000). This increase in unsaturated fatty acids appears to maintain the fluidity of biomembranes (Cossins & Bowler 1987). It is very important to maintain the fluidity of membrane phospholipids for the proper activity of membrane enzymes and diffusion within membranes (Gerson et al. 2008), especially at low T_b, where phospholipids could decrease the lipid mobility. Since PUFA can´t be synthesized by mammals, they are essential in the diet (Sprecher et al. 1995, Hulbert

2007). Ground squirrels in the wild select diets high in PUFA and more essential fatty acids during summer and fall (Frank 1994) and in rats, cold exposition was associated with a threefold increase in food intake (Ocloo et al. 2007). However, our study does not support these results. There was no increase in PUFA in mitochondrial membranes. Indeed, PUFA intake is energetically very costly for organisms, because they are more sensitive to reactive oxygen species (ROS) (Munro & Thomas 2004). Each double bond of PUFA is a target for ROS, resulting in peroxidation (Gutiérrez 2002, Gerson et al. 2008) and this could be a possible reason for the lack of a higher PUFA content in cold acclimatized mountain hares. Furthermore, the membrane fluidity does not seem to be affected very strongly due to the good isolation of the winter pelage. Although the ambient temperature decreases, the mountain hares are able to keep their body temperature almost constant.

On the other hand, Ruf and Arnold (2008) suggested that the tolerance of heart muscle to cold is not increased due to higher general PUFA content, but due to shifts in the n-6/n-3 ratio. In our study we found a very pronounced increase in n-6/n-3 ratio in the PL of heart mitochondria with altitude. This increase was proposed to upregulate the Ca^{++} pump activity in the sarcoplasmic reticulum of the heart (SERCA), avoiding high Ca^{++} concentrations in the cytosol and hence heart arrhythmia, although tissue temperature decreases (Ruf & Arnold 2008). The increased Ca^{2+} pump activity also might enhance the skeletal muscle activity. Since mountain hares do not alter their behaviour during the winter, maintaining the proper activity of skeletal muscle at low T_b is of particular importance when escaping from predators. Furthermore, high levels of n-6 PUFA might lead to higher uncoupling and hence non-shivering thermogenesis in muscle, due to the fact that muscle uncoupling proteins (UCP-2 and UCP-3) show higher affinity to n-6 PUFA (Zackova et al. 2003).

Apparently in hares cold acclimation is more related to the higher n-6/n-3 ratio than to the PUFA content.

The mean PUFA content in mitochondria of mountain hare heart muscles was very high (58.12%). Also in the heart of european hares (*L. europaeus*) a high PUFA content (65,7%) was found when compared to other mammals (Valencak et al. 2003). Valencak et al. (2003) proposed that the high amount of PUFA in heart and skeletal muscle is related to the very high maximum running speed of this species. This could also be an explanation for the high PUFA amount in mountain hares heart mitochondria. The high n-6 amount in PUFA (83%) is also evidence for high maximum running speed (Ruf et al. 2006).

There were some changes in the proportion of certain FAs within PUFA, such as C18:3n-3, which could be related to changes in the FA composition of the plants.

There were also significant changes in C22:6n-3, which decreased with increasing altitude. Infante et al. (2001) found a higher frequency of contraction in muscles with high C22:6 in PL, which suggests that this FA supports an improved and sustained muscle function. Taking this into account and the fact that the BMR is decreased in winter, there could be a decreased heart contraction rate in cold acclimatized mountain hares.

In general, there was a decreased biosynthesis of highly unsaturated FA such as C20:5 and C22:6 with increasing altitude. This is probably a mechanism to avoid oxidative stress due to peroxidation and to save metabolic energy.

In mitochondria of adult mountain hares a higher n-6/n-3 ratio was found when compared with subadults. Our study supports the results of other studies related to FA changes due to aging. In rat hearts an age-related increase in the n-6/n-3 ratio in cardiac mitochondrial membranes was found (Pepe et al. 1999). Also in rat testis

mitochondria there was an age-related increase of n-6 and a decrease of n-3. The n-6 PUFA are the preferred substrate for ROS generation (Pepe 2005). Cardiac work is especially maintained by mitochondria, and an aged heart may show a reduction in oxygen utilization efficiency and reduced mechanisms to minimize ROS production (Pepe 2005). The number of antioxidants present in mitochondria may also decrease with age, producing a lower capacity to cope with ROS (Papa & Skulachev 1997).

Mitochondrial metabolism is of special interest in questions related to cold acclimation, due to their central role in energy metabolism. When mammals are chronically exposed to cold, there is a shift in heat production from shivering to non-shivering thermogenesis based on brown adipose tissue (BAT) (Ocloo et al. 2007). The uncoupling protein (UCP-1) uncouples electron transport from the ATP synthesis for heat production in BAT (Nichols & Locke 1984). However, BAT is not present in mountain hares. Some small mammals showed changes in skeletal muscle mitochondria when exposed to cold, suggesting an enhanced thermogenesis (Ogawa et al. 1987). There was an increased cytochrome level and an enhanced cytochrome c oxidase and succinate dehydrogenase activity in cold acclimatized guinea pigs (Kinnula et al. 1983). In addition, in cold acclimatized duckling an enhanced ATP synthesis was found: the specific cox activity was unaffected, while the oxidative capacity in skeletal muscle was increased due to a higher amount of mitochondrial proteins (Chaînier et al. 2000). However, cold acclimatized mountain hares did not show a higher mitochondrial activity.

Cold acclimation in ectotherms is of special interest and is very well studied due to the direct influence of environment on their physiological processes. Fish remain swimming during cold acclimation due to their possibility to shift their thermal optimum (Guderley & St-Pierre 2002). Sustained swimming during cold requires ATP

synthesis: they increase their mitochondrial protein content or the activities of fibre muscle mitochondrial enzymes (Sänger 1993). In muscle of cold acclimatized carp a higher activity of cytochrome c oxidase (cox, complex IV) was observed, whereas the concentration of this enzyme complex remained constant (Wodtke 1991a,b). In contrast, the common frog (*Rana temporaria*), which decreases the standard metabolic rate to 25% during the winter (Donohoe and Boutilier 1998), also decreases its cox activity (St-Pierre and Boutilier 2001).

Our study does not support these results. The mitochondrial activity did not show significant changes and the mitochondrial protein content remained constant in cold acclimatized mountain hares. Since the BMR decreases in winter, it is possible that it also decreases with altitude, which is negatively correlated with Ta. Red deer (*Cervus elaphus*) (Arnold et al. 2004) and przewalski horse (*Equus ferus przewalskii*) (Kuntz et al. 2006) also show a downregulation of BMR in the winter, accompanied by a reduction of the heart rate. Hence, it is possible that the mountain hares also decrease their heart rate during the winter and when acclimatized to cold habitats. In this case one would expect that the mitochondrial activity of the ETC (electron transport chain) also decreases with decreasing temperature. It is possible that due to the good isolation mechanisms of the pelage and the low decrease in T_b of 0.4℃ (Nieminen & Mustonen 2008), it is not necessary for mountain hares to compensate the decreased T_b or to downregulate the mitochondrial enzyme activity. It is more likely that mountain hares decrease their body peripheral temperature only, while the core temperature remains stable.

Several studies demonstrated that there is an age-related decrease of respiratory chain enzyme activities (Kwong & Sohal 2000, Vázquez-Memije et al. 2005). In our study we found no correlation between aging and mitochondrial enzymatic activity.

Since the membrane environment directly affects the function of membrane proteins, changes in the PL FA composition of mitochondrial membranes might directly affect the mitochondrial enzyme activity. Hulbert and Else (1999) proposed the hypothesis that membrane fatty acids act as pacemaker for BMR. There are many considerable changes in PL FA composition occuring during thermal acclimation in order to maintain a constant membrane fluidity (homeoviscous adaptation). In cold acclimatized ducklings, the increased PUFA content was associated with an increased ATP synthesis in skeletal muscles (Chaînier et al. 2000). In cold acclimatized rainbow trout the enhanced oxidative capacity of red muscle mitochondria was accompanied with an increased UI and PUFA content, mainly C22:6 (Guderley et al. 1997). A higher polyunsaturation of membranes increases the enzymatic activity, but also increases the proton leak, generating a higher energetical cost (Hulbert & Else 1999). In mountain hares, the mitochondrial cox activity was not affected by SFA, MUFA or PUFA, but was positively influenced by UI, C20:5, C22:5 and C22:6.

A higher ratio of mitochondrial / homogenate protein content could mean either that there is an increase in mitochondrial inner membrane surface or mitochondrial proliferation, in order to increase the membrane surface for an enhanced electron transport. Increases in mitochondrial membrane surface or in the number of mitochondria and an enhanced mitochondrial activity are complementary strategies, because both enhance the oxidative capacity (Guderley & St-Pierre 2002). However, there was no increase in the mitochondrial yield due to cold acclimation in mountain hares. Since cox can be only found in the inner mitochondrial membrane, its activity can be used as a measure for the membrane surface area of mitochondria (Leary et al. 2003). There was a trend

towards higher activity and thus in the surface of the inner mitochondrial membrane. However, this increase was not significant. This could be due to the fact that an enhanced membrane surface would mean a high energetically cost, because the proton leak increases as a function of inner membrane surface area (Porter et al. 1996).

The changes in FA composition and the slight, but not significant, increase in the ETC activity in mountain hares could also be due to an adaption to hypoxia. The oxygen content decreases by 21% from 1180 to 2800 m.a.s.l. Since mitochondria are the principal organelles for oxygen consumption it has been proposed that there is a mitochondrial adaptation to the decrease of oxygen saturation (hypoxia) (Lynn et al. 2007). In rats exposed to hypoxia, a decrease in n-6 PUFA, which was compensated by an increase in n-3 PUFA, particularly 20:5n-3, 22:5n-3 and 22:6 n-3, and an increase in the UI was observed in total PL of the left and right ventricles of the heart (Jezová et al. 2002). It was also demonstrated that there is a proliferation of cardiac mitochondria and a reduction of volume in hearts due to hypoxia (Costa et al. 1988). The decrease in the n-6/n-3 ratio may be attributed to an increased ischemic tolerance when exposed to hypoxia (Jezová et al. 2002). In contrast, we found an increased n-6/n-3 ratio. In mitochondria of the right and left heart ventricle of adult rats exposed to hypoxia there was a reduction in mitochondrial size and increase in mitochondria number, with no change in total mitochondrial protein content (Nouette-Gaulain 2005). There was also a slight decrease in the ETC activity (Nouette-Gaulain 2005). In contrast, we found a trend towards increased ETC activity. Therefore, the changes observed in the FA composition, the constant mitochondrial yield and mitochondrial activity seem not to be adaptations related to hypoxic conditions, but to ambient temperature.

Summarizing, it is more likely that the cold acclimation of mountain hares is related to a higher n-6/n-3 ratio than to a higher PUFA content. The downregulation of BMR might be coupled to a higher Ca^{2+} pump activity in order to reduce the risk of heart arrhythmia. Moreover, the higher Ca^{2+} pump activity might enhance the activity of skeletal muscle with decreasing tissue temperature. The mitochondrial activity remained constant probably due to the fact that no compensation was needed: the pelage of the mountain hares isolates them very well from low T_a. In the future, we suggest to investigate if there is an increased activity of SERCA in cold acclimatized mountain hares and if there is a difference in the diet depending on the altitude of their habitat.

Literature

Altman PL and Dittmer DS (1972): Biology Data Book 2nd Edition. Federation of American Societies for Experimental Biology, Bethesda, Maryland.

Arnold W (1988): Social Thermoregulation During Hibernation in Alpine Marmots (*Marmota marmota*). *J Comp Physiol B.* 158:151-156.

Arnold W, Ruf T, Reimoser S, Tataruch F, Onderscheka K and Schober F (2004): Nocturnal hypometabolism as an overwintering strategy of red deer (*Cervus elaphus*). *Am J Physiol* 286:R174-R181.

Brookes PS, Buckingham JA, Tenreiro AM, Hulbert AJ and Brand MD (1998): The proton permeability of the inner membrane of liver mitochondria from ectothermic and endothermic vertebrates and from obese rats: correlations with standard metabolic rate and phospholipid fatty acid composition. *Comp Biochem Physiol B Biochem Mo. Biol* 119(2):325-334.

Chaînier F, Roussel D, Georges B, Meister R, Rouanet JL, Duchamp C and Barré H (2000): Cold Acclimation or Grapeseed Oil Feeding Affects Phospholipid Composition and Mitochondrial Function in Duckling Skeletal Muscle. *Lipids* 35:1099–1106.

Clouet P, Niot I, Gresti J, Demarquoy J, Boichot J, Durand G and Bézard J (1995): Polyunsaturated n-3 and n-6 fatty acids at a low level in the diet alter mitochondrial outer membrane parameters in Wistar rat liver. *J Nutr Biochem* 6:626-634.

Cossins AR and Bowler K (1987): Temperature biology of animals. Chapman and Hall, London.

Costa LE, Boveris A, Koch OR and Taquini AC (1988): Liver and heart mitochondria in rats submitted to chronic hypobaric hypoxia. *Am J Physiol* 255:123–129.

Crider BP and Xie XS (2003): Characterization of the functional coupling of bovine brain vacuolar-type H(C)-translocating ATPase Effect of divalent cations, phospholipids, and subunit H (SFD). *J Biol Chem* 278, 44281–44288.

Daum G (1985): Lipids of mitochondria. *Biochim Biophys* Acta 822:1-42.

Donohoe PH and Boutilier RG (1998): The protective effects of metabolic rate depression in hypoxic cold submerged frogs. *Respir Physiol* 111:325–336.

Eder K (1995): Review: gas chromatographic analysis of fatty acid methyl esters. *J chromatogr B.* 671:113-131.

Folch J, Lees M and Sloane-Stanley GH (1957): A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226:497-509.

Frank CL (1992): The influence of dietary fatty acids on hibernation by golden-mantled ground squirrels (S. *lateralis). Physiol Zool* 65:906-920.

Frank CL (1994): Polunsaturate content and diet selection by ground squirrels (S. *lateralis). Ecology* 75:458-463.

Geiser F and Kenagy GJ (1987): Polyunsaturated lipid diet lengthens torpor and reduces body temperature in a hibernator. *Am J Physiol* 252:R897-R901.

Geiser F, McAllan BM and Kenagy GJ (1994): The degree of dietary fatty acid unsaturation affects torpor patterns and lipid composition of a hibernator. *J Comp Physiol B* 164:299-305.

Gerson AL, Brown JCL, Thomas R, Bernards MA and Staples JF (2008): Effects of dietary polyunsaturated fatty acids on mitochondrial metabolism in mammalian hibernation. *The Journal of Experimental Biology* 211:2689-2699.

Guderley H, St-Pierre J, Couture P and Hulbert AJ (1997). Plasticity of the properties of mitochondria from rainbow trout red muscle with seasonal acclimatization. *Fish Physiol. Biochem.* 16, 531–541.

Guderley H and St-Pierre J (2002): Review. Going with the flow or life in the fast lane: contrasting mitochondrial responses to thermal change. *The Journal of Experimental Biology* 205:2237-2249.

Gutiérrez AM, Reboredo GR and Catalá A (2002): Fatty acid profiles and lipid peroxidation of microsomes and mitochondria from liver, heart and brain of *Cairina moschata*. *The international Journal of Biochemistry & Cell Biology* 34:605-612.

Hulbert AJ and Else PL (1999): Membranes as possible pacemakers of metabolism *J Theor Biol* 199, 257–274.

Hulbert AJ (2007): Membrane fatty acids as pacemakers of animal metabolism. *Lipids* 42:811-819.

Infante JP, Kirwan RC and Brenna JT (2001): High levels of docosahexaenoic acid (22:6 n-3)-containing phospholipids in highfrequency contraction muscles of hummingsbirds and rattlesnakes. *Comp Biochem Physiol* 130B:291-298.

Je žová J, Nováková O, Kolá ř F, Tvrzická E, Necká ř J and Novák F (2002): Chronic hypoxia alters fatty acid composition of phospholipids in right and left ventricular myocardium. *Molecular and Cellular Biochemistry* 232:49-56.

Kaiser, G. (2009): The effect of altitude on fatty acid composition in heart muscles of Mountain hares (*Lepus timidus varronis*). *Master-Thesis, University of Natural Resources and Applied Life Sciences, Vienna.*

Kinnula VL, Huttunen P and Hirvonen J (1983): Adaptive changes in skeletal muscle mitochondria of the guinea-pig during acclimation to cold. *Eur J Appl Physiol* 51:237-245.

Kuntz R, Kubalek C, Ruf T, Tataruch F and Arnold W (2006): Seasonal adjustment of energy budget in a large wild mammal, the Przewalski horse (*Equus ferus przewalskii*). *Journal of Experimental Biology* 209:4566-4573.

Kwong LK and Sohal RS (2000): Age related changes in activities of mitochondrial electron transport complexes in various tissues of the mouse. *Arch Biochem Biophys* 373:16-22.

Leary SC, Lyons CN, Rosenberger AG, Ballantyne JS, Stillman J and Moyes CD (2003): Fiber-type differences in muscle mitochondrial profiles. *Am J Physiol Regul Integr Comp Physiol* 285:R817-R826.

Lynn EG, Lu Z, Minerbi D and Sack MN (2007): The regulation, control, and consequences of mitochondrial oxygen utilization and disposition in the heart and skeletal muscle during hypoxia. *Antioxidants & Redox Signaling* 9(9):1353-1362.

Munro D and Thomas DW (2004): The role of polyunsaturated fatty acids in the expression of torpor by mammals: a review. *Zoology* 107:29-48.

Mühlenhoff U and Lill R (2001): Mitochondria: Methods for Preparation. *Encyclopedia of Life Sience*. Article 2601, pp. 1-8. Macmillan Reference Ltd., London.

Nieminen P and Mustonen AM (2008): A preliminary study on the seasonal body temperature rhythms of the captive mountain hare (*Lepus timidus*). *Cryobiology* 56:163-167.

Nichols D and Locke RM (1984): Thermogenic mechanisms in brown fat. *Physiol Rev* 64: 1–64, 1984.

Nodari M (2006): Ecological role of mountain hare (*Lepus timidus*) in the alpine ecosystems. *PhD Thesis, The University of Insubria.*

Nouette-Gaulain K, Malgat M, Rocher C, Savineau JP, Marthan R, Mazat JP, and Sztark F (2005): Time course of differential mitochondrial energy metabolism adaptation to chronic hypoxia in right and left ventricles. *Cardiovasc Res* 66:132–140.

Ocloo A, Shabalina IG, Nedergaard J and Brand M (2007): Cold-induced alterations of phospholipid fatty acyl composition in brown adipose tissue mitochondria are independent of uncoupling protein-1. *Am J Physiol Integr Comp Physiol* 293:R1086-R1093.

Ogawa K, Ohno T and Kuroshima A (1987): Muscle and Brown Adipose Tissue Fatty Acid Profiles in Cold-Exposed Rats. *Japanese Journal of Physiology* 37:783-796.

Papa S and Skulachev VP (1997): Reactive oxygen species, mitochondria, apoptosis and aging. *Mol Cell Biochem* 174:305–319.

Pepe S, Tsuchiya N, Lakatta EG and Hansford RG (1999): PUFA and aging modulate cardiac mitochondrial membrane lipid composition and Ca2+ activation of PDH. *Am. J. Physiol.* 276:H149–H158.

Pepe S (2005): Effect of dietary polyunsaturated fatty acids on age-related changes in cardiac mitochondrial membranes. *Experimental Gerontology* 40:751-758.

Porter RK, Hulbert AJ and Brand MD (1996): Allometry of mitochondrial proton leak: influence of membrane surface area and fatty acid composition. *Am J Physiol* 271, R1550–R1556.

Pyörnilä A, Putaala A, Hissa R and Sulkava S (1992): Adaptations to environment in the mountain hare (*Lepus timidus*): thermal physiology and histochemical properties of locomotry muscles. *Can J Zool* 70:1325-1330.

Rolfe DFS and Brown GC (1997): Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol Rev* 77:731-758.

Ruf T, Valencak T, Tataruch F, Arnold W (2006): Running speed in mammals increases with muscle n-6 polyunsaturated fatty acid content. PLoS ONE 1(1): e65.

Ruf T and Arnold W (2008): Effects of polyunsaturated fatty acids on hibernation and torpor: a review and hypothesis. *Am J Physiol Regul Integr Comp Physiol* 294:R1044-R1052.

Sänger, AM (1993). Limits to the acclimation of fish muscle. *Rev. Fish Biol. Fish.* 3:1–15.

Soveri T and Aarnio M (1983): Measuring the body condition of hares during winter. *Finn. Game Res.* 412:21-28.

Sprecher H., Luthria DL, Mohammed BS and Baykousheva SP (1995): Re-evaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *J Lipid Res* 36:2471–2477.

Staples JF and Brown JCL (2008): Mitochondrial metabolism in hibernation and daily torpor: a review. *J Comp Physiol B* 178:811-827.

St-Pierre J and Boutilier R. G (2001): Aerobic capacity of frog skeletal muscle during hibernation. *Physiol Biochem Zool* 74, 390–397.

Suchentrunk F, Willing R and Hartl GB (1991): On eye lens weights and other age criteria of the Brown hare (*Lepus europaeus* Pallas, 1778). *Zeitschrift für Säugetierkunde* 56:365-374.

Valencak TG, Arnold W, Tataruch F and Ruf T (2003): High content of polyunsaturated fatty acids in muscle phospholipids of a fast runner, the European brown hare (*Lepus europaeus*). *J Comp Physiol B Biochem Syst Environ Physiol* 173:695-702.

Vázquez-Memije ME, Cárdenas-Méndez MJ, Tolosa A and Hafidi ME (2005): Respiratory chain complexes and membrane fatty acid composition in rat testis mitochondria throughout development and aging. *Experimental Gerontology* 40:482-490.

Wodtke E (1981a): Temperature adaptation of biological membranes. The effects of acclimation temperature on the unsaturation of the main neutral and charged phospholipids in mitochondrial membranes of the carp (*Cyprinus carpio*). *Biochim Biophys Acta* 640, 698–709.

Wodtke E (1981b): Temperature adaptation of biological membranes. Compensation of the molar activity of cytochrome C oxidase in the mitochondrial energy-transducing membrane during thermal acclimation of the carp (*Cyprinus carpio* L.). *Biochim Biophys Acta* 640, 710–720.

Zabelinskii SA, Chebotareva MA , Kostkin VB and Krivchenko AI (1999): Phospholipids and their fatty acids in mitochondria, synaptosomes and myelin from the liver and brain of trout and rat: a new view on the role of fatty acids in membranes. *Comparative Biochemistry and Physiology Part B* 124:187-193.

Zackova M, Skobtsova E, Urbankova E and Jezek P (2003): Activating ω-6 PUFAs and inhibitory purine nucleotides are high affinity ligands for novel mitochondrial uncoupling proteins UCP2 and UCP3. *J Biol Chem* 278:20761-20769.

Apendix

Table 1: The effect of altitude on mitochondrial enzyme activity of complex II and IV, given in μmol/(min·mg), and mitochondrial yield

Sample number	Altitude	Weight	Age	Activity Compl IV	Activity complex II	Mitochondrial Yield
321/04	2700,000	2500,000	1,000	0,065	0,343	0,047
911/05	2740,000	2100,000	1,000	0,083	0,811	0,029
915/05	2780,000	.	1,000	0,057	0,720	0,034
931/04	2750,000	2215,000	0,000	0,066	0,188	0,044
931/05	2740,000	2080,000	0,000	0,082	0,200	0,033
932/04	2700,000	2615,000	1,000	0,061	0,271	0,034
941/04	2800,000	2600,000	0,000	0,063	0,517	0,039
942/04	2800,000	2500,000	0,000	0,070	0,575	0,053
942/05	2700,000	2280,000	.	0,095	0,434	0,032
1021/05	2800,000	2800,000	1,000	0,069	0,185	0,027
151/05	1580,000	2903,000	1,000	0,054	0,329	0,049
342/04	1530,000	2700,000	0,000	0,044	0,225	0,049
511/04	1760,000	2800,000	1,000	0,043	0,187	0,048
611/05	1510,000	3250,000	.	0,058	0,254	0,035
612/04	1590,000	2450,000	1,000	0,055	0,119	0,027
811/05	1670,000	2810,000	1,000	0,048	0,447	0,035
1025/04	1180,000	2420,000	1,000	0,041	0,233	0,036
1026/04	1695,000	2520,000	1,000	0,073	0,311	0,053
1211/05	1650,000	2800,000	0,000	0,080	0,398	0,032
1221/05	1700,000	2650,000	1,000	0,069	0,230	0,038
MV(high)				0,071	0,424	0,037
SD(high)				0,012	0,227	0,009
MV(low)				0,057	0,273	0,040
SD(low)				0,013	0,099	0,009

Table 2: The effect of altitude on fatty acid composition (given in w/w%)

	Fatty Acids									
Sample number	C 14-0	C 15-0	C 16-0	C 16-1	C 17-0	C 18-0	C 18-1	C 18-2	C 18-3	C 20-4
321/04	0,462%	0,178%	11,336%	0,819%	0,907%	27,384%	6,630%	35,079%	3,662%	10,055%
911/05	0,261%	0,171%	8,380%	0,394%	0,917%	23,392%	5,664%	42,733%	4,557%	10,195%
915/05	0,326%	0,217%	8,105%	0,474%	1,046%	23,568%	5,443%	43,155%	5,557%	9,168%
931/04	0,732%	0,288%	14,760%	0,894%	0,870%	24,598%	9,304%	30,732%	4,760%	9,660%
931/05	0,266%	0,159%	8,367%	0,426%	0,740%	20,586%	3,758%	42,138%	4,335%	13,579%
932/04	0,504%	0,242%	11,444%	0,609%	0,959%	23,086%	6,400%	42,037%	3,674%	8,510%
941/04	1,251%	0,198%	10,547%	0,301%	0,795%	28,336%	5,421%	34,759%	2,966%	11,318%
942/04	0,323%	0,245%	9,423%	0,499%	0,996%	24,112%	4,879%	43,980%	5,336%	7,385%
942/05	0,480%	0,145%	8,632%	0,364%	0,704%	23,198%	3,996%	41,250%	4,143%	12,742%
1021/05	0,394%	0,270%	10,167%	0,295%	1,030%	24,095%	4,950%	41,311%	3,578%	10,699%
151/05	0,422%	0,238%	9,108%	0,445%	0,742%	22,718%	2,309%	39,502%	8,368%	10,858%
342/04	0,604%	0,235%	9,976%	0,450%	1,047%	24,221%	5,354%	35,695%	6,754%	11,695%
511/04	0,558%	0,258%	12,042%	0,537%	0,954%	24,228%	7,789%	31,295%	7,373%	10,746%
611/05	0,423%	0,270%	10,281%	0,487%	1,067%	24,903%	2,845%	36,234%	8,163%	10,061%
612/04	1,342%	0,296%	11,598%	0,464%	1,042%	27,740%	6,087%	34,441%	5,289%	8,412%
811/05	0,431%	0,335%	10,897%	0,354%	1,081%	23,332%	5,935%	35,253%	8,137%	9,411%
1025/04	0,253%	0,247%	9,019%	0,442%	1,244%	27,883%	0,772%	42,763%	5,616%	8,469%
1026/04	0,496%	0,334%	10,696%	0,598%	1,096%	26,729%	1,222%	36,132%	8,597%	9,241%
1211/05	0,302%	0,233%	10,699%	0,394%	0,840%	20,871%	7,881%	32,346%	9,911%	10,174%
1221/05	0,394%	0,235%	9,437%	0,391%	1,047%	22,563%	4,800%	37,785%	4,939%	13,936%
MV	0,511%	0,240%	10,246%	0,482%	0,956%	24,377%	5,072%	37,931%	5,786%	10,316%
MV(high)	0,500%	0,211%	10,116%	0,507%	0,897%	24,236%	5,644%	39,717%	4,257%	10,331%
SD(high)	0,299%	0,049%	2,050%	0,207%	0,119%	2,205%	1,576%	4,498%	0,819%	1,867%
MV(low)	0,522%	0,268%	10,375%	0,456%	1,016%	24,519%	4,499%	36,145%	7,315%	10,300%
SD(low)	0,307%	0,040%	1,014%	0,072%	0,141%	2,326%	2,578%	3,327%	1,628%	1,653%

	Fatty Acids							
Sample number	C 20-5	C 22-5	C 22-6	SFA	MUFA	PUFA	n6/n3	UI
321/04	0,700%	2,446%	0,342%	40,268%	7,449%	52,283%	6,313	0,147
911/05	0,648%	2,339%	0,348%	33,122%	6,058%	60,820%	6,706	0,163
915/05	0,598%	2,073%	0,270%	33,262%	5,917%	60,821%	6,157	0,161
931/04	0,856%	2,097%	0,450%	41,248%	10,198%	48,554%	4,949	0,142
931/05	1,109%	3,910%	0,630%	30,117%	4,183%	65,700%	5,581	0,185
932/04	0,468%	1,766%	0,301%	36,236%	7,009%	56,756%	8,142	0,149
941/04	0,726%	2,900%	0,481%	41,128%	5,722%	53,150%	6,515	0,150
942/04	0,529%	2,004%	0,287%	35,099%	5,379%	59,522%	6,297	0,153
942/05	0,779%	3,147%	0,419%	33,160%	4,360%	62,481%	6,361	0,172
1021/05	0,580%	2,281%	0,350%	35,956%	5,245%	58,799%	7,660	0,158
151/05	1,341%	3,412%	0,536%	33,229%	2,754%	64,017%	3,688	0,177
342/04	1,177%	2,325%	0,467%	36,083%	5,803%	58,114%	4,419	0,165
511/04	1,148%	2,564%	0,509%	38,039%	8,326%	53,636%	3,626	0,158
611/05	1,595%	3,133%	0,538%	36,943%	3,332%	59,725%	3,447	0,167
612/04	0,734%	2,151%	0,405%	42,019%	6,550%	51,431%	4,995	0,142
811/05	1,642%	2,737%	0,455%	36,076%	6,289%	57,635%	3,443	0,163
1025/04	0,763%	2,197%	0,333%	38,646%	1,214%	60,140%	5,751	0,154
1026/04	1,247%	3,022%	0,591%	39,350%	1,820%	58,829%	3,372	0,162
1211/05	2,140%	3,556%	0,652%	32,946%	8,275%	58,779%	2,615	0,176
1221/05	1,072%	2,868%	0,534%	33,676%	5,191%	61,133%	5,495	0,174
MW	0,993%	2,646%	0,445%	36,330%	5,554%	58,116%	5,277	0,161
MV(high)	0,699%	2,496%	0,388%	35,960%	6,152%	57,889%	6,468	0,158
SD(high)	0,185%	0,647%	0,110%	3,821%	1,749%	5,218%	0,915	0,013
MV(low)	1,286%	2,796%	0,502%	36,701%	4,955%	58,344%	4,085	0,164
SD(low)	0,423%	0,492%	0,092%	2,925%	2,556%	3,591%	1,031	0,011

Table 3: Results of the ANCOVA: effect of altitude, age and weight on mitochondrial enzyme activity, mitochondrial yield and fatty acid composition of mitochondrial PL

	altitude		age		weight	
	p	$F_{1,13}$	p	$F_{1,13}$	p	$F_{1,13}$
complex IV	0,212	1,721	0,663	0,199	0,353	0,928
complex II	0,346	0,957	0,992	0,001	0,924	0,009
Mito yield	0,705	0,149	0,367	0,874	0,544	0,388
C14:0	0,906	0,014	0,633	0,239	0,917	0,011
C15:0	0,184	1,971	0,635	0,236	0,581	0,321
C16:0	0,751	0,105	0,779	0,082	0,653	0,012
C16:1	0,689	0,167	0,796	0,07	0,41	0,726
C17:0	0,181	1,997	0,165	2,166	0,529	0,418
C18:0	0,983	0,001	0,396	0,769	0,655	0,21
C18:1	0,31	1,114	0,369	0,867	0,444	0,625
C18:2	0,24	1,514	0,265	1,359	0,598	0,292
C18:3	0,002	16,028	0,211	1,731	0,513	0,452
C20:4	0,747	0,108	0,508	0,462	0,974	0,001
C20:5	0,008	9,783	0,053	4,518	0,439	0,636
C22:5	0,254	1,422	0,24	1,52	0,947	0,005
C22:6	0,032	5,786	0,065	4,07	0,786	0,077
SFA	0,955	0,003	0,644	0,223	0,92	0,01
MUFA	0,312	1,107	0,393	0,781	0,494	0,495
PUFA	0,594	0,298	0,951	0,004	0,801	0,066
n6/n3	0,001	24,307	0,047	4,813	0,849	0,38
UI	0,247	1,467	0,417	0,703	0,973	0,001

Table 4: Results of the ANCOVA: effects of mitochondrial FA composition on mitochondrial enzyme activity

	Complex IV		Complex II	
	p	$F_{1,12}$	p	$F_{1,12}$
C14:0	0,3	1,174	0,453	0,602
C15:0	0,64	0,23	0,443	0,628
C16:0	0,348	0,952	0,132	2,607
C16:1	0,424	0,685	0,209	1,763
C17:0	0,236	1,558	0,845	0,04
C18:0	0,076	3,783	0,819	0,055
C18:1	0,893	0,019	0,752	0,105
C18:2	0,839	0,043	0,448	0,615
C18:3	0,101	3,164	0,133	2,596
C20:4	0,342	0,979	0,505	0,472
C20:5	0,025	6,581	0,372	0,861
C22:5	0,025	6,599	0,767	0,092
C22:6	0,016	7,879	0,713	0,142
SFA	0,073	3,864	0,366	0,884
MUFA	0,852	0,036	0,696	0,161
PUFA	0,154	2,321	0,38	0,831
n6/n3	0,262	1,384	0,492	0,502
UI	0,032	5,847	0,515	0,449