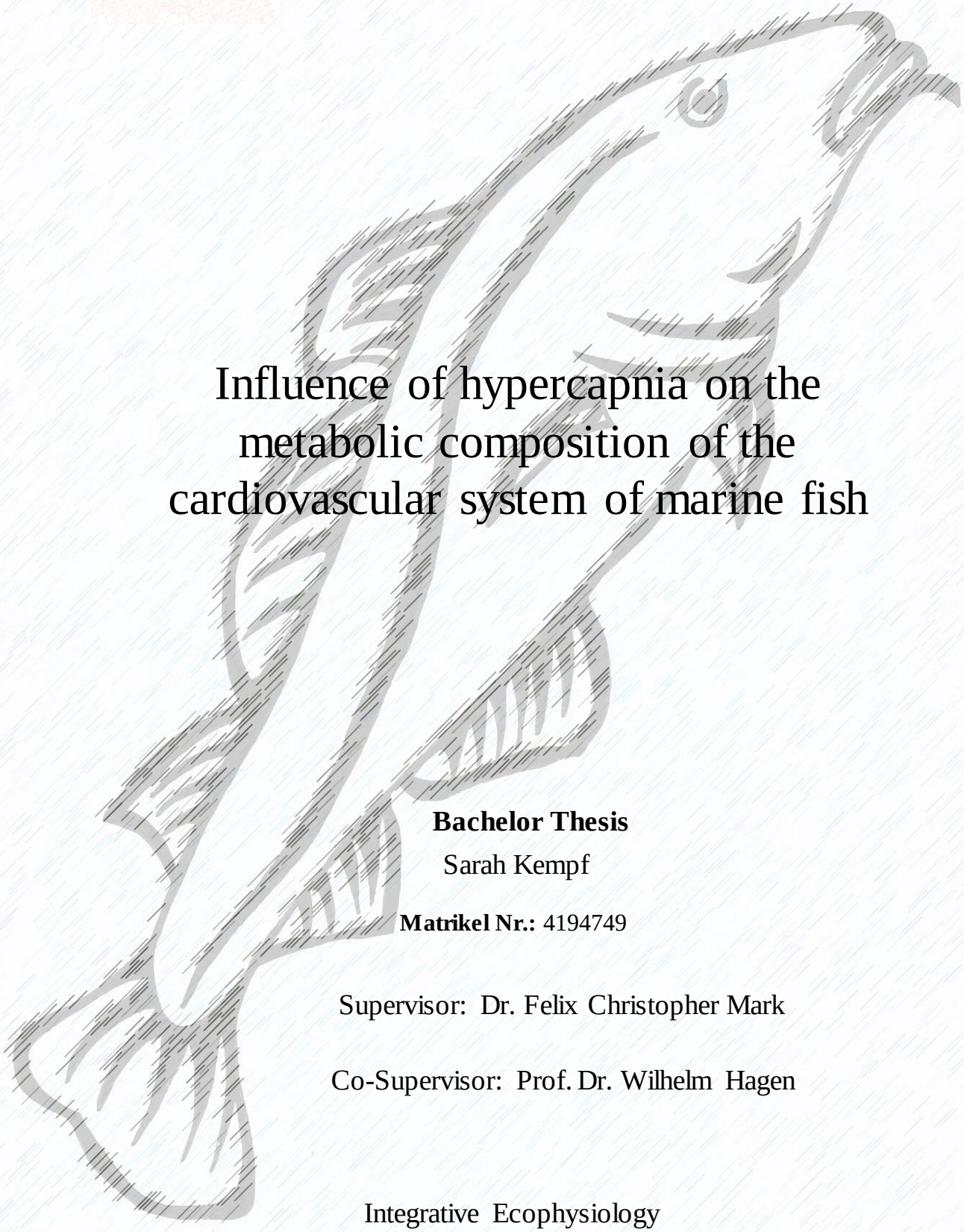

Influence of hypercapnia on the metabolic composition of the cardiovascular system of marine fish

Bachelor Thesis

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Integrative Ecophysiology

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Content

Declaration of authorship

I hereby confirm that I have independently composed this Bachelor thesis, and no other than the indicated aid and sources have been used. This work has not been presented to any other examination board. No data can be taken out of this work without prior approval of the thesis promotor.

Sarah Kempf
Bremen, 29th of June, 2017

List of abbreviations

°C	Degree Celsius
µL	Microliter
μatm	Micoatmosphere
Acetyl-CoA	Acetyl coenzyme A
ADP	Adenosinediphosphate
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosinetriphosphate
Cl^-	Chloride ions
CO_2	Carbon dioxide
CO_2(aq)	Carbon dioxide dissolved in water
CO_2(g)	Gaseous carbon dioxide
CO_3^{2-}	Carbonate ions
CoA-SH	Coenzyme A
CS	Citrate synthase
Cys	Cystein
D_2O	Deuterium oxide
DIC	Dissolved inorganic carbon
FAD^+	Flavin-Adenine-dinucleotide
FADH	Flavin-Adenine-dinucleotide (reduced form)
Fig.	Figure
fructose-1,6-P2	Fructose-1,6-bisphosphate
fructose-6-P	Fructose-6-phosphate
GaMme	Glutaric acid monomethyl ester
GDH	Glutamate dehydrogenase
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
H^+	Hydrogen ions
H_2CO_3	Carbonic acid

H_2O	Water
HCO_3^-	Bicarbonate ions
His	Histidine
IDH	Isocitrate dehydrogenase
Ile	Isoleucine
LC	Lethal concentration
Leu	Leucine
-log	Negativ logarithm
Lys	Lysine
MHz	Megahertz
Met	Methionine
Min	Minute
mM	Milimole per litre
mmol	Milimole
mmol/kgSW	Milimole per kilogram Seawater
n	Sample size
Na^+	Sodium ions
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
nmol	Nanomole
NMR	Nuclear magnetic resonance
-P	Phosphate
PCO2	Carbon dioxide partial pressure
PDG	Phosphatedependent glutaminase
PDH	Pyruvate dehydrogenase
PEPCK	Phosphoenolpyruvat-Carboxykinase
pH	Potential of hydrogen
pHe	Extracellular pH
pHi	Intracellular pH
Phe	Phenylalaine
Pi	Inorganic phosphate
PK	Pyruvate kinase
ppm	Parts per milion
Pro	Proline

Rcf	Relative centrifugal force
Rpm	Rounds per minute
S	Second
Ser	Serine
Thr	Threonine
Trp	Tryptophane
Tyr	Tyrosine
Val	Valine
α –KGDH	α -ketoglutarate dehydrogenase
δ	chemical shift
µatm	micro atmosphere

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Summary

Marine organisms and entire ecosystems are influenced by increasing temperatures and increasing CO_2 partial pressure (hypercapnia). The experimental organism of this thesis, the marine teleost *Gadus morhua*, inhabits regions that are supposed to experience some of the largest climatic changes on the globe. The aim of this study was to investigate the effects of ocean acidification and increasing temperature on the physiological mechanisms in the heart of *Gadus morhua* in order to draw conclusions for the whole organism. The fish were divided into two groups, one was incubated under ambient pCO_2 (390 μatm) and the other group under future pCO_2 levels (1170 μatm; scenario after IPCC: RCP 8.5). Both groups were split into four different temperature levels (3, 8, 12 and 16 °C) with 12 animals in each treatment. The main focus was on the metabolic products of glycolysis, citric acid cycle, lactic acid cycle, amino acid metabolism and amino acid derivatives.

Results show that environmental hypercapnia led to a significant decrease of glucose-6-phosphate (glycolysis), on amino acids and their derivatives alanine, glutamine,isoleucine, creatine phosphate, glucarate and taurine. The elevation of temperature led to a significant increase of creatine (amino acid derivatives) and lactate in the treatment groups with 390 μatm CO_2. Hypercapnic accumulation did not significantly influence the metabolites of the citric acid cycle. Furthermore, similar ATP concentrations through all treatments indicated that *Gadus morhua* is able to cope with environmental changes and to maintain its supply of energy.

Zusammenfassung

Marine Organismen und komplette Ökosysteme werden durch steigenden CO_2 Partialdruck (Hyperkapnie) und damit korrelierte, steigende Temperaturen beeinflusst. In dieser Studie wurde der Knochenfisch *Gadus morhua* untersucht. Es wird erwartet, dass die Regionen die er besiedelt in Zukunft starke klimatische Veränderungen durchlaufen werden.

Das Ziel dieser Studie war es die Effekte von Ozeanversauerung und steigenden Temperaturen auf die physiologischen Mechanismen im Herzgewebe von *Gadus morhua* zu untersuchen. Dies gibt einen ersten Einblick in den Einfluss auf den ganzen Organismus. Die Fische wurden in zwei Gruppen eingeteilt: eine wurde unter aktuellem pCO_2 (390 μatm) inkubiert, die andere unter dem prognostizierten pCO_2 von 1170 μatm (Szenario nach IPCC: RCP 8.5). Beide Gruppen wurden jeweils in vier unterschiedliche Temperatur-Gruppen (3, 8, 12 und 16 °C), mit jeweils 12 Tieren pro Temperatur, eingeteilt. Bei der Untersuchung des Einflusses auf physiologische Mechanismen wurde spezielles Augenmerk auf die Stoffwechselproduckte von Glykolyse, Citrat Zyklus, Milchsäuregärung sowie auf den Aminosäure Metabolismus und die zugehörigen Derivate gelegt.

Es konnte gezeigt werden, dass Hyperkapnie eine signifikante Abnahme von Glucose-6-Phosphat (Glykolyse), Aminosäuren und deren Derivate Alanin, Glutamin, Isoleucin, Kreatinphosphat, Glucarat und Taurin bewirkt. Steigende Temperaturen führten zu einer signifikanten Zunahme von Kreatin und Laktat in den Inkubationen unter 390 μatm CO_2. Die Metabolite des Citrat Zyklus wurden durch die erhöhte CO_2 Konzentration nicht signifikant beeinflusst. Des Weitern konnte durch ähnliche ATP Konzentration über alle Inkubationen hinweg gezeigt werden, dass *Gadus morhua* in der Lage ist mit den untersuchten ökologischen Veränderungen umzugehen und seine Energieversorgung aufrecht zu erhalten.

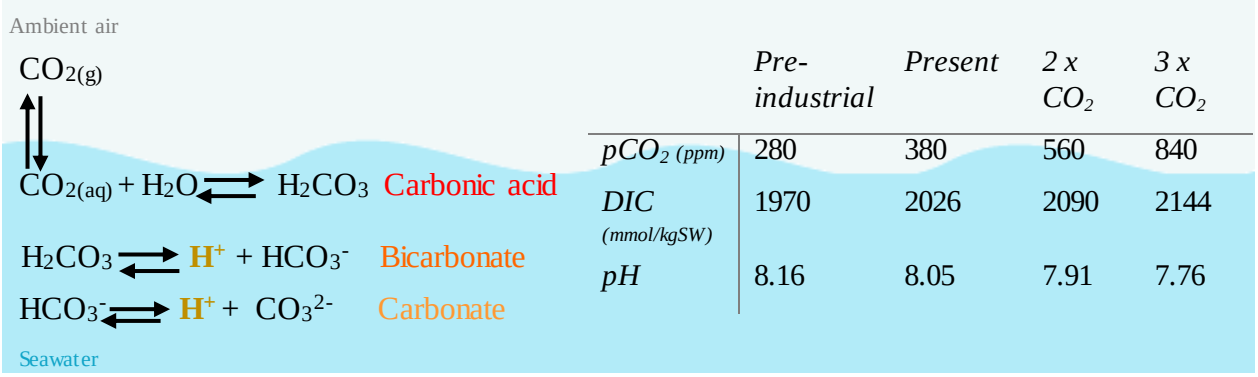
1. Introduction

1.1. Ocean acidification and CO_2 pressure

Over the past 250 years, atmospheric carbon dioxide (CO_2) levels increased by nearly 40%, from preindustrial levels of approximately 280 ppm (parts per million) to 380 ppm today (Fig. 1). This increase is mostly driven by human fossil fuel combustion, deforestation and industrialization (Doney and Schimel, 2007).

During the next hundred years, the physical conditions in the world's oceans will change dramatically. The carbon dioxide (CO_2) pressure levels may rise up to 1170 μatm, that leads to increased radiative forcing, due to this global water surface temperatures are expected to increase by 2–3 °C as well as the ocean surface pH will decrease 0.3–0.5 units by the year 2100 (Caldeira, 2005; Houghton, 1996; Meinshausen et al., 2011; Pörtner et al., 2014).

The oceans act as buffers of the atmospheric CO_2 content, due to the good solubility of CO_2 in the seawater. About 30-50% of the anthropogenic CO_2 released into the atmosphere is taken up by the oceans (Sabine et al., 2004). This leads to a drop of pH since carbonic acid (H_2CO_3) is formed which dissociates to bicarbonate ions (HCO_3^-) and further to carbonate ions (CO_3^{2-}) (Fig. 1). During the dissociation process, hydrogen ions (H^+) are released, which cause the decrease in pH (pH = -log [H^+]) (Fabry et al., 2008). The process of rising CO_2 level and consequent dropping pH is called ocean acidification (OA).



	Pre-industrial	*Present*	*2 x CO_2*	*3 x CO_2*
pCO_2 (ppm)	280	380	560	840
DIC (mmol/kgSW)	1970	2026	2090	2144
pH	8.16	8.05	7.91	7.76

Figure 1: CO_2 solution in seawater
Left: dissociation processes of CO_2 in seawater. Right: pH values, pCO_2 concentrations (ppm) and DIC concentrations (mmol/kgSW) at pre-industrial and current conditions as well as two times and three times pre-industrial CO_2 levels. pH is based on the seawater scale (modified after Fabry, Seibel et al. 2008).

Fish are able to regulate their acid-base household by accumulating HCO_3^- in body fluids to compensate for CO_2 induced acid-base disturbance (Hu et al., 2016; Toews et al., 1983). Therefore, it was assumed that fish are very tolerant towards OA, but further studies made clear that climate variability affects the population dynamics of fish stocks, such as cod.

It has been found that growth, distribution, condition, maturity as well as physical processes and properties of cod can be linked to atmospheric and ocean climate indices (Drinkwater, 2009; Hu et al., 2016; Ottersen et al., 2006; Peck et al., 2006).

In order to investigate the effects of climate change and OA on the Atlantic cod, changes in the metabolic composition in heart tissue, under various temperatures and CO_2 conditions, were examined in this study. Therefore the samples were analysed by NMR spectroscopy. Special attention was paid on the metabolites of glycolysis, citric acid cycle, lactic acid.

1.2. pH regulation in fish during elevated PCO_2

The regulation of the intracellular pH (pH_i) involves Cl^-/HCO_3^- exchange or acid extrusion via Na^+/H^+ exchange (Glass and Wood, 2009; Heuer and Grosell, 2014) in most tissues and in the red blood cells also catecholamine activated Na^+/H^+ exchange (Parks et al., 2010; Roos and Boron, 1981). When the fish tries to regulate its intracellular pH during exposure to moderately elevated pCO_2 a pH_i overshoot associated with the compensation of the extracellular pH (pH_e) can occur (Heuer and Grosell, 2014). Such an overshoot has been reported for red blood cells, brain, liver and heart cells of the temperate white sturgeon exposed to 1500 μatm for 48 hours (Baker et al., 2009). The acid-base regulatory capacity of fish is generally attributed to the simultaneous transport pathways for H^+ and HCO_3^- via the basolateral and apical membranes of the gill epithelium. They although have the ability to regulate their pH_i at protein level by phosphorylation and protein decomposition (Perry and Gilmour, 2006).

1.3. Study organism

The Atlantic cod (*Gadus morhua*, Figure 2) is a benthopelagic fish of the family *Gadidae*.

http://especiesmarinasdeinteresbromatologic.blogspot.de/2011/05/gadus-morhua-bacalao-torsk-cod.html

Figure 2: *Gadus morhua*, Atlantic cod (Linneaus, 1758)

It has a pan-Atlantic distribution and inhabits regions with temperature ranges from below -1 °C up to 20 °C, but it is usually found in regions with a temperature range of 0 -12 °C. Therefore the Atlantic cod is supposed to be a thermal tolerant species. The regions inhabited by cod (Fig. 3) are supposed to experience some of the largest climatic changes on the globe (Drinkwater, 2005). It is also used commercially, with regard to that it is one of the most important species in the North Atlantic, despite substantial declines which have occurred in several regions during recent decades (O'Brien et al., 2000).

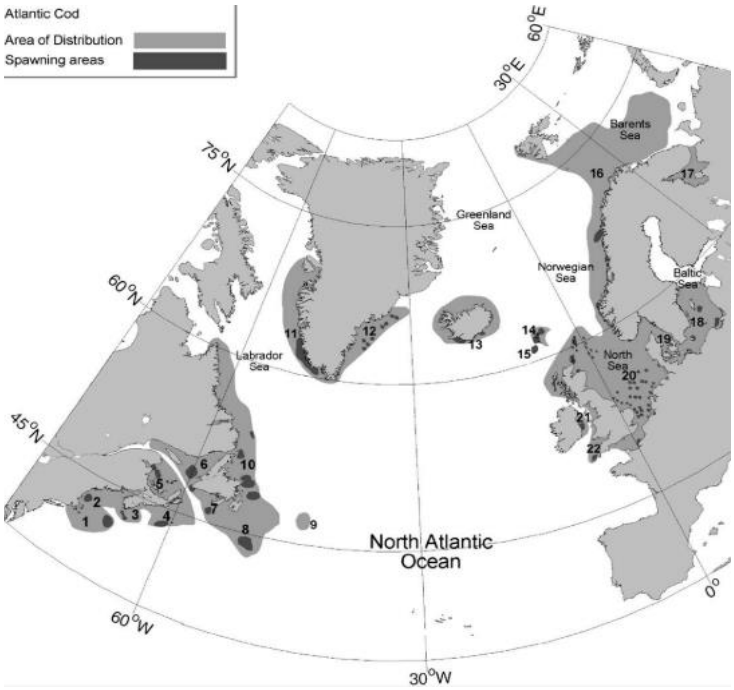


Figure 3: Distribution of Atlantic cod
The area of distribution (grey) and the spawning area (dark grey) of Atlantic cod is shown (Drinkwater, 2005).

1.4. Metabolic pathways in the cardiovascular system

1.3.1. Glycolysis

During this metabolic pathway one molecule glucose ($C_6H_{12}O_6$) is converted into two pyruvate (CH_3COCOO^-) molecules in the cytosol (Figure 3).

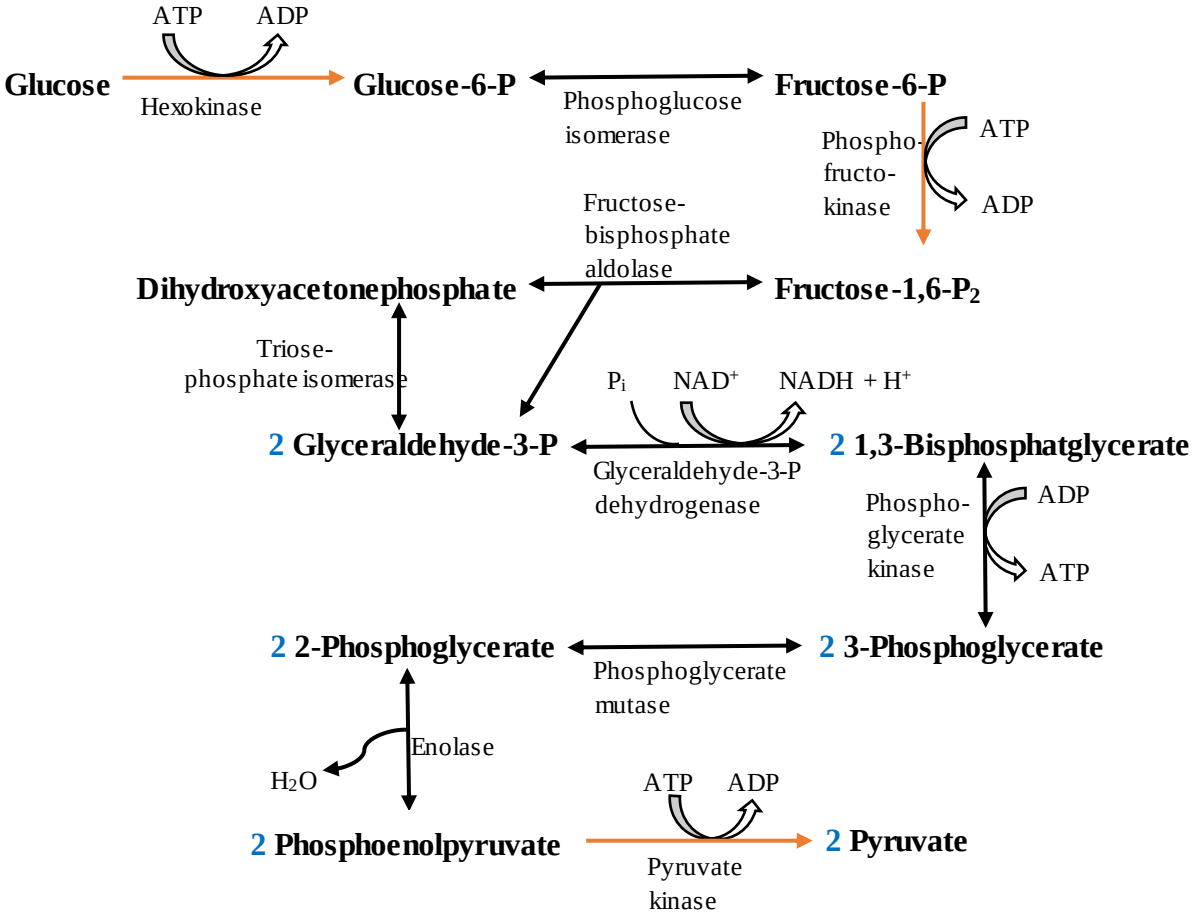


Figure 4: Schematic depiction of the glycolysis. The orange arrows symbolize the irreversible reactions of the pathway (modified after Relman, 1972).

The glycolysis is one of the metabolic pathways that can be affected by changes in intracellular pH (Relman, 1972). It is assumed that the sensitivity of the phosphofructokinase (PEK) reaction is the reason for the pH sensitivity of the glycolysis. During its phosphorylation of fructose-6-P to fructose-1,6-P_2 ATP is converted to ADP (Figure 3) The ATP consumption makes the reaction irreversible and it is one of the reactions that pushes substrate to the synthesis of pyruvate (Relman, 1972). Several experiments permit the conclusion that the PFK reaction in the glycolysis is lowered in alkaline medium (Halperin et al., 1969; Ui, 1966).For example in an experiment by Ui (1966) proved that the rate of inhibition of phosphofructokinase activity by excess ATP was dependent upon the pH. At pH 7.3 an increase in ATP concentration

resulted in a sudden inhibition of PFK activity, whereas at pH 7.6 such an effect was not observed. Consequently PFK activity was profoundly influenced by a change in pH when larger amounts of ATP were present in the glycolytic system. Furthermore the glucose-6-phosphate which accumulated during PFK inhibition was inhibitory to hexokinase the pH-induced stimulation of PFK caused an exaggerated acceleration of total glycolysis.

1.3.2. Citric acid cycle

The citric acid cycle (Figure 5) is responsible for the aerobic processing of glucose derivatives with the complete oxidation of the glucose derivatives to CO_2 and generates the reduction equivalents needed to generate ATP in the respiratory electron transport system (Vohwinkel et al., 2011). It takes place in the mitochondria (Krebs, 1953). Furthermore, the citric acid cycle is an important pathway to provide intermediates for the synthesis of amino acids (Vohwinkel et al., 2011). Under aerobic conditions, the product of the glycolysis, pyruvate, is oxidatively decarboxylated by the pyruvate dehydrogenase and its acetyl residue is transferred to CoA. After that the acetyl residue is transferred to oxaloacetate and the cycle is able to start (Krebs, 1953).

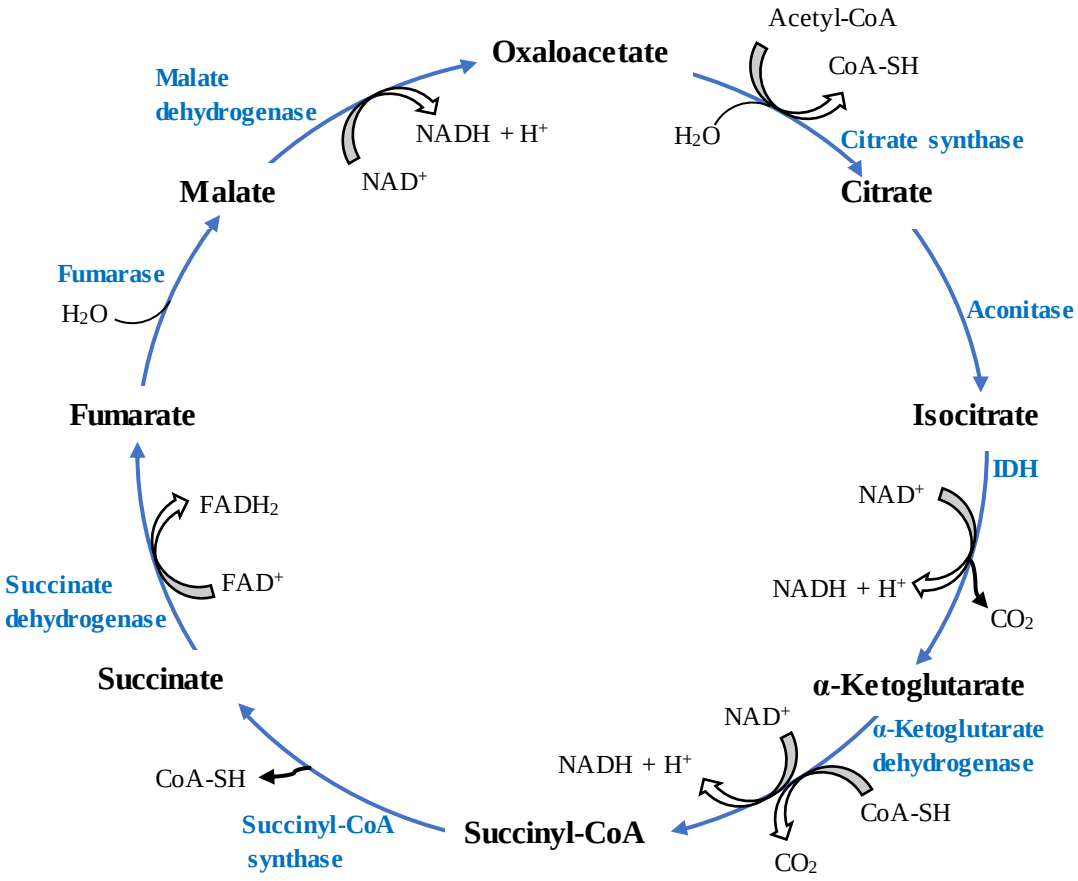


Figure 5: Schematic depiction of the citric cycle. The blue arrows and the blue labeling represent the involved enzymes (modified after Krebs, 1953).

It was found that the isocitrate dehydrogenase (IDH) is a key enzyme of the citric acid cycle, since it catalyzes the conversion of isocitrate to α-ketoglutarate and thereby generating one molecule of CO_2 and a reduction equivalent in the process (Vohwinkel et al., 2011). It has been confirmed that there is a connection between cell growth and rising CO_2-level. The studies by Vohwinkel et al. (2011) showed evidence that high CO_2 decreases cell proliferation (independently of pH and hypoxia) by causing mitochondrial dysfunction. This is due to a release of the gene regulatory sequence, miR-183, which decreases IDH-levels what leads to mitochondrial dysfunction and decreasing cell proliferation (Vohwinkel et al., 2011).

1.3.3. Lactic acid fermentation

Under anaerobic conditions, or if pyruvate is building up faster (by glycolysis) than it can be metabolized (this occurs when the glycolysis is increased by physiological stress and the increased production of pyruvate overloads the capacity of the aerobic energy supply), lactic acid fermentation takes place. It is used to provide new cellular energy. The lactate dehydrogenase is catalyzing the interconversion of pyruvate and lactate with a simultaneous conversion of NADH and NAD^+ (Reddy et al., 2008)(Figure 5).

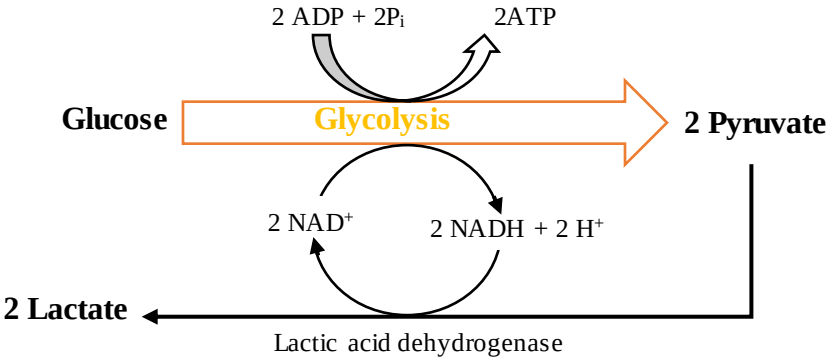


Figure 6: Schematic depiction of the lactic acid fermentation. The orange arrow symbolizes the whole glycolysis, after which the product pyruvate is transformed to lactate by lactic acid dehydrogenase. During this reaction, NAD is regenerated (modified after Reddy, Altaf, Naveena, Venkateshwar, & Kumar, 2008)

The physiological mechanisms, which are affected by OA, lead to a shift of acid-base status, including a shift of pH_e. This shift of pH_e likely reduces the functional capacity of affected mechanisms and of the whole organism in due course. As a result, pO2 levels in the body fluids drop and reach limiting levels earlier than during normocapnia (Pörtner, 2008). That means less oxygen supply in the body fluids and a shift to anaerobic metabolism. The range of estimates of cod tolerance to hypoxia is quite wide, due to different temperatures and exposure durations (Plante et al., 1998). Using lethal concentration(LC)50 methodology, Plante et al. (1998) reported a lethal threshold of 21% air saturation for a 96-h exposure at 2–6 C, but cod were more tolerant to short exposures using LC50 for a 3-h exposure was 9% air saturation.

This inverse relationship linking hypoxia tolerance and exposure duration is due to fish's ability to survive for some time, using anaerobic metabolism, when ambient dissolved oxygen does not permit them to sustain standard metabolic rate. Besides OA, the ecosystem is affected by declines in the dissolved O_2 (ocean deoxygenation) since O_2 is less soluble in warmer water, due to the fact that gas in a solvent is directly proportional to the partial pressure of that gas above the solvent which is temperature-dependent. However, the solubility changes, driven by

sea surface warming, are responsible for only about 20% of the modeled decrease in oxygen. The reduction in the rate of transport into deeper waters due to changes in ocean circulation and convection were identified as the main reason for the simulated reduction in sub-surface dissolved oxygen and for the increase in the net sea-to-air flux (Bopp et al., 2002; Matear et al., 2000). Global warming is supposed to increase the upper ocean stratification and thereby restrict the O_2 supply to the ocean interior (Bopp et al., 2002; Keeling et al., 2010).

1.3.4. Amino acid metabolism

The functions of amino acids in fish are numerous, for example they are building blocks of proteins. The synthesis of these proteins during the growth of fish can account for 20–42 % of their energy expenditures (Ballantyne, 2001). Furthermore, amino acids are catabolic substrates to generate ATP as they provide 14 – 85 % of the energy requirements of teleost fish (Van Waarde, 1983). In comparison to mammals, with 20 % of amino acid catabolism, this is a substantially higher rate (Fauconneau and Arnal, 1985). Glutamine is the only amino acid that has no function as a nitrogen storage under normal conditions in fish. Due to this, the circulating levels of glutamine are lower than the levels of other amino acids. This impacts the metabolism of other amino acids, but the exact regulating factors for the flow of amino acids into anabolic or catabolic pathways are little known despite many years of research (Ballantyne, 2001).

All tissues have some ability to synthesize non-essential amino acids, remodel amino acids and to convert non-amino acid carbon skeletons into amino acids and other derivatives that contain nitrogen. The carbon skeletons are generally conserved as carbohydrate, via gluconeogenesis, or as fatty acids via fatty acid synthesis pathways (Ballantyne, 2001). Fig. 6 depicts a diagram of the pathway for amino acid catabolism in fish muscle.

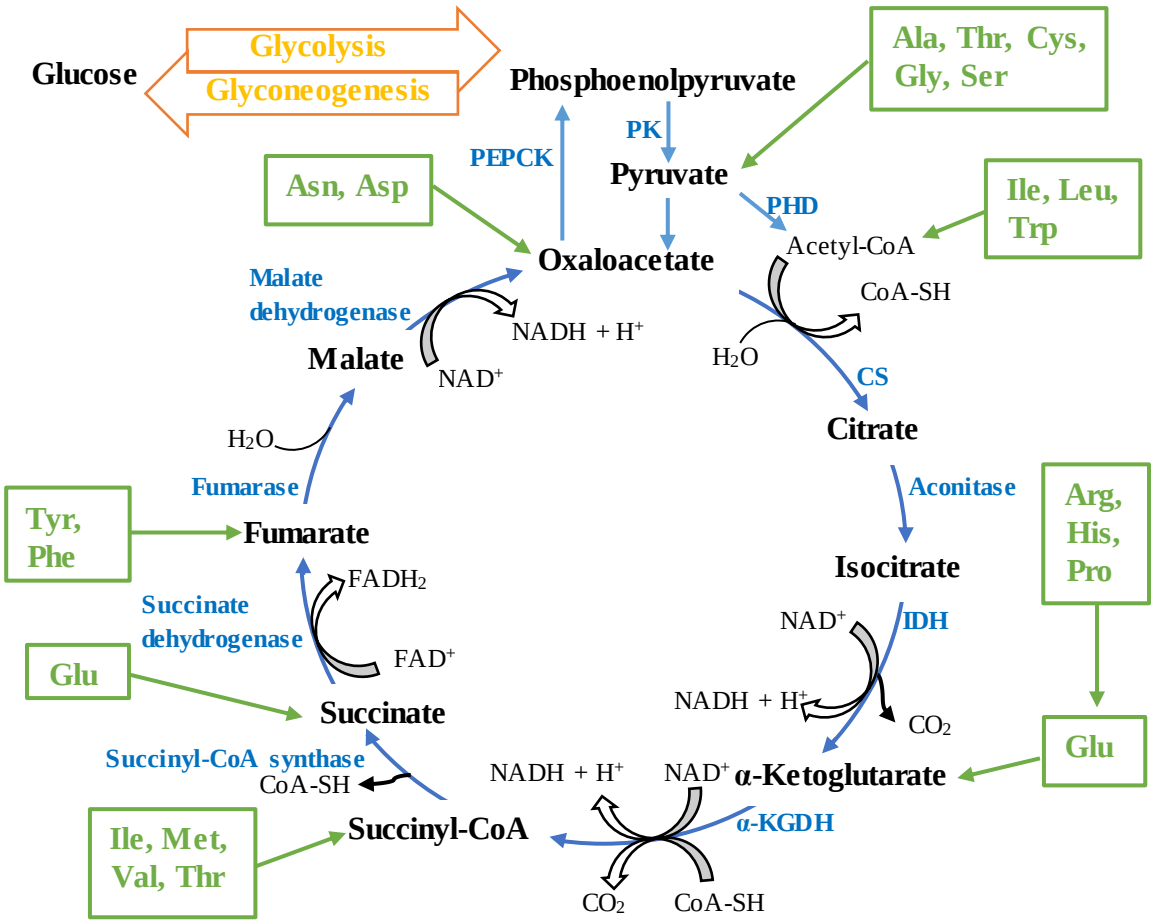


Figure 7: Schematic depiction of the pathway for amino acid catabolism in fish muscle. The abbreviations refer to the following enzymes: PDH: pyruvate dehydrogenase; PK: pyruvate kinase CS: citrate synthase; IDH: isocitrate dehydrogenase; GDH: glutamate dehydrogenase; PDG: phosphatedependent glutaminase; α–KGDH: α-ketoglutarate dehydrogenase; PEPCK: phosphoenolpyruvat-carboxykinase (modified after Needham 1930; Krebs 1953; Ballantyne 2001)

2. Materials and Methods

2.1 Sample collection

The Atlantic cod were caught byR/V Heincke at several locations surrounding Svalbard: Rijpfjorden (80° 15.420N22° 12.890E), Hinlopenstretet (79° 30.190N18° 57.510E), and Forlandsundet (78° 54.600N11° 3.660E) (Kunz et al., 2016).

The fish were directly transferred in a thermostatted recirculating tank system (4 m^3) at the aquaria of the Alfred Wegener Institute, Bremerhaven. There they were kept for several months at 5 °C (Kunz et al., 2016). The mortality during the capture was low. However, cannibalism significantly reduced the number of individuals. They were fed twice a week with a mixture of frozen copepods, baby krill and high-protein feed pellets (Kunz et al., 2016).

2.2 Experimental design

The fish were incubated at four different stable temperature/pCO_2 conditions. For each treatment 12 single aquaria (approx.. 24 L each) were used and the fish were randomly allocated to the temperature and pCO_2 incubation set-up with a 12 h day and night rhythm (Leo et al., 2017). The respective pCO_2 conditions were pre-adjusted in a header tank containing ~200 L of seawater, supplying the individual aquariums. The differed temperatures (3, 8, 12, 16 °C) were chosen based on the natural temperature range of their habitat. Each temperature was treated with the current pCO_2, 390 μatm (control pCO_2) and with the pCO_2 predicted for the year 2100, 1170 μatm (high pCO_2) (Kunz et al., 2016) according to the Representative Concentration Pathway (RCP) 8.5 of the Intergovernmental Panel on Climate Change (IPCC). A mass flow controller (4 and 6 channel MFC system, HTK, Hamburg, Germany) was used to set the desired pCO_2 by mixing almost CO_2-free pressurized air with pure CO_2 (Kunz et al., 2016; Pörtner et al., 2014). At the end of April 2014, 96 Atlantic cod were transferred to the experimental setup. They differed in body size from 14.2 - 24.8 cm (mean total length 18.5 cm ± 2.2 SD) and their weight was 15.3 – 103.8 g (mean weight 43.4 g ± 17.3 SD) (Kunz et al., 2016). The water quality was ensured by the use of biological filter systems, protein skimmers (Sander, Germany) and a daily water exchange of 600 L (Kunz et al., 2016).

After an incubation of four months, many experiments were carried out, as "respiration measurements" and "growth experiment" (Kunz et al., 2016). After all experiments were over the fish were anaesthetized with 0.2 g L^{-1} tricaine methane sulphonate (MS222) and killed by

a spinal cut behind the head plate. Hearts were rapidly excised and frozen in liquid nitrogen and stored at -80 °C for further experiments (Leo et al., 2017).

2.3 Sample preparation and NMR spectroscopy

To extract the metabolites from the samples, ranging from 5 to 55 mg wet weight, the frozen heart tissues were mixed with 400 µL ice cold methanol and 125µL ice cold Milli-Q water in a 2 mL homogenization tube containing ceramic beads. The filled tubes were immediately mixed for one cycle of 25 s at 5500 rpm at 0-4 °C in a Precellys tissue grinder. After that, 400 µL ice cold Milli-Q water and 400 µL chloroform were added. Subsequently the tubes were vortexed for 15 s and incubated on ice for 10 min. After incubation, they were centrifuged for 10 min and 3000 rcf at 4 °C. Afterwards three phases were observed: the upper layer containing methanol and polar metabolites, a thin protein-layer in the middle and a lower layer containing chloroform and lipids. The upper and lower layers were transferred to separate 1.5 mL tubes. The chloroform-layer was dried in a fume hood at room temperature and the methanol-layer dried by vacuum centrifuge (Speedvac) at room temperature overnight. The dried polar metabolites were resuspended with an individual amount of D_2O, to reach at least 70 µL to fill the NMR rotor. The D_2O contains trimethylsilylpropionat (TSP) as marker that acts as an internal standard and as a chemical shift reference (δ = 0.0 ppm). After adding D_2O the tube was vortexed and 70 µL of the resuspension was transferred to the NMR rotor. The system was chilled to 20 °C and calibrated according to manufacturer's instructions (Manual for TopSpin, 2.1 Version 2.1.1, Brucker BioSpin). All samples remained for 256 measurements (26:56 min) in the spectroscope to equalize noise.

2.4 NMR data analysis

As a first step, all spectra were automatically corrected with the programm Chenomx|nmr suite 8.1 professional. Therefore, the Chenomx Processor converts various spectrum formats into the Chenomx file format. For the assignment of the metabolites, Chenomx Profiler was used, since it also quantifies their concentrations based on data in an NMR spectrum. The Chenomx Compound Library of metabolites at 400 MHz was chosen as catalog of metabolites. The metabolites were sorted by hand and the correction system of Chenomx helped to fit the metabolites correctly.

The metabolic products of the abovementioned metabolic pathways (glycolysis, citrate acid cycle, lactic acid cycle, amino acid metabolism) were placed in the spectra. After that, the remaining pikes were assigned.

2.5 Statistical analysis

One Way Analyses of Variance (ANOVA) were performed to test for significant effects of the treatments on 22 metabolites (Table 1). Metabolites with a significant decrease compared to control are colored blue in the table and are marked with a minus sign (-) and a significant increase is indicated by red color and a plus sign (+), whereas all non-significant changes are marked with "0" and colored in green. All data were tested onnormal distribution and homogeneity of variances and were graphically represented in RGui. The significance level was set to $\alpha = 0.05$. All statistical analyses were performed with RGui (Version 3.1.3, R Development Core Team (2008)).

Table 1: Table of significance
Shown are the 22 tested metabolites. Significant decrease: colored blue and marked with a minus sign (-); significant increase: red color and a plus sign (+); all not significant changes are marked with "0" and colored in green. The significance level was set to $\alpha = 0.05$.

Metabolites	Elevated temperature (390 μatm CO_2)	Elevated temperature (1170 μatm CO_2)	CO_2
Alanine	0	0	-
Asparagine	0	0	0
Glutamate	0	0	0
Glutamine	0	0	-
Glycine	0	0	0
Isoleucine	0	0	-
Leucine	0	0	0
Valine	0	0	0
Creatine	+	0	0
Creatine phosphate	0	0	-
Creatinine	0	0	0
Glucarate	0	0	-
N-Acetylglutamine	0	0	0
Taurine	0	0	-
Citrate	0	0	0
Fumarate	0	0	0
Succinate	0	0	0
Fructose	0	0	-
Glucose-6-phosphate	0	0	-
Lactate	+	0	0
AMP	0	0	0
ATP	0	0	0

3. Results

3.1. Contained metabolites

Metabolites were assigned to the NMR spectra by means of Chemomx, their concentrations in the respective sample were determined by including the dilution of the sample with D_2O. Example spectra with marked metabolites are shown below in Figure 7-9. Not all expected metabolic products of the abovementioned metabolic pathways could be assigned to the sample spectra, because the spectra of both, expected metabolite and sample, were not always compatible. Therefore, we concentrated on metabolites that could be clearly assigned. All contained metabolites are shown in Table 2 in the appendix.

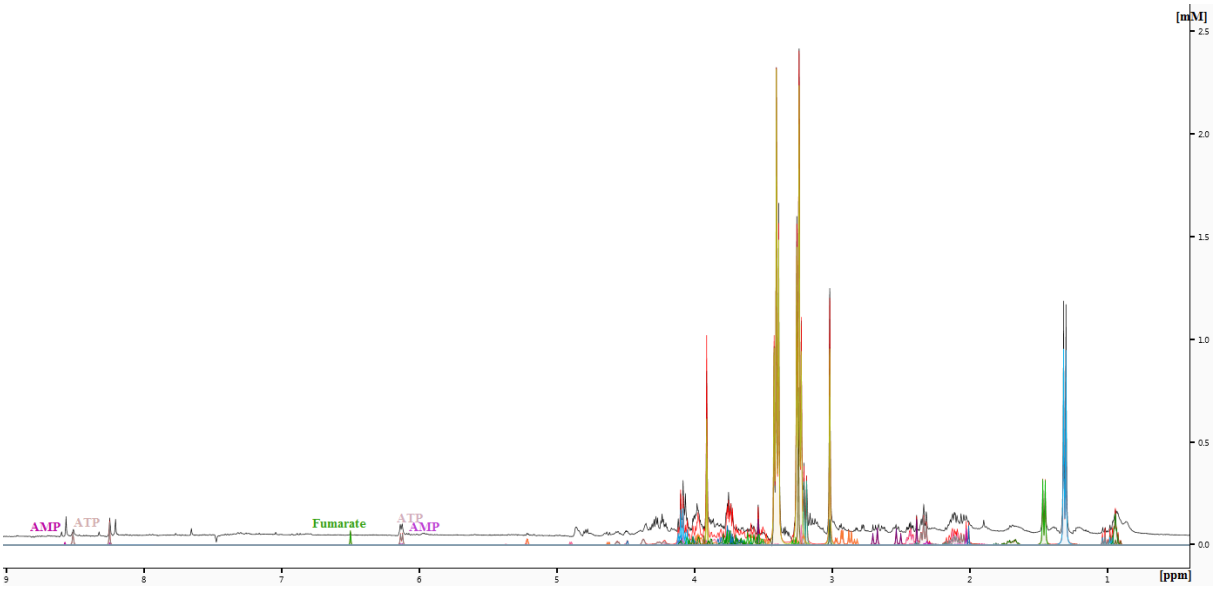


Figure 8: NMR-spectra after adding metabolites by the Software Chenomx. Metabolites in the chemical shift range between 9 ppm and 0 are shown. Metabolites are marked by different colors and their names are written in the color of the peak.

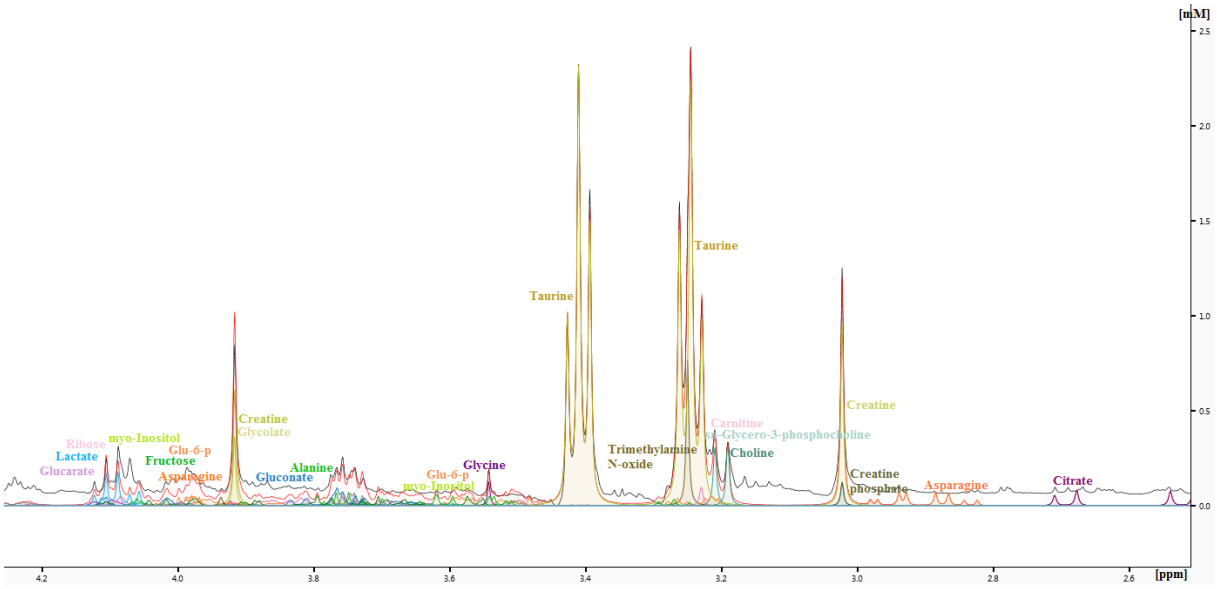


Figure 9: NMR-spectra (extract) after adding metabolites by the Software Chenomx. Metabolites in the chemical shift range between 4.2 ppm and 2.5 are shown. Metabolites are marked by different colors and their names are written in the color of the peak.

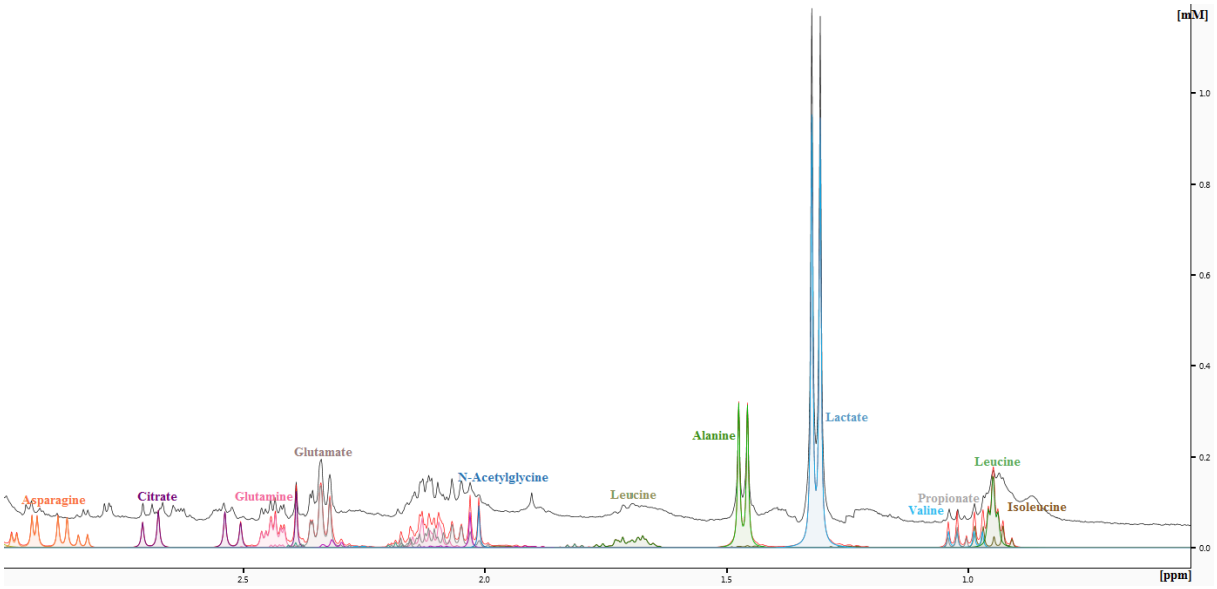


Figure 10: NMR-spectra (extract) after adding metabolites by the Software Chenomx. Metabolites in the chemical shift range between 2.6 ppm and 0 are shown. Metabolites are marked by different colors and their names are written in the color of the peak.

3.2. Glycolysis

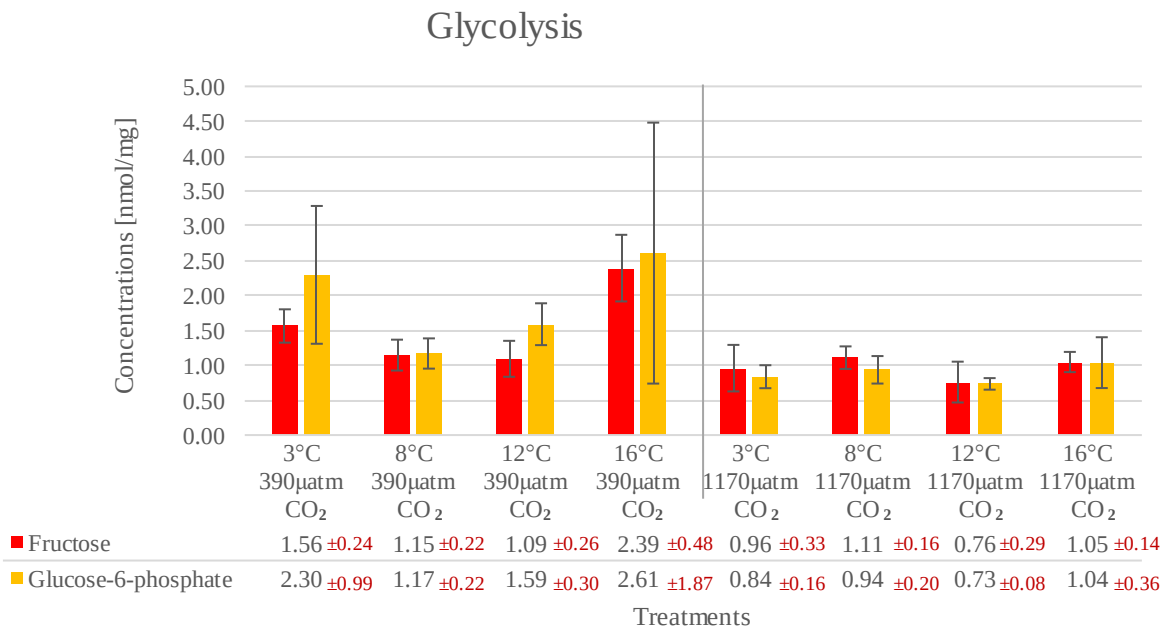


Figure 11: Glycolysis
Concentrations of fructose (red) and glucose-6-phosphate (orange) in nmol/mg for the two pCO_2 groups 390 and 1170 μatm, Values represent mean of (390 μatm CO_2: $n_{3°C} = 4$, $n_{8°C} = 6$, $n_{12°C} = 5$, $n_{16°C} = 3$; 1170 μatm CO_2: $n_{3°C} = 6$, $n_{8°C} = 6$, $n_{12°C} = 6$, $n_{16°C} = 3$) measurements (± standard errors of the means (red)).

Rising CO_2 had a significant negative effect on fructose ($p = 0.03$) and glucose-6-phosphate ($p = 0.01$) concentrations, whereas temperature had no significant effect (fructose: $p = 0.56$; glucose-6-phosphate: $p = 0.81$) (Table 1 within the CO_2-groups (390 and 1170 μatm) (Fig. 10) The highest concentration of fructose (red bars) occurred in the treatment with 16 °C and 390 μatm CO_2 with 2.39 ± 0.48 nmol/mg and the lowest concentration was in the treatment with 12 °C and 1170 μatm CO_2. It stands out that all concentrations for the same temperature in the group of 390 μatm CO_2 are higher than in the group with high CO_2 level (1170 μatm), for both fructose and glucose-6-phosphate (orange bars).

The highest concentration (2.61 ± 1.87 nmol/mg) of glucose-6-phosphate appeared in the treatment of 16 °C and 390 μatm CO_2, their standard error of means was ± 1.87. Similar to fructose, the lowest concentration of glucose-6-phosphate occurred at 12 °C and 1170 μatm CO_2.

3.3. Citric acid cycle

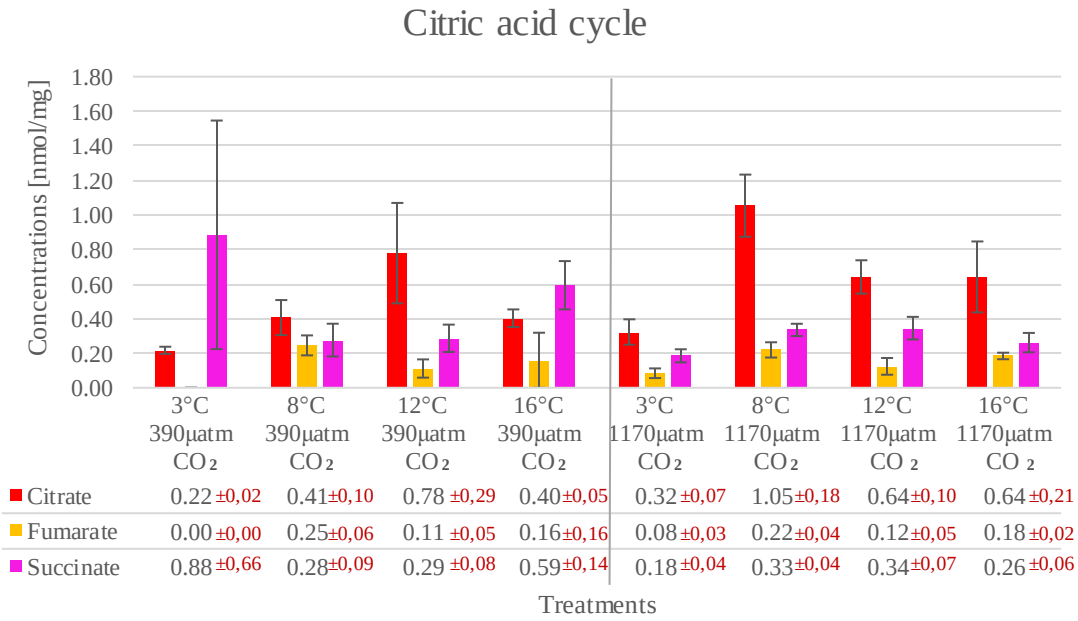


Figure 12: Citric acid cycle
Concentrations of citrate (red), fumarate (orange) and succinate (pink) in nmol/mg for the two pCO_2 groups 390 and 1170 μatm, Values represent mean of (390 μatm CO_2: $n_{3°C} = 4$, $n_{8°C} = 6$, $n_{12°C} = 5$, $n_{16°C} = 3$; 1170 μatm CO_2: $n_{3°C} = 6$, $n_{8°C} = 6$, $n_{12°C} = 6$, $n_{16°C} = 3$) measurements(± standard errors of the means (red)).

No significant temperature- or CO_2 effect was detected for any metabolite of the citric acid cycle (citrate: $p_{temp} = 0.11$,$p_{CO2} = 0.12$; fumarate: $p_{temp} = 0.20$,$p_{CO2} = 0.78$; succinate: $p_{temp} = 0.79$, $p_{CO2} = 0.20$) (Table 1).

However, there was a trend between the concentrations of the metabolites shown in the figure above (Figure 12). Fumarate (orange bars) tended to be the metabolite with the lowest concentrations in all treatments (mean of all treatments 0.14 ± 0.05 nmol/mg), whereas the highest concentrations alternated in the treatment group of the low CO_2 level between succinate (pink bars) and citrate (red bars) and in the high CO_2 concentration group citrate tended to be the metabolite with the highest concentration. The highest concentration of succinate occurred in the group of 3 °C and 390 μatm CO_2 with 0.88 ± 0.66 nmol/mg and the lowest concentration was at 3 °C and high CO_2 with 0.18 ± 0.04 nmol/mg.

3.4. Lactic acid fermentation

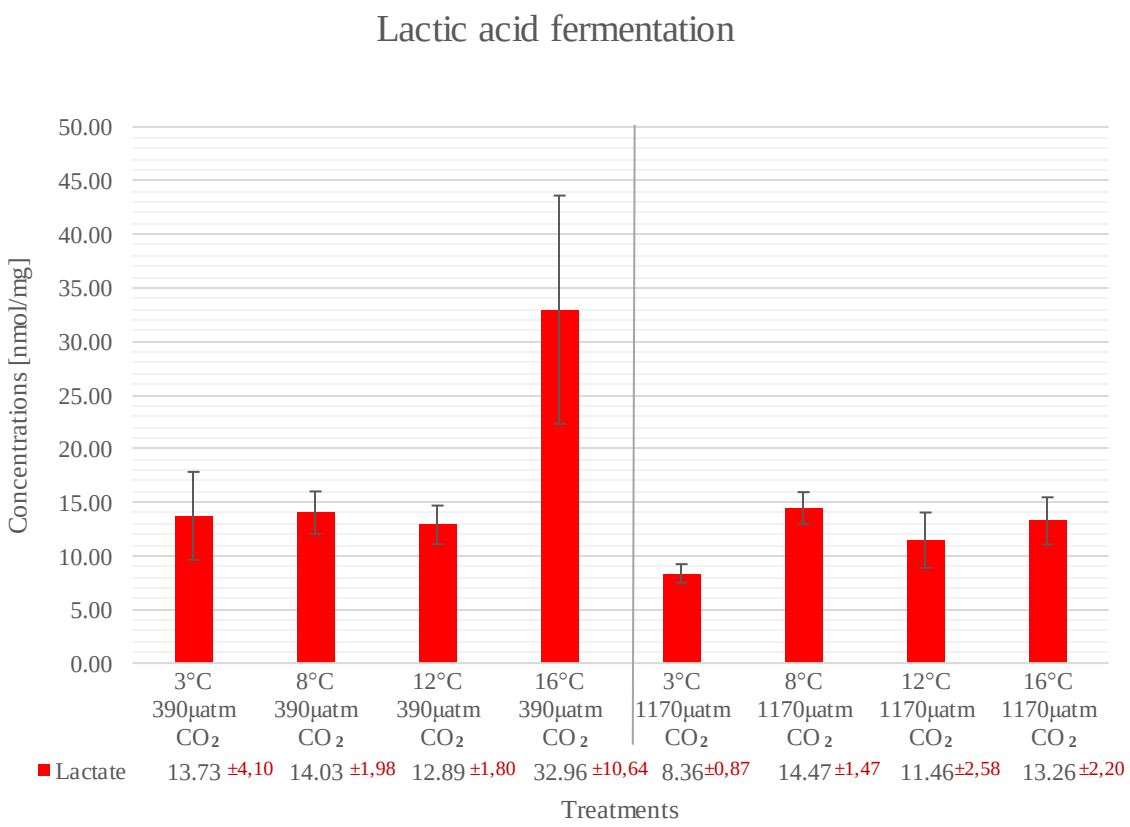


Figure 13: Lactic acid fermentation
Concentrations of lactate (red) in nmol/mg for the two pCO_2 groups 390 and 1170 μatm, Values represent mean of (390 μatm CO_2: $n_{3°C} = 4$, $n_{8°C} = 6$, $n_{12°C} = 5$, $n_{16°C} = 3$; 1170 μatm CO_2: $n_{3°C} = 6$, $n_{8°C} = 6$, $n_{12°C} = 6$, $n_{16°C} = 3$) measurements (± standard errors of the means (red)).

A significant positive temperature effect ($p = 0.02$) occurred at the metabolite lactate (Table 1). As shown in figure 12, the highest concentration was measured at 16 °C and low CO_2 level with 32.96 ± 10.64 nmol/mg and the lowest at 3 °C and the high CO_2 concentration with 8.36 ± 0.87 nmol/mg. All other concentrations did not significantly differ.

3.5. Amino acid metabolism

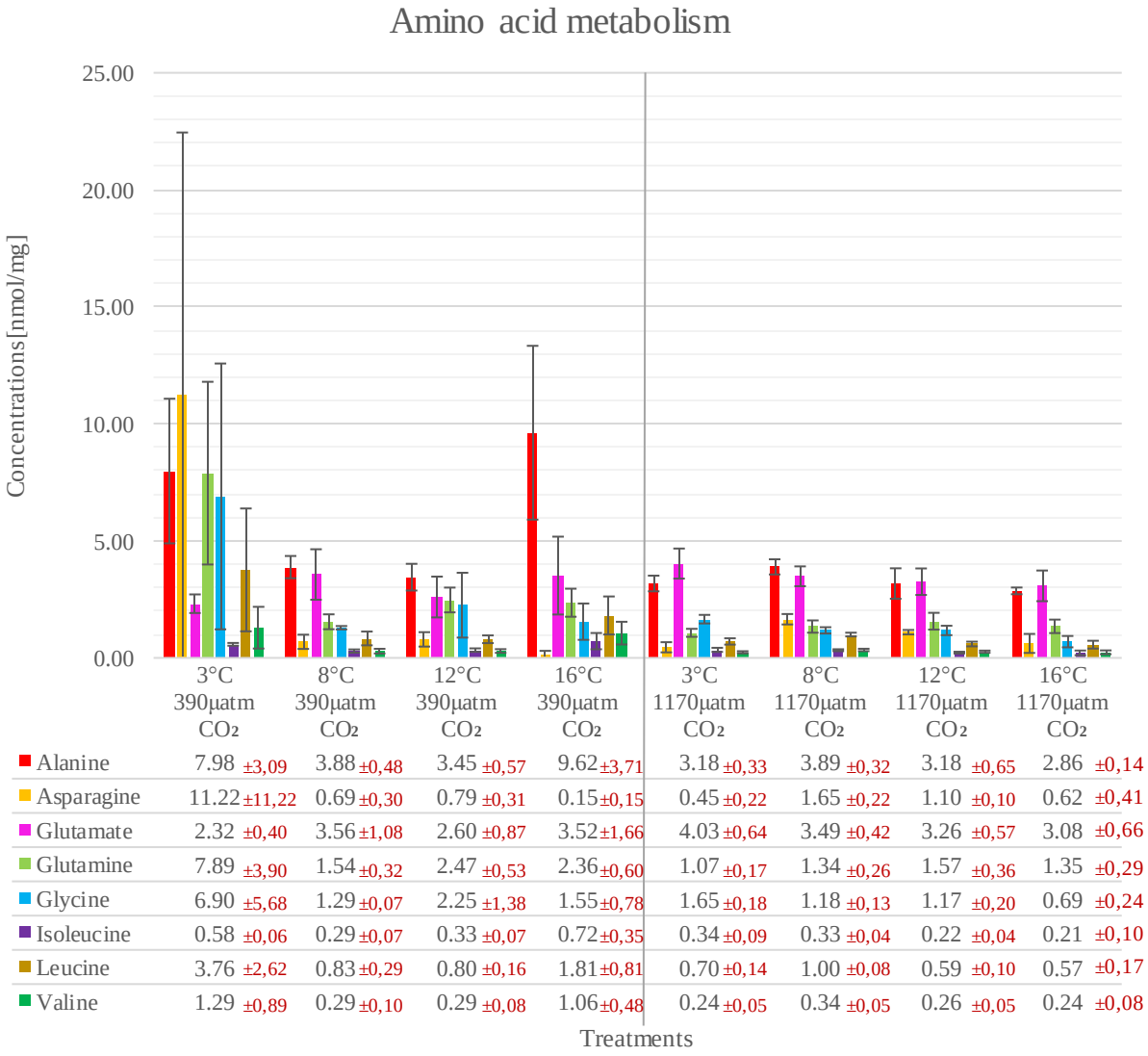


Figure 13: Amino acid metabolism
Concentrations of alanine (red), asparagine (orange), glutamate (pink), glutamine (light green), glycine (blue), isoleucine (purple), leucine (brown) and valine (dark green) in nmol/mg for the two pCO_2 groups 390 and 1170 μatm, Values represent mean of (390 μatm CO_2: $n_{3°C} = 4$, $n_{8°C} = 6$, $n_{12°C} = 5$, $n_{16°C} = 3$; 1170 μatm CO_2: $n_{3°C} = 6$, $n_{8°C} = 6$, $n_{12°C} = 6$, $n_{16°C} = 3$) measurements (± standard errors of the means (red)).

CO_2 had a significant negative effect on the concentrations of alanine ($p = 0.03$), glutamine ($p = 0.04$) and isoleucine ($p = 0.05$). No significant effect was detected for all other metabolites (Table 1).

With the exception of alanine (red bars), glutamine (light green bars) and isoleucine (purple bars) it is noticeable that the concentrations of the other substances were remarkably high in the

control treatment at 3 °C. It is striking that alanine rose in the treatment group 16 °C at low CO_2 to 9.62 ± 3.71 nmol/mg and was thus the highest concentration. In comparison to the control group, the values in the high CO_2 group were very similar for the respective substances and no significant changes with an increasing temperature could be determined.

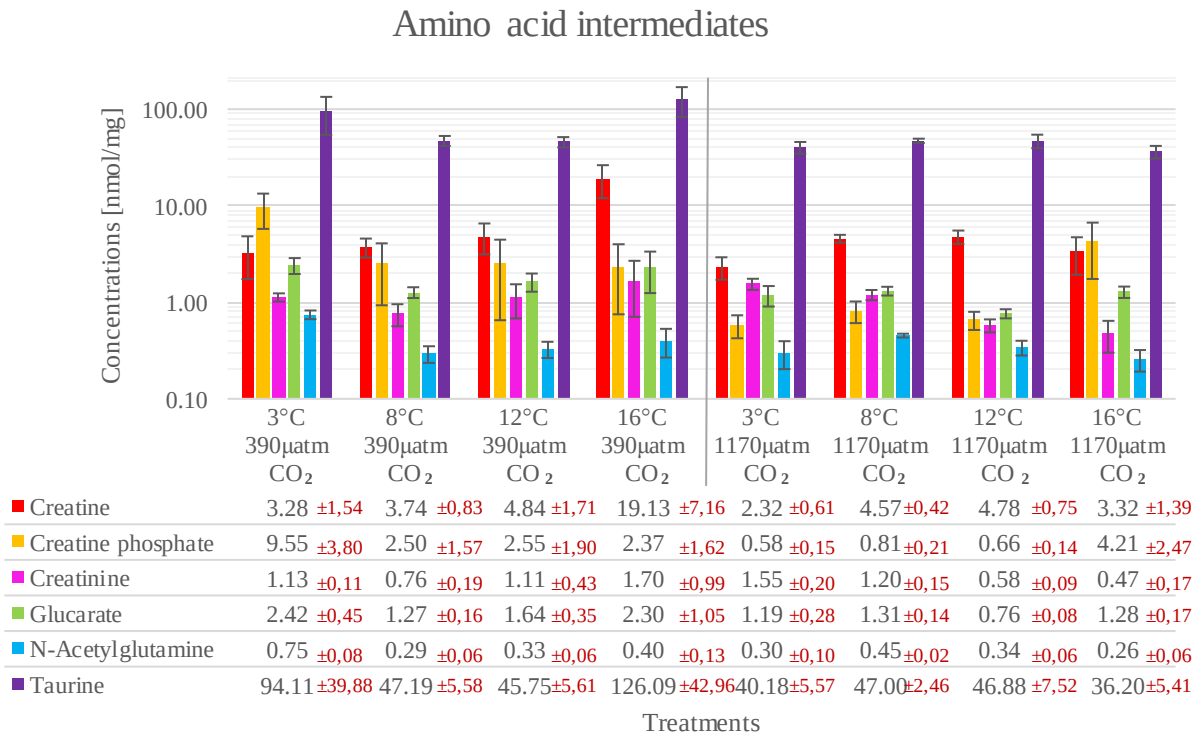


Figure 14: Amino acid intermediates
Concentrations of creatine (red), creatine phosphate (orange), creatinine (pink), glucarate (light green), n-acetylglutamine (blue) and taurine (purple) in nmol/mg for the two pCO_2 groups 390 and 1170 μatm, Values represent mean of (390 μatm CO_2: $n_{3°C} = 4$, $n_{8°C} = 6$, $n_{12°C} = 5$, $n_{16°C} = 3$; 1170 μatm CO_2: $n_{3°C} = 6$, $n_{8°C} = 6$, $n_{12°C} = 6$, $n_{16°C} = 3$) measurements) ± (standard errors of the means (red)).

Rising temperature had a significant positive effect on creatine ($p = 0.01$). On all other metabolites temperature had no significant effect. CO_2 concentrations had a significant negative effect on the metabolites creatine phosphate ($p = 0.03$), glucarate ($p = 0.01$) and taurine ($p =$

0,03), whereas all other metabolites were not significantly affected by changes in the CO_2 concentration (Table 1).

The creatine (red bars) concentration of 19.13 ± 7.16 nmol/mg in the group 16 °C and low CO_2 stands out because it exceeds the other creatine concentrations ten-fold. Also taurine (purple bars) had such variabilities, most concentrations shifted from 36.2 ± 5.14 nmol/mg to 47.19 ± 5.58 nmol/mg, whereas the taurine concentrations in the treatment group of the control CO_2 level at 3 °C was 94.11 ± 39.88 nmol/mg and at 16 °C 129.09 ± 42.96 nmol/mg. N-acetylglutamine (blue bars) was detected in the least concentrations of this group of amino acid intermediates (0.26 0.06 nmol/mg to 0.75 ± 0.08 nmol/mg). The glucarate concentrations (green bars) varied from 0.76 ± 0.08 nmol/mg to 2.42 ± 0.45 nmol/mg.

3.6. AMP and ATP

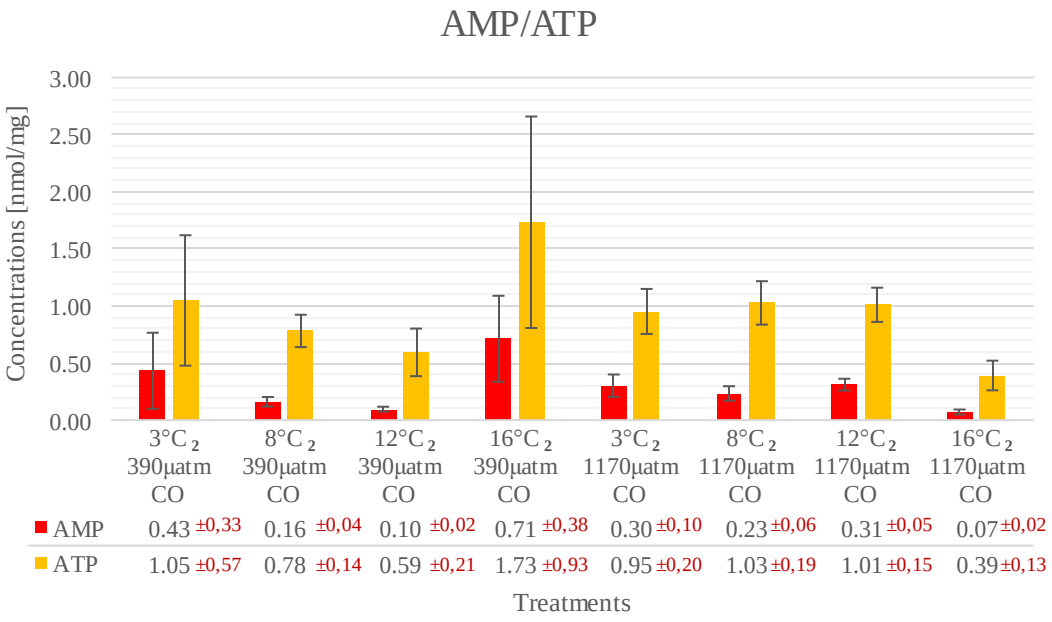


Figure 15: AMP and ATP
Concentrations of AMP (red) and ATP (orange) in nmol/mg for the two pCO_2 groups 390 and 1170 μatm, Values represent mean of (390 μatm CO_2: $n_{3°C} = 4$, $n_{8°C} = 6$, $n_{12°C} = 5$, $n_{16°C} = 3$; 1170 μatm CO_2: $n_{3°C} = 6$, $n_{8°C} = 6$, $n_{12°C} = 6$, $n_{16°C} = 3$) measurements) ± standard errors of the means (red).

None of the tested experimental factors had a significant effect on AMP ($p_{Temp} = 0.90$, $p_{CO2} = 0.68$) and ATP ($p_{Temp} = 0.94$, $p_{CO2} = 0.87$).

As there were a decrease in ATP in the low CO_2 level group from 3 °C to 12 °C followed by an increase from 0.59 ± 0.21 nmol/mg (12 °C) to 1.79 ± 0.93 nmol/mg at 16 °C. The lowest and highest ATP concentration occurred in the treatments of 16 °C, the lowest at 1170 μatm CO_2 (0.39 ± 0.13 nmol/mg) and the highest at 390 μatm CO_2 (1.79 ± 0.93 nmol/mg). All other concentrations were did not differ significantly. The AMP concentrations varied from 0.07 ± 0.02 nmol/mg to 0.71 ± 0.48 nmol/mg.

4. Discussion

4.1.Anaerobic metabolism

It is noticeable that in the treatments with 3 °C and 16 °C with 390 μatm CO_2 comparable effects were observed for the concentrations of succinate, alanine and ATP (Figures 12, 14 and 16). The higher succinate concentrations compared to citrate and fumarate in the treatments 3 °C and 16 °C in the control CO_2 group (Figure 12) may be explained by an oxygen deficiency while removal of the heart. The myocardium tries to increase anaerobic production of energy by increasing glucose consumption and lactate production (Neely and Morgan, 1974). Alanine is increasingly synthesized under anaerobic conditions while glutamate and aspartate concentrations decline (Jefferson et al., 1971; Taegtmeyer et al., 1977). This alanine increase is shown as well in Figure 14 in the treatment groups of 3 °C and 16 °C with low CO_2 levels, whereas no significant change of the glutamate concentrations was detected (Figure 14). However, the glutamate concentrations in the samples were very low, even ten times lower than values reported in the literature (Lyndon et al., 1993; Özden, 2005)(Table 7 and 8 in the appendix). Increased alanine synthesis is related to an increase of pyruvate (Needham, 1930). The pyruvate concentrations were not detected in the spectra, but they can be compared to the glucose-6-phosphate concentrations when one assumes that this glucose-6-phosphate was produced via glycolysis. In comparison to that, the glucose-6-phosphate concentrations (Figure 11) were also high in the same treatment groups as alanine (Figure 14) and succinate (Figure 12). In addition, succinate is an intermediate of the degradative pathway of glutamate (Chang and Goldberg, 1978) and the synthesis of succinate helps restoring the oxidation-reduction equilibrium of the glycolytic pathway which is disrupted by the synthesis of alanine. This is coupled to two energizing reactions catalyzed by α-ketoglutarate dehydrogenase and succinate dehydrogenase (Figure 7) (Needham, 1930). Because other conditions influence this reaction, it is not clear if this is the only reason for the rise of succinate and alanine in the samples. One of these factors include transamination of aspartate and glutamate by substrate specific transaminases (Taegtmeyer et al., 1977). Their presence could not have been tested with this method. Furthermore, substrate phosphorylation in the conversion of succinyl-CoA to succinate and the following oxidation of NADH by fumarate (Figure 5) in the myocardial mitochondria are energy sustaining mitochondrial reactions. These non-glycolytic energy sources are likely to contribute to the adaptation of aquatic vertebrates to hypoxia (Hochachka and Storey, 1975). Therefore, 16 % of the ATP synthesis, compared to ATP obtained from lactic acid fermentation (Figure 6) through anaerobic glycolysis, would be produced through anaerobic alanine

formation by glycolysis and succinate formation in the citric acid cycle (Taegtmeyer et al., 1977). This additional 16 % ATP through anaerobic driven alanine formation may explain the higher concentrations of ATP (Figure 16) in the treatments with 3 °C and 16 °C at low CO_2 levels.

Looking at Figure 13, the significant positive temperature effect ($p = 0.02$) on lactate fits to the poorer solubility of oxygen in warmer water (Bopp et al., 2002; Keeling et al., 2010) and the finding of possible anaerobic metabolism products in the treatment with the highest temperature. The higher the temperature got, the more lactate was produced by the anaerobic lactic acid fermentation. Relating to Plante et al. (1998), who found out that anaerobic metabolism is not possible over longer incubation times than 96h, the detected effect has to be related to oxygen deficiency during the removal of the tissue, and not to the incubation.

4.2. Glycolysis

The lowered PFK reaction kinetics in the glycolysis in more alkaline medium (Halperin et al., 1969) could not be proved, since the concentrations of glucose-6-P were not lower in the low CO_2 medium than in the high CO_2, medium - it was the other way around (Figure 11)($p = 0,01$). Also the up-regulating effects of ocean acidification of glycolytic enzymes was missing (Enzor et al., 2017).

4.3. Citric acid cycle

High bicarbonate levels and therefore increased pCO_2 was identified as an inhibitor of citrate synthase (complex II) in fish (Simpson, 1967; Strobel et al., 2013) with following stimulation of the mitochondrial anaplerotic pathways, like amino acid metabolism or gluconeogenesis, to overcome this inhibition (Langenbuch and Pörtner, 2003). The fact that CO_2 had no significant effect (Table 1) on the metabolites of the citric acid cycle and the citrate concentrations were actually not decreasing in the high pCO_2 treatments (Figure 12) lets assume that the mitochondria maybe increased the citrate synthase capacity to compensate the inhibition. Kreiss et al. (2015) found that the cytochrome c oxidase and citrate synthase ratio in liver tissues of *Gadus morhua* increased continuously with increasing pCO_2 for the fish at 10 °C. About 50 % higher ratios were observed in fish reared at 18 °C. This increase was significant at medium pCO_2 (1200 μatm) compared to control pCO_2 (550 μatm) at 10 °C (Kreiss et al., 2015). It is

possible that such a mechanisms are the reason for the continuous high citrate concentration within the treatments. Furthermore, Leo et al. discovered that in *Gadus morhua*, different incubation temperature leads to variable mitochondrial response patterns under elevated pCO_2. As a result of the degree of cold acclimated it benefits from a lower rate of metabolism and a higher plasticity to acclimate to increasing temperature. Consequently, mitochondrial function of its hearts may be less constrained by rising temperatures (Leo et al., 2017). The results of this study fit into the data collected here.

The expected increase of the products catalyzed by the isocitrate dehydrogenase, succinate and fumarate (Vohwinkel et al., 2011), with increasing pCO_2 was not as clearly detected as expected. However, it stands out that the citrate concentrations in the treatments with high CO_2 levels were on average higher than these in the low ones and therefore the fumarate concentrations nearly remained the same, as well as the succinate whose concentrations even decreased in the treatments with 16 °C from low to high CO_2 level (Figure 12). This could be associated with an increased activity of the isocitrate dehydrogenase. It would require further tests to prove this hypothesis.

4.4. Amino acid metabolism and intermediates

Amino acids can also be used as catabolic substrates for the generation of ATP and as such provide between 14 – 85 % of the energy requirements of teleost fish (Van Waarde, 1983). A decrease of amino acids with increasing temperature and/or CO_2 would lead to an increased energy supply by amino acid catabolism. Elevating CO_2 had such an significant negative effect on alanine, glutamine and isoleucine (Figure 14). However, glutamine is the only amino acid that has no function as a nitrogen storage under normal conditions in fish (Ballantyne, 2001) and cannot be used for energy supply. Isoleucine contributes to the provision of acetyl-CoA and succinyl-CoA by its catabolic reactions (Figure 7). This is also a possible explanation for the higher citrate concentration in the treatments with the higher CO_2 level (Figure 14). The valine concentrations in the treatments with 3 °C (1.29 ± 0.89 nmol/mg) and 16 °C (1.06 ± 0.48 nmol/mg) at low CO_2 level were notably higher than the other concentrations of valine. This can be linked to the high succinate concentration (Figure 12) in the same treatments, since it seems that the higher valine supply led to catabolic reactions and the succinate synthesis was improved. The enrichment of succinate is an indication of anaerobic metabolism, which must have taken place during the removal of the heart. (Plante et al., 1998)It has to be mentioned that asparagine (yellow bars) was identified in only one of four individual samples for the group 3

°C and 390 μatm CO_2 and that explained the high standard error of the means (11.22 ± 11.22 nmol/mg).

Creatine is a naturally occurring guanidine-derived compound, which, in its phosphorylated forms, functions in the maintenance of cellular ATP homoeostasis (Ellington, 1989; Harris et al., 1992). Creatine and its associated phosphotransferase, phosphocreatine kinase, are able to support higher ATP/ADP ratios at equilibrium (Ellington, 1989). Creatine reacts to creatine phosphate (by phosphocreatine kinase) which provides the phosphoryl group which is used to reconstitute ATP out of ADP during muscle contractions (Harris et al., 1992). Creatinine is a degradation product of creatine (Mora et al., 2008). This means that better supply of creatine and its phosphates could increase the ATP availability, or at least, enhance its turnover. It can be assumed that ATP and creatine phosphate therefore keep their balance in a functioning organism.Comparing Figure 14 (amino acid intermediates) and Figure 15 (AMP/ATP), one realises that the creatine and creatine phosphate concentrations fit to those of ATP. A decrease in ATP occured in the low CO_2 level group from 3 °C to 12 °C followed by an increase from 0.59 ± 0.21 nmol/mg (12 °C) to 1.79 ± 0.93 nmol/mg at 16 °C, compared to creatine the same distribution of the highest and lowest concentration was observed (Fig. 14). It was striking, that the lowest and highest ATP concentration occurred in the treatments of 16 °C, the lowest at 1170 μatm CO_2 (0.39 ± 0.13 nmol/mg) and the highest at 390 μatm CO_2 (1.79 ± 0.93 nmol/mg), due to that their number of samples were low (390 μatm CO_2 n = 4 , 1170 μatm CO_2 n = 3) and may be the reason for the occurring effect . Otherwise since all other concentrations were similar and there was no statistically significant CO_2 or temperature effect, this result remains unclear. In addition to the energy rich phosphates the 400 MHz NMR spectroscope was not able to filter these substances clearly, since only the hydrogen nucleus were excited with this spectroscope. In this case a different spectroscope would be necessary to excite the phosphates. With the 400 MHz spectroscopy it is not guaranteed that the measured concentration really belong to ATP, ADP or creatine phosphate.

Taurine (aminoethane sulfonic acid) is found in very high concentrations in cardiac and skeletal muscle (Schaffer et al., 2010), There appears to be a correlation between taurine levels and heart rate, since the highest taurine levels were found in species with the highest heart rates (Kocsis et al., 1976). This may explain the high concentrations in the treatments 3 °C (94.11 ± 39.88 nmol/mg) and 16 °C (129.09 ± 42.96 nmol/mg) at low CO_2 level. As mentioned above, there are indications for an insufficient oxygen supply in these two treatment groups during the heart removal, which may have caused a higher carbon dioxide excess. In consideration to this,

it would cause in turn an increase in ventilation volume by virtue of a greater depth of breathing, while the ventilation frequency is decreasing slightly. Consequently the heart rate will go up with increasing carbon dioxide concentrations (Randall and Shelton, 1963).

Potential effects of the tested parameters temperature and CO_2 on the metabolite concentrations of Atlantic cod were possibly not detected due to a too small sample size. Also the linear model was very robust against the presupposition of the presuppositions. However, the models explained on average only 12% of the variance, and this speaks against the reliability of the models (see appendix Figure 16).

4.5. Methological considerations

Possible significant differences between the treatments might be masked by small sample size. Therefore more study organisms are needed.

In order to fully assess acclimation capacity of the metabolic pathways of Atlantic cod to environmental hypercapnia, measurements on other parameters, such as pH_e and pH_i would be needed. Furthermore, treatment with more different CO_2 levels would improve the significance of the data. Also the temperature range (3-16 °C) was chosen close to the natural thermal range (0-12 °C) of *Gadus morhua* (Drinkwater, 2005). This temperature range could be expanded to higher temperatures to prove the influence of elevated temperature beyond the ideal temperature for Atlantic cod.

Due to the detected oxygen deficiency in some samples / treatment groups it should be ensured that the time between anesthetizing, removal and freezing is minimized in future experiments.

In addition to the energy rich phosphates the 400 MHz NMR spectroscope was not able to filter these substances clearly, since only the hydrogen nucleus were excited with this spectroscope. In this case a different spectroscope would be necessary to excite the phosphates. With the 400 MHz spectroscopy it is not guaranteed that the measured concentration really belong to ATP, ADP or creatine phosphate.

Compared to the literature values, the concentrations of the metabolites were very low in all samples. This indicates that the method used, was not able to extract all substances completely out of the tissue. In the future another extraction method should be used.

In conclusion, a correlation analysis could improve the meaningfulness of the data and this should be done with more time budget.

5. Conclusion

In this study the impact of two environmentally relevant water pCO_2 and four temperatures on metabolites of glycolysis, citrate acid cycle, lactic acid fermentation, amino acid metabolites and amino acid intermediates of Atlantic cod (*Gadus morhua*) were investigated. After four months of acclimation, environmental hypercapnia led to a significant decrease of glucose-6-phosphate (glycolysis), on the amino acids and their intermediates alanine, glutamine and isoleucine, creatine phosphate, glucarate and taurine. An elevation of temperature led to a significant increase of creatine (amino acid intermediate) and lactate within the treatment groups with 390 μatm CO_2. Hypercapnic accumulation did not significantly influence the metabolites of the citric acid cycle, but an influence of decreasing water pH on the isocitrate dehydrogenase can be assumed and therefore an influence of increasing CO_2 not excluded. Some concentration dissimilarities could be traced back to tissue hypoxia during sampling of the treatment groups 3 °C and 16 °C with 390 μatm CO_2 by comparison of all metabolic pathways and their products. Furthermore, except for creatine and lactate, no significant temperature effect was detected. The very similar ATP concentrations through all treatments indicates that *Gadus morhua* is able to cope with environmental changes and maintain its supply of energy.

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First I would like to thank Dr. Felix Mark for the past year, in which he has staked me through his enthusiasm and fascinating nature for the physiology of marine organisms more and more. Furthermore I want to thank him for his competent supervision and corrections, his great way to teach and motivate during relaxing coffee brakes.

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8. Appendix

Table 2: raw data

The row „File“ shows the NMR-data file, the sample number and the animal number is given in the second row, following the temperature (Temp) (°C), CO_2 (µatm), weight of the heart tissue (mg) and D_2O (µL) are shown. All metabolites are given in mmol/L.

File	sample_no -animal	Temp [°C]	CO_2 [µatm]	Weight [mg]	D2O [µl]	Alanine	AMP	Ascorbate	Asparagine	ATP
6	1731-1	3	390	11.8	83	2.40	0.20	0.21	6.38	0.39
7	1781-3	3	390	18.9	95	0.72	0.01	0.21		0.07
8	1806-4	3	390	11.5	83	1.04	0.03	0.21		0.08
9	1856-6	3	390	18	94	0.76	0.01	0.08		0.11
41	1506-33	8	390	14.3	86	0.85	0.02	0.24	0.09	0.12
42	1531-36	8	390	24.2	97	0.89	0.09	0.45	0.45	0.18
43	1556-31	8	390	12.5	88	0.58	0.02	0.34	0.06	0.05
44	1581-35	8	390	39.8	80	1.42	0.03	0.34	0.68	0.26
14	6-29	8	390	16.2	81	1.05	0.02	0.13		0.24
15	31-30	8	390	58.3	117	1.19	0.08	0.21		0.59
10	1881-57	12	390	15.4	93	0.52	0.01	0.31		0.02
11	1906-59	12	390	14.9	88	0.78	0.01	0.17		0.06
31	906-49	12	390	12.5	88	0.55	0.03	0.25	0.24	0.19
32	931-51	12	390	18.2	91	0.90	0.01	0.25	0.27	0.18
34	981-53	12	390	27.9	84	0.37	0.03	0.23	0.31	0.08
45	1631-81	16	390	18.8	94	0.57	0.00	0.28	0.09	0.09
4	1656-82	16	390	8.4	76	1.73	0.09	0.06		0.13
5	1706-84	16	390	6.5	72	0.94	0.12	0.13		0.32
16	306-13	3	1170	17.4	87	0.75	0.01	0.09		0.12
17	331-14	3	1170	16	80	0.77	0.02	0.25		0.09
18	356-16	3	1170	13	91	0.53	0.04	0.24		0.19
19	381-17	3	1170	11.6	82	0.24	0.06	0.13	0.08	0.07
20	406-18	3	1170	20.1	81	0.75	0.06	0.03	0.26	0.34
35	1006-19	3	1170	5.8	76	0.23	0.06	0.03	0.08	0.11
27	756-37	8	1170	15.8	95	0.68	0.07	0.21	0.42	0.26
28	781-41	8	1170	22.5	90	0.95	0.03	0.29	0.36	0.23
29	806-42	8	1170	20.7	83	1.30	0.08	0.19	0.36	0.32
30	831-43	8	1170	19.6	98	0.58	0.07	0.12	0.36	0.24
36	1156-44	8	1170	14.2	86	0.67	0.01	0.52	0.30	0.16
37	1181-46	8	1170	12.5	88	0.47	0.02	0.12	0.13	0.03
21	606-61	12	1170	32.1	97	0.96	0.04	0.52	0.36	0.20
22	631-63	12	1170	24	96	1.53	0.08	0.23	0.27	0.32
23	656-62	12	1170	33.1	100	1.06	0.07	0.61	0.35	0.36
24	681-66	12	1170	85	85	1.26	0.37	0.13	0.68	0.53
25	706-64	12	1170	24.2	97	0.70	0.08	0.28	0.34	0.36
26	731-65	12	1170	23.3	94	0.69	0.12	0.25	0.32	0.27
38	1406-91	16	1170	35.7	108	0.93	0.03	0.52	0.53	0.21
39	1431-92	16	1170	38.4	116	1.06	0.04	0.24	0.08	0.15
12	1931-95	16	1170	25.9	104	0.64	0.01	0.06		0.02

Table 3: raw data part 2

The row „File“ shows the NMR-data file, sample number and the animal number temperature, CO_2, weight of the heart tissue and D_2O were omitted but can be found Table 2. All metabolites are given in mmol/L.

File	Carnitine	Choline	Citrate	Creatine	Creatine phosphate	Creatinine	Dimethyl-amine	Fructose	Fumarate
6	0.31	0.10	0.04	0.34	2.93	0.18		0.22	
7	0.33	0.10	0.04	0.34	0.98	0.18		0.22	
8	0.33	0.19	0.04	1.09	1.16	0.14		0.31	
9	0.62	0.08	0.04	0.22	0.82	0.25		0.26	
41	0.20	0.04	0.09	0.77	0.20	0.07		0.35	0.08
42	0.14	0.17	0.14	0.69	0.23	0.31	0.39	0.27	0.04
43	0.26	0.07	0.05	0.88	0.23	0.19		0.16	0.02
44	0.33	0.11	0.36	2.21	0.37	0.21		0.50	0.12
14	0.12	0.10	0.04	0.05	2.06	0.18	1.20	0.22	0.07
15	0.36	0.15	0.04	2.07	0.13	0.11	1.34	0.21	0.05
10	0.12	0.08	0.04	1.37	0.02	0.02		0.32	
11	0.20	0.14	0.04	1.47	0.04	0.26		0.09	
31	0.07	0.06	0.28	0.06	1.57	0.12	0.12	0.20	0.02
32	0.05	0.12	0.18	1.36	0.18	0.06	0.05	0.24	0.06
34	0.02	0.10	0.20	0.02	0.17	0.91	0.16	0.12	0.03
45	0.09	0.10	0.10	1.32	0.06	0.08		0.29	0.10
4	0.19	0.17	0.04	2.15	0.13	0.40	0.20	0.32	
5	0.13	0.11	0.04	2.83	0.50	0.09		0.26	
16	0.41	0.18	0.04	0.12	0.06	0.45	0.37	0.22	0.03
17	0.14	0.08	0.04	0.74	0.06	0.18	0.34	0.40	0.03
18	0.09	0.14	0.04	0.62	0.11	0.25	0.48	0.22	0.02
19	0.03	0.04	0.04	0.16	0.10	0.15		0.00	
20	0.11	0.06	0.15	0.63	0.06	0.36	0.38	0.27	0.02
35	0.07	0.07	0.04	0.12	0.09	0.14		0.00	
27	0.10	0.16	0.22	0.74	0.12	0.22		0.08	0.06
28	0.15	0.16	0.11	1.36	0.12	0.29	0.79	0.26	0.04
29	0.13	0.14	0.41	1.28	0.12	0.33	0.37	0.40	0.04
30	0.07	0.05	0.26	0.76	0.36	0.35	0.11	0.26	0.04
36	0.06	0.15	0.14	0.93	0.09	0.11	0.51	0.15	0.06
37	0.09	0.08	0.11	0.42	0.11	0.14		0.19	0.02
21	0.08	0.13	0.30	1.70	0.15	0.12	0.31	0.11	
22	0.09	0.20	0.14	1.90	0.19	0.17	0.55	0.46	0.09
23	0.13	0.19	0.14	1.84	0.18	0.25	0.35	0.00	0.04
24	0.47	0.11	0.36	2.12	0.15	0.29	0.30	0.16	0.05
25	0.03	0.17	0.22	1.15	0.25	0.14	0.04	0.26	0.02
26	0.06	0.18	0.19	0.91	0.26	0.20		0.29	0.04
38	0.04	0.13	0.31	1.94	0.35	0.18	0.18	0.46	0.07
39	0.21	0.21	0.28	1.33	0.44	0.26		0.28	0.07
12	0.22	0.10	0.04	0.02	2.55	0.02		0.23	0.03

Table 4: raw data part 3

The row „File“ shows the NMR-data file, sample number and the animal number temperature, CO_2, weight of the heart tissue and D_2O were omitted but can be found Table 2. All metabolites are given in mmol/L.

File	Glucarate	Gluconate	Glu-6-P	Glutamate	Glutamine	GaMme	Glycine	Glycolate	Ile
6	0.25	0.23	0.46	0.47	2.78	0.47	3.39	5.65	0.09
7	0.65	0.23	0.15	0.43	0.94	0.11	0.03	0.47	0.09
8	0.21	0.22	0.64	0.19	0.46	0.04	0.36	0.16	0.10
9	0.60	0.08	0.10	0.46	0.76	0.04	0.19	0.54	0.10
41	0.32	0.17	0.25	0.53	0.21	0.03	0.26	0.86	0.04
42	0.34	0.31	0.12	0.69	0.31	0.03	0.27	0.96	0.05
43	0.14	0.15	0.12	0.09	0.21	0.01	0.17	0.11	0.02
44	0.62	0.49	0.69	1.74	0.70	0.03	0.56	0.13	0.27
14	0.27	0.21	0.18	1.71	0.61	0.01	0.27	1.36	0.09
15	0.39	0.23	0.95	1.38	0.39	0.02	0.69	0.03	0.09
10	0.37	0.18	0.16	0.36	0.46	0.03	0.03	0.02	0.03
11	0.10	0.17	0.31	0.10	0.69	0.03	1.42	0.05	0.04
31	0.34	0.38	0.39	0.60	0.20	0.01	0.15	0.59	0.07
32	0.44	0.26	0.27	1.09	0.64	0.03	0.24	0.29	0.12
34	0.27	0.10	0.34	0.19	0.28	0.01	0.15	1.07	0.06
45	0.06	0.24	0.15	0.16	0.23	0.03	0.02		0.02
4	0.31	0.22	0.70	0.72	0.34	0.03	0.31	0.17	0.08
5	0.34	0.09	0.07	0.29	0.26	0.05	0.16	0.02	0.12
16	0.34	0.23	0.11	1.09	0.13	0.05	0.37	1.25	0.06
17	0.27	0.29	0.25	0.92	0.30	0.06	0.39	0.08	0.05
18	0.03	0.23	0.05	0.50	0.16	0.03	0.27	0.32	0.03
19	0.12	0.10	0.08	0.18	0.10	0.01	0.11	0.10	0.02
20	0.21	0.41	0.30	1.36	0.21	0.04	0.48	0.51	0.09
35	0.16	0.06	0.09	0.30	0.12	0.02	0.12	0.30	0.06
27	0.23	0.07	0.29	0.57	0.33	0.03	0.20	0.46	0.03
28	0.23	0.27	0.29	0.71	0.24	0.03	0.33	0.57	0.06
29	0.23	0.52	0.08	1.36	0.37	0.02	0.33	0.59	0.11
30	0.31	0.15	0.13	0.66	0.10	0.02	0.14	0.66	0.07
36	0.24	0.21	0.13	0.56	0.36	0.01	0.27	0.65	0.07
37	0.24	0.11	0.13	0.35	0.14	0.01	0.13	0.39	0.05
21	0.24	0.20	0.35	0.73	0.61	0.01	0.30	0.77	0.06
22	0.24	0.61	0.17	1.28	0.70	0.02	0.49	0.86	0.08
23	0.24	0.63	0.26	1.42	0.75	0.06	0.45	1.09	0.05
24	0.40	0.52	0.48	1.78	0.51	0.04	0.50	0.95	0.10
25	0.23	0.42	0.15	1.00	0.20	0.02	0.28	0.58	0.07
26	0.21	0.29	0.20	0.53	0.30	0.02	0.29	0.94	0.08
38	0.56	0.79	0.62	0.71	0.65	0.03	0.26	0.81	0.15
39	0.39	0.55	0.12	1.55	0.44	0.04	0.38	0.97	0.02
12	0.25	0.19	0.22	0.60	0.19	0.01	0.04	0.04	0.03

Table 5: raw data part 4

The row „File“ shows the NMR-data file, sample number and the animal number temperature, CO_2, weight of the heart tissue and D_2O were omitted but can be found Table 2. All metabolites are given in mmol/L.

File	Lactate	Leucine	myo-Inositol	N-Acetyl-glutamine	N-Acetyl-glycine	O-Phospho-ethanolamine	Propionate	Ribose
6	0.86	1.65	0.28	0.12	0.05	0.39	0.12	8.65
7	2.20	0.22	0.28	0.12	0.04	0.39	0.12	0.41
8	3.51	0.22	0.22	0.12	0.05	0.20	0.12	0.32
9	2.39	0.14	0.36	0.12	0.05	0.39	0.09	0.38
41	2.64	0.18	0.24	0.07	0.06	0.34	0.10	0.47
42	4.42	0.16	0.20	0.11	0.08	0.87	0.04	0.42
43	1.56	0.05	0.07	0.04	0.05	0.35	0.05	0.20
44	4.99	0.15	0.31	0.17	0.16	0.44	0.17	1.07
14	4.18	0.44	0.23	0.04	0.05	0.63	0.04	0.37
15	4.33	0.22	0.28	0.04	0.13	0.39	0.14	1.12
10	1.83	0.06	0.18	0.06	0.03	0.29	0.02	0.27
11	2.37	0.14	0.17	0.03	0.04	0.29	0.07	0.08
31	2.38	0.12	0.13	0.06	0.05	0.24	0.07	0.26
32	3.32	0.28	0.20	0.11	0.08	0.50	0.07	0.21
34	2.00	0.17	0.02	0.06	0.07	0.24	0.07	0.19
45	2.34	0.12	0.19	0.03	0.06	0.22	0.02	0.41
4	4.91	0.37	0.18	0.06	0.05	0.29	0.14	0.54
5	3.86	0.14	0.51	0.04	0.04	0.03	0.12	0.57
16	1.86	0.20	0.28	0.05	0.10	0.54	0.09	0.33
17	1.60	0.17	0.28	0.02	0.09	0.64	0.09	0.20
18	1.69	0.05	0.11	0.04	0.05	0.39	0.05	0.40
19	0.78	0.03	0.03	0.02	0.01	0.23	0.01	0.16
20	2.02	0.18	0.17	0.08	0.06	0.47	0.08	0.24
35	0.56	0.08	0.08	0.06	0.02	0.39	0.06	0.24
27	2.58	0.14	0.16	0.09	0.06	0.39	0.04	0.36
28	4.68	0.24	0.11	0.09	0.06	0.39	0.13	0.28
29	2.85	0.26	0.16	0.11	0.06	0.66	0.07	0.16
30	2.93	0.16	0.08	0.09	0.06	0.17	0.07	0.19
36	2.87	0.22	0.07	0.07	0.06	0.47	0.03	0.18
37	1.30	0.14	0.12	0.07	0.05	0.13	0.04	0.09
21	2.94	0.17	0.09	0.10	0.07	0.47	0.07	0.33
22	5.45	0.20	0.32	0.09	0.09	0.45	0.05	0.59
23	4.71	0.24	0.32	0.13	0.11	0.19	0.03	0.60
24	3.19	0.17	0.12	0.10	0.07	0.92	0.07	0.58
25	2.10	0.13	0.24	0.09	0.12	0.57	0.10	0.19
26	3.04	0.20	0.09	0.14	0.09	0.31	0.05	0.33
38	5.55	0.23	0.32	0.08	0.15	0.39	0.11	0.60
39	4.98	0.28	0.18	0.13	0.10	0.53	0.10	0.33
12	1.98	0.04	0.16	0.03	0.05	0.27	0.06	0.20

Table 6: raw data part 5

The row „File“ shows the NMR-data file, sample number and the animal number temperature, CO_2, weight of the heart tissue and D_2O were omitted but can be found Table 2. All metabolites are given in mmol/L.

File	sn-Glycero-3-phosphocholine	Succinate	Taurine	Theophylline	Threonate	Trimethylamine	Trimethyl-amine N-oxide	Valine
6	0.51	0.41	30.17		4.59		2.99	0.56
7	0.56	0.01	8.47		1.25		0.13	0.06
8	0.69	0.03	10.05	0.02	0.68		0.39	0.08
9	0.03	0.08	9.41	0.07	0.87		0.03	0.06
41	0.11	0.05	8.91				0.89	0.02
42	0.27	0.12	10.25	0.01			0.34	0.05
43	0.06	0.01	7.91			0.01	0.16	0.02
44	0.19	0.01	16.56				0.27	0.09
14	0.46	0.12	13.35	0.02	0.39	0.09	1.25	0.15
15	0.16	0.09	16.32	0.01	0.76	0.08	0.50	0.16
10	0.07	0.06	8.24	0.01	0.25		0.03	0.06
11	0.09	0.04	8.89	0.01	0.04		0.15	0.02
31	0.24	0.08	7.51			0.03	0.42	0.04
32	0.33	0.04	10.56			0.01	0.31	0.12
34	0.24	0.02	6.90			0.01	0.30	0.04
45	0.19	0.08	8.50				0.10	0.04
4	0.07	0.10	16.64	0.04	0.55	0.07	1.57	0.12
5	0.07	0.05	16.72	0.09	0.24		3.85	0.17
16	0.41	0.05	9.57	0.01	0.34	0.03	2.20	0.07
17	0.09	0.05	10.30		0.43	0.03	3.10	0.06
18	0.40	0.05	7.55		0.22	0.04	1.58	0.03
19	0.13	0.02	2.57				0.56	0.01
20	0.29	0.03	7.51			0.03	1.51	0.05
35	0.13	0.01	3.08				0.94	0.03
27	0.30	0.05	8.63				0.69	0.09
28	0.37	0.11	12.88			0.08	1.04	0.04
29	0.68	0.10	12.52			0.05	0.81	0.09
30	0.21	0.08	8.71			0.02	0.96	0.05
36	0.19	0.04	8.01			0.03	0.61	0.08
37	0.18	0.03	5.17				0.53	0.04
21	0.20	0.09	12.56			0.02	0.59	0.08
22	0.48	0.14	18.72			0.05	0.71	0.11
23	0.42	0.17	17.81			0.03	0.84	0.11
24	0.19	0.13	18.92			0.02	0.67	0.11
25	0.25	0.07	12.06				0.59	0.06
26	0.12	0.08	11.75				0.35	0.06
38	0.34	0.09	14.07				0.16	0.11
39	0.19	0.12	14.27				0.10	0.11
12	0.26	0.03	5.71	0.01	0.26		0.28	0.01

Table 7: Concentrations of amino acids [mg/g]
Shown are the concentrations (mg/g) of amino acids for the means for each treatment group.

	Alanine [mg/g]	Glutamate [mg/g]	Glycine [mg/g]	Isoleucine [mg/g]	Leucine [mg/g]	Valine [mg/g]
3°C 390µatm CO_2	0.71	0.34	0.52	0.08	0.49	0.15
8°C 390µatm CO_2	0.35	0.52	0.10	0.04	0.11	0.03
12°C 390µatm CO_2	0.31	0.38	0.17	0.04	0.10	0.03
16°C 390µatm CO_2	0.86	0.51	0.12	0.09	0.24	0.12
3°C 1170µatm CO_2	0.28	0.59	0.12	0.05	0.09	0.03
8°C 1170µatm CO_2	0.35	0.51	0.09	0.04	0.13	0.04
12°C 1170µatm CO_2	0.28	0.48	0.09	0.03	0.08	0.03
16°C 1170µatm CO_2	0.26	0.45	0.05	0.03	0.07	0.03

Table 8: Literature values of amino acids
Amino acid concentrations (mg/g) (Özden, 2005)

	[mg/g]
Alanine	7.51
Glutamate	12.14
Glycine	5.88
Isoleucine	6.42
Leucine	9.49
Valine	6.90

Figure 16: exemplary R-plot