Temperature adaptability of lipid composition in polar fish: a comparison between sub- and high-Antarctic species

Master thesis

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CONTENTS

Abbreviations

°C	degree Celsius
µg	microgram
µl	microliter
µm	micrometer
^{13}C	carbon isotope (mass of ~13 u)
^{1}H	hydrogen isotope (mass of ~1 u)
^{31}P	phosphorous isotope (mass of ~31 u)
ANOVA	analysis of variance
bar	unit of pressure
BWT	body weight
$CDCl_3$	deuterated chloroform ($CHCl_3$)
cer	ceramide
cf.	confer (compare)
chol	cholesterol
CL	cardiolipin
cm	centimeter
CTD	conductivity temperature depth sensor
D_2O	deuterated water (H_2O)
DCM	dichloromethane
DHA	docosahexaenoic acid
e.g.	exempli gratia (for example)
ELSD	evaporative light scattering detector
et al.	et alii (and others)
FA	fatty acid
FFA	free fatty acids
FID	free induction decay
g	gram
g_a	gravitational acceleration
h	hour
HPLC	high performance liquid chromatography
hr MAS	high resolution magic angle spin
Hz	Hertz
i.a.	inter alia (among other things)
i.e.	id est (that is)
J	Joule
k	kilo (SI prefix thousand)
KCl	potassium chloride
$KClO_4$	potassium perchlorate
KOH	potassium hydroxide
LWT	liver wet weight
M	molar concentration
m	meter
M/C	methanol/chloroform
MAG	monoacylglycerol
mg	milligram
MHz	Megahertz
min	minutes
ml	millilitre
M_{O2}	oxygen consumption
n	number of replicates
n.d.	not detected
nm	nanometer
NMR	nuclear magnetic resonance
no	numero (number)
O_2	oxygen
p	probability constant
PA	phosphatidic acid
PC	phosphatidylcholine
PCA	perchloric acid
PE	phosphatidylethanolamine
pH	measure of acidity
PI	phosphatidyl inositol
P_{O2}	oxygen partial pressure
POL	phospholipids
ppm	parts per million
PUFA	polyunsaturated fatty acid
rel. units	relative units
rf	radio frequency
rpm	revolutions per minute
s	second
S	south
SD	standard deviation
SL	standard length
T	temperature
TAG	triacylglyceride
TMS	tetramethylsilane
UFA	unsaturated fatty acid
UV	ultra violet
v	volume
W	west
WE	wax ester

Abstract

Despite evidence for distribution shifts of single species and ecosystem changes as a reaction to climatic warming, little is known about direct implications of temperature changes on the role of physiology and adaptability for species distribution. In this study the effect of a short-term temperature increase on metabolic and lipid composition of liver tissue in the sub-Antarctic fish species *Lepidonotothen squamifrons* and the high-Antarctic species *Trematomus hansoni* were assessed using high-performance liquid chromatography and nuclear magnetic resonance spectroscopy.

Data of this study seems to be the first data available for liver lipid composition of the two investigated species. Composition of succinate likewise phospholipid levels indicated temperature induced changes between 0 and 6 °C for *T. hansoni* and 2 and 9 °C for *L. squamifrons*. This complies well with observations of temperature dependent respiration, which indicated a rise in energy demand around 4 °C for the high- and sub-Antarctic species alike. Although changes in anaerobic metabolites found here were not significant, combination of results from this study and respiration experiments suggest that experimental temperature of 6 °C might be close to upper critical temperatures of *T. hansoni*, while *L. squamifrons* might be able to tolerate acute temperatures above 9 °C.

Considering interspecies differences, higher total lipid and triacylglyceride levels support the suggestion of a higher degree of metabolic cold adaptation in high-Antarctic *T. hansoni* compared to sub-Antarctic *L. squamifrons*.

The results of this study comply well with current ideas about temperature dependence of lipids and other metabolic compounds in fish. Recent suggestions about metabolic cold adaptation in Antarctic fish were confirmed and results indicated the existence of different degrees of cold adaptation in Antarctic fish, depending on variability of habitat temperature.

1. Introduction

Temperature is one of the abiotic key factors in the marine environment. In ectotherm fish, body temperature is determined by ambient temperature, affecting metabolic processes and shaping distribution limits.

The concept of oxygen and capacity limited thermal tolerance in marine water breathers reveals how temperature and hypoxia are interlinked, with specific implications for the whole organism functioning (Pörtner et al. 1998). Systemic hypoxemia can be caused by thermal stress. Thermally induced hypoxemia together with the impact of temperature are considered to be driving factors shaping acclimation responses from molecular to whole organism levels (Pörtner 2002a; Mark et al. 2005).

The temperature window of an organism is defined by the so-called optimal temperature, the pejus temperatures and the upper and lower critical temperatures. At the optimal temperature, an organism meets best conditions, enhancing growth and reproduction. At upper and lower critical temperatures, oxygen supply can no longer match the increasing demand and metabolism reverts to anaerobic pathways, which is indicated by the accumulation of anaerobic metabolites (Zielinski & Pörtner 1996). Further, those temperatures, which mark a decreasing P_{O_2} in body fluids and therefore lead to a deterioration of conditions for the animal, are defined as upper and lower pejus temperatures (Frederich & Pörtner 2000).

Former studies revealed various statistical relations between temperature and organismic traits (Pauly 1980; Fonds et al. 1989; Brodte et al. 2006a; Hildebrandt et al. 2011). Pörtner and Knust (2007) showed that thermally restricted oxygen delivery in eelpout (*Zoarces vivparus*) complies with ambient temperatures beyond which growth performance and abundance decline (Pörtner & Knust 2007). Growth and reproduction of an organism depend upon surplus aerobic energy being available after baseline costs of maintenance have been met. As this energy is only available within the window of aerobic scope (range of possible oxidative metabolism) (Pörtner et al. 2005), optimal temperature, characterised by low maintenance costs was found to go in hand with high growth rates e.g. in the boreal eelpout (*Zoarces viviparus)* (Brodte et al. 2006a), which in turn supports population abundances.

Declining width of thermal windows with decreasing habitat temperature variation have been observed for different eelpout species (Knust et al. unpublished; Mark et al. 2002; van Dijk et al. 1999). Antarctic *Pachycara brachycephalum*, living at temperatures between -1 and 2 °C, displays a quite narrow thermal window. In contrast, the boreal eelpout *Zoarces viviparus* (acclimated to 12 °C) from the German Wadden Sea, where temperatures vary widely, has a much broader thermal tolerance between 0 and 24 °C.

Understanding the processes enrolled in physiological acclimation and adaptation to temperature becomes more and more urgent because of potential effects of global warming on both, aquatic and terrestrial ecosystems. Various studies have recently evaluated the effects of rising temperatures on ecosystem level, but implications of a temperature increase for single species are still mostly unclear. However, physiological adaptation to temperature seems to play an essential role in governing distribution patterns e.g. of fish (Pörtner 2002b; Perry et al. 2005; Pörtner & Knust 2007).

Antarctica is considered to have been a thermally very stable environment over the last million years. As a consequence, a lot of Antarctic species have become highly stenothermal which is reflected in very narrow temperature windows (Somero & DeVries 1967; Pörtner & Knust 2007).

Today, the Western Antarctic Peninsula is considered as one of the planet's "hot spots" of climatic warming. Waters surrounding the Western Antarctic Peninsula are warming more rapidly than other parts of the world's oceans (Meredith & King 2005). Over the past 50 years, surface waters near the Antarctic Peninsula have risen in temperature about 1 °C and another increase of 2 °C is predicted for the next century (Murphy & Mitchell 1995; Meredith & King 2005). In the surrounding ecosystems cascading effects of rapid regional warming have already become evident. Changes in species abundance and shifts in distribution have been reported, going in hand with alterations in community structure and food web dynamics (Aronson et al. 2009; Schofield et al. 2010; Walther 2010). Besides broad scale ecological impacts of climate change, scientific interest in the effects of temperature elevation on individual species increased. The thermal tolerance of a species defines its thermal window and yields insight into physiological plasticity and adaptability regarding changes in ambient temperature.

Fish play an important role in Antarctic ecosystems. Being prey and predators alike, they are a crucial link between lower and higher trophic levels. The Antarctic fish fauna is dominated by the endemic perciform suborder *Notothenioidei*. Notothenioid fish display a variety of adaptations at molecular level to maintain essential body functions in their extreme thermal habitat (Eastman 1993; Clarke & Johnston 1996). Besides, different physiological processes seem to be slower in Antarctic fish, compared to their temperate counterparts. Polar fish show slower growth rates, obtain smaller maximal sizes and grow older than comparable temperate species (Brodte et al. 2006a; Hildebrandt et al. 2011; La Mesa & Vachi 2001; Kock & Everson 1998).

In many fish species a general trend towards increased lipid accumulation with increasing latitude has been found (Sidell 1991; Brodte et al. 2008). Generally, higher lipid levels in Antarctic species are thought to be a consequence of cold-adaptation. High mitochondrial densities were found in Antarctic fish muscles (Johnston et al. 1998; Peck 2002). The development of an increased mitochondrial density is accompanied by a shift from carbohydrate to lipid metabolism, going in and with the preferred use of lipids by mitochondria as well as enhanced lipid storage on intracellular and whole-organism levels (Crockett & Sidell 1990; Sidell & Hazel 2002; Pörtner 2002b; Pörtner et al. 2004). Thus, lipids play a crucial role in Antarctic fish, being essential storage compound likewise important structural components in membranes.

The hypothesis of homeoviscous adaptation suggests that optimal membrane function is limited to a certain range of membrane fluidities. At warmer temperatures membranes are suggested to become "hyperfluid", while fluidity decreases with lower temperatures, constraining membrane related activities in both cases. Exposure to temperatures above or below the thermal range of optimal fluidity, are thought to initiate alterations in lipid composition of membranes to offset temperature effects and keep membrane fluidity in the optimal range (Miller et al. 1976; Hazel 1995; Pruitt 1988). At lower temperatures, proportions of unsaturated fatty acids stabilize membrane fluidity, thus keeping cell transport mechanisms going (Hazel 1995). Saturated fatty acyl chains are supposed to confer rigidity while unsaturated fatty acids, especially fatty acyl chains with *cis*-double bonds confer fluidity to lipid assemblages in membranes (Cribier et al. 1993). Temperature induced reordering of the membrane's lipid phase is considered to influence activity of membrane bound enzymes and therefore reaction rates (Bell et al. 1986).

Moreover, the composition and ratios of phospholipids such as phosphatidylethanolamine and phosphatidylcholine, especially regarding fatty acid composition and head group structure, affect the biophysical attributes of membranes (Hazel 1995). Phosphatidylcholine with a large cylindrically shaped head group allows tight packing, while the conically shaped phosphatidylethanolamine has a

disordering impact on bilayer structures and presumably impacts trans-membrane processes (Hazel 1995).
Besides, Hulbert and Else (1999) proposed polyunsaturated fatty acids in membranes, particularly docosahexaenoic acid, to play a pacemaker role for metabolism. They suggested that an increased polyunsaturation of the membrane bilayer could increase metabolic activity by stimulating the activity of membrane proteins, thus acting as a form of pacemaker.

In the framework of this study, temperature dependent lipid and metabolite profiles were analysed to check for changes in cellular composition induced by a short-term temperature increase and possible parallels to the whole animal level (oxygen consumption). To assess possible differences in the potential of species from different environmental temperature regimes, a sub- and a high-Antarctic species, *Lepidonotothen squamifrons* (figure 1) and *Trematomus hansoni* (figure 2), were compared.
The grey notothen, *L. squamifrons*, as well as the striped notothen, *T. hansoni*, are periform fish, belonging to the family *Nototheniidae*. *L. squamifrons* is a benthopelagic species living at depths between 10 and 900 m. It is common in temperate waters around 45 °S–56 °S, in the Southern Ocean near South Georgia and Bouvet Island as well as close to some sub-Antarctic islands. The grey notothen feeds mainly on macrozooplankton, such as crustaceans, cnidarians and thaliaceans, as well as small midwater fish.
Similarly, *T. hansoni* is a demersal species, living in a depth range of 5 to 549 m. But in contrast, this species is found in polar regions between 53 °S and 78 °S, in the Southern Ocean. The striped notothen feeds on small fish, krill and other euphausiids, polychaetes, copepods, amphipods, isopods and small gastropods. (Gon & Heemstra 1990)
These two species were chosen as model organisms, due to their comparable lifestyle and the fact, that they were easily attainable by bottom trawl, which is an important practical criterion for model organisms from remote regions, such as Antarctica.

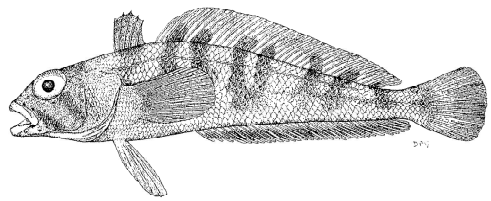

Figure 1: Grey notothen, *Lepidonotothen squamifrons* (copyright by Gon & Heemstra 1990)

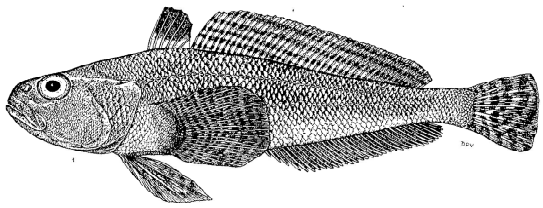

Figure 2: Striped notothen, *Trematomus hansoni* (copyright by Gon & Heemstra 1990)

2. Material & methods

2.1. Model organisms

Grey notothen, *Lepidonotothen squamifrons*, were caught near South Georgia (53° 24.63' S, 42° 40.40' W) at a depth of 309 m by bottom trawl. Striped notothen, *Trematomus hansoni*, were caught in the Eastern Weddell Sea (70° 51.30' S, 10° 35.35' W) at a depth of 223.5 m (bottom trawl). All animals were directly transferred to aquarium tanks. *L. squamifrons* were kept at habitat temperature of 2 °C (temperature determined at trawl depth with CTD), while *T. hansoni* were kept at 0 °C, as habitat temperature of -1.9 °C was technically not feasible. Fish were allowed to recover from trawling for 14 days. During this time animals were starved, since some fish were used for respiration experiments before tissue sampling and postprandial activity is known to raise oxygen consumption (Jobling 1983). Sampling was done on board of research vessel *Polarstern* from February until May 2011 during expedition ANTXXVII-3/4.

Due to only a limited number of *T. hansoni* caught on this cruise, samples of animals 1–6 were derived from a cruise in December 2003. Those *T. hansoni* were caught in the Eastern Weddell Sea (70° 56.57' S, 10° 31.98' W) by bottom trawl at a depth of 299.6 m. Treatment after catch was done as stated above.

For *L. squamifrons*, mean standard length of fish used in this experiment was 25.0 ±1.8 cm, while mean body weight was 269.1 ±51.5 g (table 1). For *T. hansoni*, mean standard length was 26.0 ±2.5 cm. Mean body weight of *T. hansoni* was 230.8 ±70.8 g (table 2). Standard length and weight of control and temperature group individuals of *T. hansoni* did not show any significant differences. For *L. squamifrons*, t test revealed significant differences in standard length between control and temperature group ($p = 0.0149$), while no significant differences were found in body weight. However, as only a limited number of individuals were available, differences in standard length were not avoidable. Interspecies comparison of length and weight did not show any significant differences between *L. squamifrons* and *T. hansoni*.

Mean liver wet weight of *L. squamifrons* was 4.3 ±1.1 g, while *T. hansoni* had a mean liver wet weight of 5.5 ±2.3 g.

Table 1: Total body weight (BWT), standard length (SL), liver wet weight (LWT) and water temperature of control and temperature group of *L. squamifrons*

L. squamifrons					
Fish no.	**SL [cm]**	**BWT [g]**	**LWT [g]**	**T [°C]**	
1	24.6	264.5	4.8	2	Control group
2	21.7	203.4	4.5	2	
3	25.0	231.2	6.5	2	
4	24.4	322.6	3.3	2	
5	22.0	215.4	4.7	2	
6	24.3	238.8	3.5	5	Temperature group
7	26.9	302.2	4.3	5	
8	25.6	261.5	5.1	6	
9	28.4	394.0	5.9	7	
10	24.5	233.5	3.2	7	
11	25.1	253.3	4.0	7	
12	25.8	302.2	2.8	7	
13	26.7	275.8	3.8	9	
Mean	25.0	269.1	4.3		
SD	1.8	51.5	1.1		

Table 2: Total body weight (BWT), standard length (SL), liver wet weight (LWT) and water temperature of control and temperature group of *T. hansoni*

T. hansoni					
Fish no.	**SL [cm]**	**BWT [g]**	**LWT [g]**	**T [°C]**	
1	23.3	203.8	4.2	0	Control group
2	26.0	165.8	3.3	0	
3	24.4	157.6	3.4	0	
4	32.2	386.2	10.3	0	
5	28.9	222.5	5.5	0	
6	25.1	140.5	2.8	0	
7	25.0	275.5	8.2	4	Temperature group
8	25.0	225.6	4.7	4	
9	23.8	213.2	4.7	5	
10	25.8	247.2	7	5	
11	26.2	300.8	6.2	6	
Mean	26.0	230.8	5.5		
SD	2.5	70.8	2.3		

2.2. Temperature incubation

Samples of temperature incubated animals were taken from fish which have been used for temperature dependent routine oxygen consumption measurements before. For respiration experiments, the fish were placed in respiration chambers at acclimation temperature of 2 °C (*L. squamifrons*) and 0 °C (*T. hansoni*). Subsequently, temperature was raised by 1 °C per 24 hours continuously. Experimental temperatures for *L. squamifrons* ranged from 2 °C to up to 9 °C for one individual, while *T. hansoni* were treated with temperatures from 0 °C to 6 °C. Measurements were stopped, when a drastic rise in oxygen consumption was observed, the animal lost balance or seemed to be extraordinary stressed, as e.g. indicated by irregular movement of opercula.
A control group of each species was kept at 2 °C (*L. squamifrons*) and 0 °C (*T. hansoni*) in the aquarium tanks for a similar period as the temperature group (cf. table 1 & 2) until fishes were killed for tissue sampling. For a more detailed description of the experimental setup for respiration experiments see project report by Sandersfeld (2011).

2.3. Tissue sampling

Liver tissue was chosen for analysis of lipid and metabolic profiles. The livers plays a central role in metabolic processes and has a high turnover rate (Houlihan et al. 1988) and storage potential for lipids (Larsen et al. 2001).
For tissue sampling, fish from the control group were taken directly from the aquarium container, while temperature incubated fish were taken from respiration chambers. After body weight and length were determined, the animals were killed and liver tissue was quickly removed, weighed and immediately frozen in liquid nitrogen. During the whole dissection procedure the dead animal was kept on ice, to keep metabolic changes in the tissue at a minimum. Tissue samples were stored at -80 °C until extraction.

2.4. Extraction procedures

In the framework of this study, lipids and water soluble compounds were analysed. For lipid extraction, the common procedure according to Folch et al. (1957) was applied. Two techniques were used for extraction of water soluble compounds, perchloric acid (PCA) extraction and the water soluble phase of Folch's extraction protocol, tested with a number of samples (*L .squamifrons*, control group, fish no. 1-5) and compared. Subsequently, Folch's extraction protocol was used for analysis of water soluble compounds for all samples.
Before extraction, liver tissue was ground to a fine powder with mortar and pestle while being kept under liquid nitrogen. This step ensured a thorough homogenization of the samples and allowed better mixing with extraction reagents.

Folch extraction

The following extraction protocol was modified according to Folch et al. (1957).
About 0.2 g ground tissue were transferred to glas centrifuge tubes and 6 ml of reagent-grade methanol:dichloromethane (2:2, v/v) were added. After thorough mixing of the solution, the

emulsion was sonicated for 10 min at 20 °C. Subsequently, about 2 ml of 0.88 % KCl were added by a Pasteur pipette, the solution mixed and centrifuged at 2000 rpm for 5 min. After centrifugation, two phases were visible. The lower phase, containing the lipid compounds, was transferred to a glas vial using a Pasteur pipette. The upper phase, containing water soluble compounds, was kept in the tube, diluted with another 2 ml of dichloromethane (DCM), thoroughly shaken and centrifuged again (2000 rpm, 5 min). This step was repeated once, so that finally one extraction with methanol:dichloromethane (2:2, v/v) was followed by two extractions with DCM. The lower phase from all 3 extraction steps was pooled in the same vial. Both phases, lipid and water soluble compounds, were dried under nitrogen. To accelerate the process, lipid extracts were warmed while desiccation. All extracts were stored at -20 °C until analysis.
For lipid analysis, only samples of *L. squamifrons* (no. 1 to 10) were used, while all samples were taken into account for analysis of water soluble compounds.

PCA extraction

For PCA extraction, 0.1 ml of 0.6 M PCA were prepared in tubes and frozen in liquid nitrogen. About 0.2 ground frozen tissue was added to the frozen PCA. Subsequently, 0.6 M PCA was added to the tube until the total volume of PCA was 5 times the mass of the ground tissue (e.g. 0.2 g tissue + 1 ml PCA in total). The suspension was sonicated for 2 min (360 Watt) at 0 °C. Afterwards samples were centrifuged for 2 min at 0 °C and 600 g_a for phase separation. Water soluble phase was transferred to a new tube and weighed. An amount of 5 M KOH equaling 10 % of the supernatant's weight was added and the pH adjusted to 7.5 by using 5 M and 1 M KOH and 0.6 M PCA. The neutralized solution was placed in a freezer for several minutes and centrifuged again for 10 min at 0 °C (6000 g_a) to remove precipitated $KClO_4$. The supernatant was lyophilized and stored at -20 °C.

2.5. Analysis of tissue extracts

One dimensional ^{1}H-NMR spectroscopy was used for the analysis of water soluble compounds in tissue extracts, whereas for lipid analysis chemical methods were combined with NMR spectroscopy. With NMR analysis complete lipid profiles can be measured and analysed for any possible variances and changing compounds can be characterised afterwards. This "profiling"-approach, which also accounts for techniques such as for mass spectrometry, could be a great help for e.g. investigations of temperature or ocean acidification effects on animals.
In this study, the potential of an existing NMR spectroscopy setup for the measurement of lipid profiles in fish liver tissue was tested. To review the obtained results, HPLC measurements were carried out simultaneously to check validity of NMR data.

2.5.1. Nuclear magnetic resonance spectroscopy

Principle

Nuclear magnetic resonance (NMR) spectroscopy has become a method of choice to determine the structure of organic compounds. The method is based on the nuclear spin characteristics of atoms with uneven numbers of protons and/or neutrons in their nucleus.

The nuclei of most elemental isotopes posses a characteristic spin (I), which may be integral (e.g. $I = 1, 2, 3\ldots$) or fractional (e.g. $= \frac{1}{2}, \frac{2}{3}, \frac{5}{2}$). However, there are also elemental isotopes whose nuclei have no spin ($I = 0$) such as ^{12}C or ^{16}O. As the isotopes of interest for the determination of organic compounds such as ^{1}H, ^{13}C and ^{31}P all have a spin of $I = \frac{1}{2}$, the following part will focus on this case.

Most nuclei have an angular momentum (P). When the assumed to be ball-like nuclei spins around its own axis, this quantised angular momentum is generated:

$$P = \sqrt{I\,(I+1)}\,h$$

Here, h is the Planck's constant ($h = \frac{h}{2\pi} = 6.6256\,Js$) and I is the intrinsic angular momentum, the spin. The nuclei's spinning charge generates a magnetic field. Thus, there is a magnetic momentum (μ), which is proportional to the angular momentum (P).

In Figure 3 below, the spinning nucleus is positioned in a Cartesian coordinate system, with the external magnetic field oriented (B_0) at the z-axis. The frequency of precession, also called Larmor frequency (ω_0) behaves proportional to the strength of the magnetic field.

$$\omega_0 = \gamma B_0$$

The proportionality constant, γ, is characteristic for every isotope or element and gives information about the nuclei's detection sensitivity in NMR experiments. The proportionality constant together with the natural occurrence of an isotope define its usefulness for NMR experiments. ^{1}H, with a high proportionality constant likewise high natural occurrence in biological samples is a particularly suitable nuclei for NMR spectroscopy.

For nuclei with spin of $I = \frac{1}{2}$, there are two possible spin states, $+\frac{1}{2}$ and $-\frac{1}{2}$ when an external magnetic field (B_0) is present. While the lower energy $+\frac{1}{2}$ state is adjusted to the external field, the higher energy $-\frac{1}{2}$ state is opposed to the external field. The strength of the external magnetic field determines the difference in energy between the two spin states. When the external field $B_0 = 0$ the two spin states have the same energy, but diverge as the strength of the field increases (figure 3).

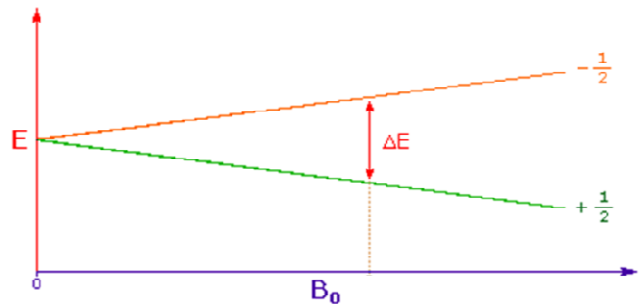

Figure 3: The two spin states have the same energy when the external field is zero, but diverge as the field increases. (copyright by Rogers 2011).

In NMR-experiments, the energy difference (ΔE) of the two spin states is usually given as a frequency of units MHz. Depending on magnetic field strength and the nucleus studied, ΔE commonly ranges from 20 to 900 MHz.

In a sample containing large numbers of spin $\frac{1}{2}$ nuclei at equilibrium in a strong external magnetic field (B_0), a slight excess of $+\frac{1}{2}$ spin states moves in the direction of the external field while a smaller number of $-\frac{1}{2}$ spin state nuclei move in the opposite direction. Thus, the general net magnetization lies along the z-axis.

When energy of the Lamor precission frequency, like a radio frequency (rf) irradiation is introduced at the right angle to the external magnetic field, the net magnetization shifts from the z-axis towards the y-axis (figure 4). After the energy input the nuclear spins return to equilibrium, which is called relaxation.

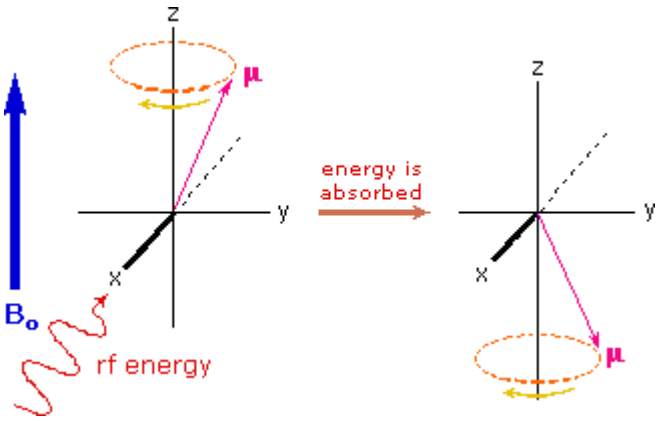

Figure 4: If rf energy with a frequency matching the Larmor frequency is introduced at a right angle to the external field (here along the x-axis), the precessing nucleus absorbs energy and the magnetic moment flips to its $-\frac{1}{2}$ state. (copyright by Rogers 2011).

Relaxation can be divided into two fractions: First, the spin-lattice relaxation (T_1), which is dependent on factors influencing molecular movements in the lattice such as viscosity and temperature. Second, the transversal or spin-spin relaxation (T_2), which is based on energy exchange between spins of high and low energy levels without energy transfer to the atom's environment.
As the relaxation mechanism is a kinetically first order process, the rf signal emitted by the sample decays exponentially, which is called a free induction decay (FID).

In a given strong magnetic field, every structurally distinct group of e.g. hydrogen protons displays a characteristic resonance frequency. In NMR spectroscopy, the sample is exposed to a short burst of rf energy to excite all e.g. protons in the sample simultaneously. The combined and overlapping resonance signals generated as the protons relax are collected by a computer and converted into the frequency domain spectrum using Fourier transformation.
Magnetic resonances are characteristically influenced by the chemical environment of the nucleus measured. The electrons around a nucleus will circulate in a magnetic field and create a secondary induced magnetic field. By opposing the applied field, surrounding electrons and atoms can shield the nucleus. As a consequence, the effective magnetic field (B_{eff}) stimulating the nucleus is much smaller than the applied magnetic field (B_0). The decrease of the effective magnetic field can be quantified by the shielding constant σ.

$$B_{eff} = B_0 - \sigma B_0 = (1 - \sigma)B_0$$

Important factors influencing the effective magnetic field are electron density, electronegativity of neighbouring groups and anisotropic induced magnetic field effects. Thus, the shielding effect influences the resonance of a nucleus depending on its molecular environment. This phenomenon is called chemical shift. Due to the shielding constant various ^{1}H resonances of different hydrogen compounds can be distinguished and measured separately. The detected frequencies are usually referenced against an internal standard, which is assigned the chemical shift of zero.
The difference between the resonance frequency of the measured nucleus (υ_s) and the standard (υ_{ref}) is used to calculate the chemical shift (δ), which is usually assigned in parts per million (ppm). (Friebolin 1992; Rogers 2011)

In NMR spectroscopy, most experiments are performed with liquid state samples. High resolution magic angle spinning (hr-MAS) is a technique for analyzing solid state samples, such as intact biological tissue, but can also be applied for liquid samples. It was first described by Andrew and co-workers (1985) as well as Lowe (1959). Here, the sample is spinned at the magic angle of ~54.74° with respect to the direction of the magnetic field.

Measurements by nuclear magnetic resonance spectroscopy

For NMR spectroscopy water soluble extracts were dissolved in deuterated water (D_2O) containing tetramethylsilane (TMS) as an internal standard, while lipid extracts were dissolved in deuterated chloroform ($CDCl_3$) without internal standard. All extracts were dissolved in a ratio of 1 ml solvent to 0.3 g extracted tissue.
All spectra were measured using a 9.4 T Bruker 400 wide bore Avance NMR spectrometer at a proton frequency of 400 MHz in combination with TOPSPIN 2.1 software and a high resolution magic angle probe (hr MAS). One dimensional ^{1}H-NMR spectrometry was used for all samples. For lipid measurements, the total relaxation delay was 5 s, while using a sweep width of 8012 Hz and 102 k data points. For water soluble compounds, measurements were carried out with a relaxation delay of 12 s, a sweep width of 5000 Hz and 32 k data points. All measurements were carried out at a temperature of 14 °C.

Analysis of NMR spectra

Signals in the NMR spectra were assigned by using chemical shift data from literature as well as from measurements of standard solutions.
Lipids were mainly identified based on data from Gribbestad and co-workers (Gribbestad et al. 2005). They measured lipid extracts (extracted by methanol and chloroform) of muscle of Atlantic salmon (*Salmo salar*) by NMR spectrometry using a DRX 500 Bruker Instrument at a frequency of 500.13 MHz. To ensure accurate peak identification, a second study of Srivastava et al. (2010) was taken into account. They identified lipid compounds in serum extracts (using methanol and chloroform) using a 400 MHz Bruker Biospin spectrometer. The advantage of this study was the identification of phosophatidylethanolamine (PE) as a single substance. Moreover, data recorded here allowed a more accurate assignment of peaks for identification of cholesterol (chol) and triglycerides (TAG). For some compounds, identification was possible by using peaks at different spectral positions. In this case, all possible peaks were analysed and results compared. The peak that was to be determined the most accurate and with the smallest standard deviation was chosen for analysis. Table 3 shows all chemical shifts, resonance protons and data sources used in this study for the identification of lipid compounds in NMR spectra. Chemical shift of phosphatidylethanolamine (PE), phosphocholine (PC) and cholesterol (chol) were checked and confirmed by measurements of standard solutions (5 mg/ml, 1,2-diplamitoyl-rac-glycero-3-phosphoethanolamine (Sigma-Aldrich), L-α-phosphotidylcholine, cholesterol (both derived from Larodan, USA)).
As no standard was available for NMR-measurement of lipid extracts, all spectra were referenced to the water signal at a chemical shift of 4.7 ppm. In this way, comparisons with results of Gribbestad et al. were alleviated as they applied the same procedure. However, due to the lacking internal standard, lipid measurements in this study cannot be related to each other without objections.

Therefore, evaluation of lipid data was done using ratios of compounds to each other rather than results of single compounds. As relative ratios of the different compounds are assumed to be independent of the extract concentration, errors of a lacking internal standard can be avoided. For evaluation of phospholipids (POL), results obtained for PE and PC were added and the sum stated as POL (POL = PE+PC). This procedure allowed comparison to HPLC results as here measured phospholipids were mostly made up by PE and PC.

Table 3: Chemical shifts used for the identification of lipid compounds in NMR spectra.

Chemical shift [ppm]		Compound	Source
0.68		Cholesterol	Srivastava et al. 2010
0.85	-0.89	All FA	Gribbestad et al. 2005
2.38		Docosahexaenoic acid (22:6 FA)	Gribbestad et al. 2005
2.81	-2.84	PUFA	Gribbestad et al. 2005
3.27		Phosphatidylethanolamine	Srivastava et al. 2010
3.35		Phosphatidylcholine	Gribbestad et al. 2005
4.14		Triacylglyceride	Srivastava et al. 2010
5.34	-5.36	UFA	Gribbestad et al. 2005

For analysis of cytosolic compounds in liver extracts, chemical shift data recorded by Warne et al. (2001) as well as Gribbestad et al. (2005) were used. Table 4 shows all chemical shifts and data sources used for the identification of cytosolic compounds in NMR spectra. For measurements of cytosolic fractions, TMS was used as an internal standard and all spectra were referenced to the TMS peak at 0.0 ppm and a relative integral area of 1. Thus, results of cytosolic compounds are presented individually and independent from each other.

Table 4: Chemical shifts used for the identification of water soluble metabolic compounds in NMR spectra.

Chemical shift [ppm]	Compound	Source
1.93	Acetate	Gribbestadt et al. 2005
2.42	Succinate	Warne et al. 2001

For final evaluation, peaks were chosen manually and integral areas calculated by TOPSPIN 2.1 software in relative units. Values of integrals were related to the amount of tissue extracted.

2.5.2. High-performance liquid chromatography

Principle

In general, chromatography is a method for the separation of the components of a mixture. The components to be separated are distributed between two phases. The sample or mixture is dissolved in a fluid, the mobile phase, which carries it through a structure, the stationary phase.
Chromatographic methods are classified depending on the type of mobile and stationary phase, the form in which they are present (e.g. gas chromatography or thin-layer chromatography) as well as the different mechanisms which control separation (e.g. frontal analysis, displacement and elution).
Classical chromatographic separation might be explained starting with a glass column, filled ¾ with a suitable adsorbent. The sample is dissolved in an appropriate solvent, which is applied to the column head in an even band. Pure solvent is allowed to percolate down the column under gravity. By interacting with the mobile phase, the band containing the sample starts to move down the column and its components start to separate. Components only weakly interacting with the adsorbent pass rapidly through the bed, while those interacting with the adsorbent strongly pass more slowly, resulting in a complete separation of the components of a mixture. Thus, solute bands migrate

through the chromatographic bed at different velocities, with theses migration rates being a function of the equilibrium distribution of the components between the stationary and the mobile phase.
Basically, high-performance liquid chromatography (HPLC) is merely an extension of the technique described above, comprising small particles of narrow size distribution, higher mobile phase flow rates through under pressure-pumping and a system to detect when the separated compounds elute off the column.

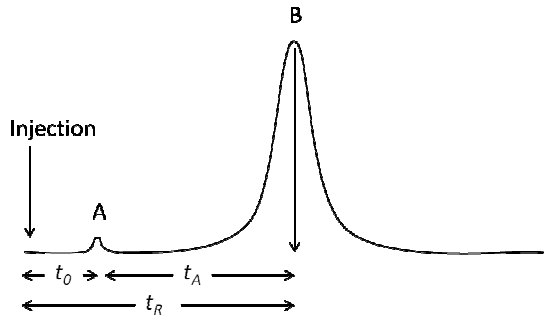

Figure 5: Illustration of chromatographic terms (modified according to Brown 1973)

A typical separation of two compounds is shown in figure 5. The time from the injection to the moment when the maximum of the solute peak appears, is called the retention time t_R. A component that is not adsorbed on the stationary phase appears at point A on the chromatogram and is referred to as the holdup time t_0 or the void volume V_0. The void volume or holdup time is equal to the total volume of solvent eluting from the column between the time of injection and the appearance of the unadsorbed compound. Thus, the retention time of a compound is defined as the retention time (t_R) minus the holdup time (t_0).

$$t_A = t_R - t_0$$

By drawing tangents to the slope at the inflexion point of the peak and determining the distance between the place where the tangents intersect the baseline, the base of the solute peak (B) is determined. The height of the solute peak is calculated from the maximum to the baseline.
To provide effective separation, a column for liquid chromatography must possess the capacity to retain samples efficiently as well as the ability to separate sample components efficiently. The capacity factor of a column is described by the following equation:

$$k'_r = \frac{t_R - t_0}{t_0} = \frac{V_R - V_0}{V_0}$$

If the capacity factor k'_r is too low, the components elute too quickly. Thus, the solvent is too strong.
The separation factor α, is a indice for the column's ability to the separation of two compounds. It is expressed as a ratio of the capacity factors of the compounds.

$$\alpha = \frac{k'_1}{k'_2}$$

If α equals 1, there is no separation and the peaks coincide. Column and solvent efficiencies are described by the term resolution in terms of both, narrowness of the peaks and separation of the maxima of two peaks. The efficiency of columns is related to peak-broadening of an initial compact band as it passes through the column. The design of the column likewise its operating conditions affect broadening.
Common solvents for HPLC are various combinations of water and organic liquids, such as methanol or acetonitrile. To support sample separation, buffers, salts or other compounds, such as trifluoroacetic acid, which acts as an ion paring agent, can be added to the water. Travelling velocity

of the single substances in a sample depends on their interactions with both phases, which basically are adsorption, dispersion, ion-exchange, exclusion or affinity processes.
The mobile phase can be varied during analysis. The change of solvent composition during a separation in which the solvent strength increases from the beginning to the end of the separation is called gradient elution. Especially for the analysis of samples of unknown complexity, gradient solution is very well suited since good resolution is automatically provided for a wide range of sample polarities such as in lipid samples. For example in lipid class analysis, a gradient might start with a non-polar solvent mixture and end with a polar solvent, containing e.g. 15 % water. The gradient helps to separate the components of the sample as a function of their affinity. In this example, more hydrophobic components will elute when the mobile phase consists mostly of non-polar solvents. (Brown 1973; Simpson 1976)

For the detection of substances separated by HPLC various detectors, such as electrochemical, UV or fluorescence detectors are available. For lipid analysis, evaporative light scattering detectors (ELSD) are a mean of choice, as ELSD is not limited to compounds that contain UV absorbing chromophores and is immune to mobile phase variations as used for gradient elution. The functional principle of ELSD can be summarized into three stages: nebulisation, mobile phase evaporation and detection. A flow of air or an inert gas atomizes the column eluent and produces an aerosol of minute droplets. These droplets are allowed to evaporate, leaving behind a particulate form of the sample compound. The dried particles pass through a light beam, leading to scattering of the light which is detected by a photodiode or a photomultiplier. (Young & Dolan 2003)

Measurements by high-performance liquid chromatography

In this study an improved HPLC method in combination with light scattering detection (ELSD) using monolithic silica columns according to Graeve and Janssen was used for lipid class analysis (Graeve & Janssen 2009).
As chromatographic equipment a LaChrom Elite HPLC system consisting of an L2200 injector, an L2300 column oven (40 °C; VWR, Darmstadt, Germany) and a Sedex 75 evaporative lights scattering detector (gain 7, 40 °C, 3.5 bar; Sedere, France) was used. Lipid class separation was carried out on a Chromolith Performance-Si (100 x 4.6 I.D.) column (VWR) with a monolithic macropore size of 2 µm and a mesopores size of 13 nm (total porosity >80 %).
A gradient programme was applied to achieve the separation of lipid classes by using a combination of three solvent mixtures: isooctane:ethylacetate (99.8:0.2, v/v, eluent A), acetone:ethylacetate (2:1, v/v) containing 0.02 % acetic acid (v/v, eluent B) and 2-propoanol:water (85:15, v/v) containing 0.05 % acetic acid and 0.05 % ethanolamine (v/v, eluent C). The gradient programme started with 100 % of solvent A, changing to a mixture of 50 % solvent A and 50 % solvent B followed by 46 % A, 39 % B and 15 % C. Eluent A (Isooctane/ethylacetate) due to its low polarity, supports the separation of squalen, sterolesters and wax esters, while separation of diacylglcerol ethers and triacylglycerol was achieved by an increase of eluent B (acetone/ethylacetate/acetic acid). A high proportion of eluent B favoured separation of neutral lipids. Increasing proportions of eluent C (2-propoanol/water/acetic acid/ethanolamine) towards the end of the program helped to separate polar lipids. Finally, polar impurities and water were flushed out by solvent B and A (for further details cf. table 5).

Table 5: Gradient mobile phase composition (solvent A, B & C) according to Graeve & Janssen 2009. A: isooctane:ethylacetate (99.8:0.2, v/v); B: acetone:ethylacetate (2:1, v/v) containing 0.02 % acetic acid (v/v); C: 2-propoanol:water (85:15, v/v) containing 0.05 % acetic acid and 0.05 % ethanolamine (v/v)

Time [min]	Solvent [%]			Flow rate [ml/min]
	A	B	C	
0.0	100	0	0	0.0
0.1	100	0	0	1.4
1.5	100	0	0	1.4
1.6	97	3	0	1.4
6.0	94	6	0	1.4
8.0	50	50	0	1.4
0.1	46	39	15	1.4
14.0	43	30	27	1.4
14.1	43	30	27	1.4
18.0	30	0	60	1.4
23.0	40	0	60	1.4
23.1	0	100	0	3.0
25.0	0	100	0	3.0
25.1	100	0	0	3.0
35.0	100	0	0	3.0
35.1	100	0	0	0.0

For calibration of the detector response, stock solutions (5 mg/ml) were prepared and combined to two standard mixtures. Mixture 1 consisted of 0.1 to 0.2 mg/ml of each neutral lipid and mixture 2 of 0.2 to 0.4 mg/ml of each phospholipid. By injecting different volumes between 5 and 60 µl of neutral and polar lipids dissolved in solvent A, calibration curves of lipid classes were obtained, yielding in total amounts of 0.5 to 30 µg for neutral lipids and 1.0 to 60 µg for phospholipids. Replicate injections of standard solutions confirmed reproducibility of the method. Lipids were kept in dichloromethane:methanol (2:1, v/v) and stored at -20 °C. Squalene, wax ester and nonadecanoic acid methylester were derived from Sigma-Aldrich. Trioleate, oleyl alcohol, cholesterol, palmitic acid, cerebrosides, phosphatdiylethanolamine, phosphatidylinosilol, phosphatidylserine, cardolipine, phosphatidylcholine as well as lysophosphatidylcholine were bought from Larodan, USA. Diacylglycerol ethers (1-O-alkyldiacyl-sn-glycerol) were extracted from shark liver oil. Used solvents were of HPLC grade (Merck, Darmstadt).

For all samples an injection volume of 2 µl was used. Data acquisition was performed by using LaChrom Elite software (3.1.7; VWR, Darmstadt, Germany). Absolute results of lipid class contents in samples were related to the amount of initially extracted tissue.

2.6. Statistical analysis

Results are represented as means ±standard deviation of the mean. For statistical analysis, results were tested using one-way ANOVA (with Tukeys post test for multiple comparisons) and t tests by GraphPad Prism software (version 5.00 for Windows, GraphPad Software, San Diego California USA). Only for comparison of extraction techniques, two-way ANOVA was used. Differences were considered to be significant at $p < 0.05$.

3. Results

3.1. Lipids

Lipid extraction

The used, slightly modified method according to Folch et al. (1957) proofed to be a clean and convenient mean for lipid extraction in liver tissue. After centrifugation the water soluble and lipid phases could be distinguished neat and clear, allowing clean separation of the different compounds. Thus, following the extraction protocol described above, lipids and water soluble compounds were extracted quickly and effectively.

Comparison of chromatographic (HPLC) and spectroscopic (NMR) analysis

In this study, the use of an existing ^{1}H NMR spectroscopy protocol for lipid analysis was tested. For validation of results obtained by NMR spectroscopy, HPLC analysis was used. In the following, results measured for phospholipid/triacylglycerides (POL/TAG) and phosphatidylethanolamine/phosphatidylcholine (PE/PC) ratios by HPLC and NMR spectroscopy were opposed to show comparability of results obtained with both methods. Detailed values, means and standard deviations are displayed in table 6 to 9.

Table 6: Relative lipid compound ratios measured by NMR spectroscopy and HPLC in liver tissue of *L. squamifrons* individuals of the control group. SD %: relative SD of mean; n.d. : signal below detection limit

Relative ratio of compounds	*L. squamifrons* control group (2 °C)							
	Fish no.					Mean	SD	SD %
	1	2	3	4	5			
POL/TAG (NMR)	3.39	2.72	4.28	4.75	4.31	3.89	0.82	21.0
POL/TAG (HPLC)	3.52	2.41	4.62	13.71	6.55	6.16	4.49	72.9
PE/PC (NMR)	0.11	n.d.	0.06	0.11	0.10	0.10	0.03	28.2
PE/PC (HPLC)	0.34	0.32	0.35	0.31	0.35	0.34	0.02	5.4

Table 7: Relative lipid compound ratios measured by NMR spectroscopy and HPLC in liver tissue of *L. squamifrons* individuals of the temperature group. Experimental temperatures are displayed in brackets next to fish no.

Relative ratio of compounds	*L. squamifrons* temperature group				
	Fish no.				
	6 (5 °C)	7 (5 °C)	8 (6 °C)	9 (7 °C)	10 (7 °C)
POL/TAG (NMR)	4.49	1.77	2.15	2.63	3.65
POL/TAG (HPLC)	5.96	1.20	1.39	2.10	4.00
PE/PC (NMR)	0.10	0.14	0.12	0.15	0.14
PE/PC (HPLC)	0.25	0.32	0.29	0.27	0.34

Table 8: Relative lipid compound ratios measured by NMR spectroscopy and HPLC in liver tissue of *T. hansoni* individuals of the control group. SD %: relative SD of mean

Relative ratio of compounds	*T. hansoni* control group (0 °C)								
	Fish no.						Mean	SD	SD %
	1	2	3	4	5	6			
POL/TAG (NMR)	1.14	2.43	0.33	4.50	0.48	2.00	1.81	1.55	85.6
POL/TAG (HPLC)	0.63	2.30	0.23	5.05	0.37	1.13	1.62	1.84	113.9
PE/PC (NMR)	0.08	0.10	0.06	0.09	0.10	0.22	0.11	0.06	51.1
PE/PC (HPLC)	0.31	0.37	0.35	0.32	0.36	0.38	0.35	0.03	7.5

Table 9: Relative lipid compound ratios measured by NMR spectroscopy and HPLC in liver tissue of *T. hansoni* individuals of *the* temperature group. Experimental temperatures are displayed in brackets next to fish no.

Relative ratio of compounds	*T. hansoni* temperature group				
	Fish no.				
	7 (4 °C)	8 (4 °C)	9 (5 °C)	10 (5 °C)	11 (6 °C)
POL/TAG (NMR)	0.89	0.53	0.22	1.20	0.08
POL/TAG (HPLC)	0.33	0.27	0.08	0.43	0.34
PE/PC (NMR)	0.23	0.12	0.11	0.31	0.82
PE/PC (HPLC)	0.30	0.27	0.37	0.27	0.26

Relative ratio of PE/PC is shown in figure 6 and figure 7. Measurements by NMR as well as HPLC indicated a PE/PC ratio to be independent of temperature in liver tissue of *L. squamifrons*. However, values obtained by HPLC were significantly higher ranging between 0.25 and 0.35, than values measured by NMR spectroscopy between 0.06 and 0.15 ($p < 0.0001$).

Values in liver tissue of *T. hansoni* seemed stable according to both methods, with an exception of the data point at 6 °C measured by NMR, which indicated an increasing development. However, this point is based on one sample only. For liver of *T. hansoni*, only measurements at 0 °C revealed significant differences between NMR (0.11 ±0.06) and HPLC (0.35 ±0.03) ($p = 0.0002$). Relative standard deviations were lower for PE/PC ratio measurements by HPLC of 5.40 and 7.54 % compared to 28.21 and 51.13 % of the mean measured by NMR spectroscopy (cf. table 6-9).

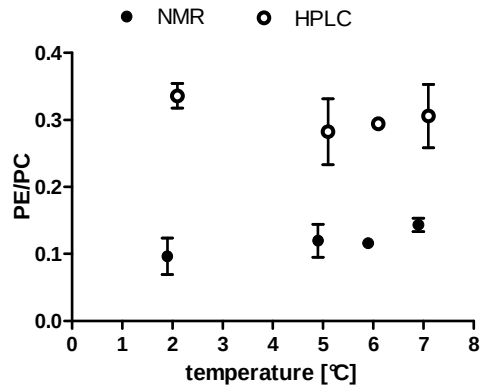

Figure 6: Temperature dependent ratio of PE/PC in liver tissue of *L. squamifrons* measured by NMR spectroscopy (closed circles) and HPLC (open circles). Values are means, black bars show SD. (*L. squamifrons*: 2 °C n=5, 5 °C n=2, 6 °C n=1, 7 °C n=2)

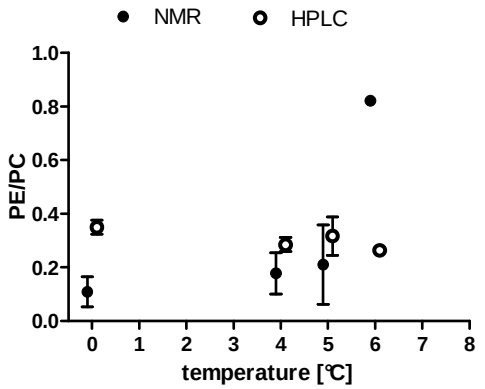

Figure 7: Temperature dependent ratio of PE/PC in liver tissue of *T. hansoni* measured by NMR spectroscopy (closed circles) and HPLC (open circles). Values are means, black bars show SD. (*T. hansoni*: 0 °C n=6, 4 °C n=2, 5 °C n=2, 6 °C n=1)

Figure 8 and figure 9 show relative ratios of POL/TAG measured by NMR spectroscopy and HPLC. In liver tissue of both species there was a decreasing trend with increasing temperature measured by both methods alike. Values measured by both methods were comparable and no significant differences were detected. Regarding figure 8 and figure 9 standard deviation of NMR spectroscopy was lower compared to HPLC, e.g. with a deviation of 21.03 % compared to 72.91 % for *L. squamifrons* at 2 °C and 85.65 % compared to 113.87 % of mean for *T. hansoni*.

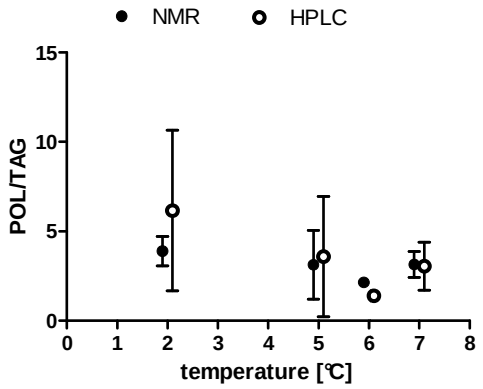

Figure 8: Temperature dependent ratio of POL/TAG in liver tissue of *L. squamifrons* measured by NMR spectroscopy (closed circles) and HPLC (open circles). Values are means, black bars show SD. (*L. squamifrons*: 2 °C n=5, 5 °C n=2, 6 °C n=1, 7 °C n=2)

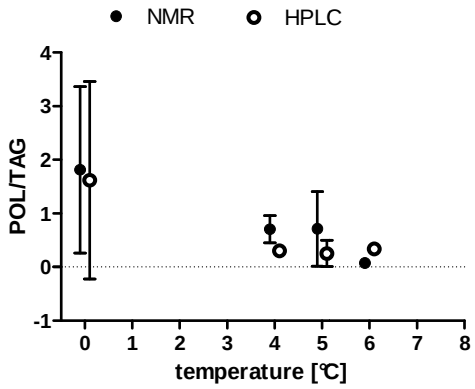

Figure 9: Temperature dependent ratio of POL/TAG in liver tissue of *T. hansoni* measured by NMR spectroscopy (closed circles) and HPLC (open circles). Values are means, black bars show SD. (*T. hansoni*: 0 °C n=6, 4 °C n=2, 5 °C n=2, 6 °C n=1)

Temperature dependent lipid composition

Figure 10 and figure 11 show exemplarily a spectrum measured by NMR spectroscopy and a chromatogram obtained by HPLC. Detailed results are shown in table 10 and table 11 for *L. squamifrons* and table 12 and table 13 for *T. hansoni* respectively. As NMR spectroscopy did not allow quantification in this study, results for lipid classes were based on HPLC measurements, while results of fatty acids were based on NMR measurements.

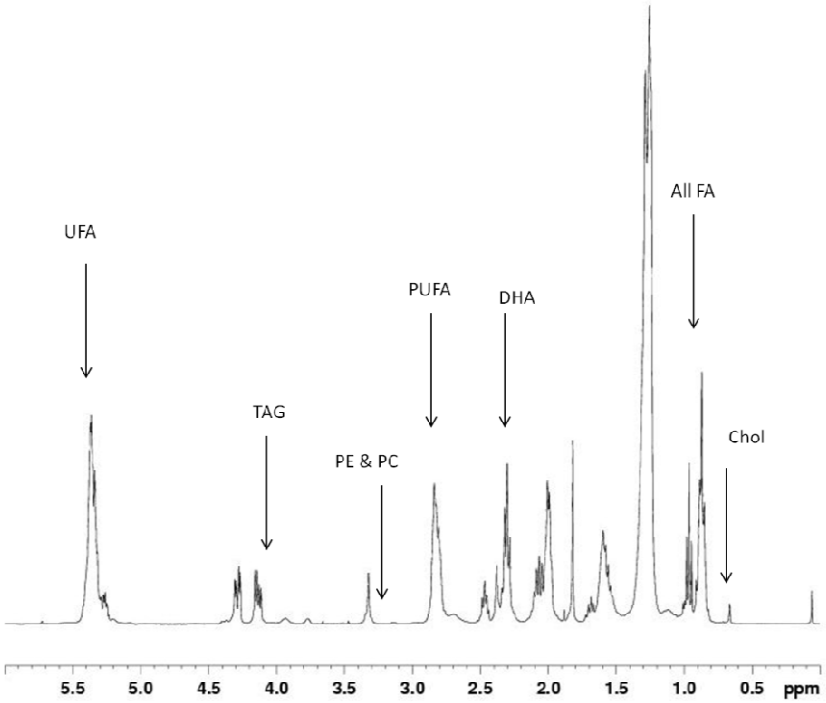

Figure 10: Exemplary NMR spectrum of lipid extract of *T. hansoni* (fish no. 11). All FA: all fatty acids; Chol: cholesterol; DHA: docosahexaenoic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PUFA: polyunsaturated fatty acids; UFA: unsaturated fatty acids, TAG: triacylglyceride

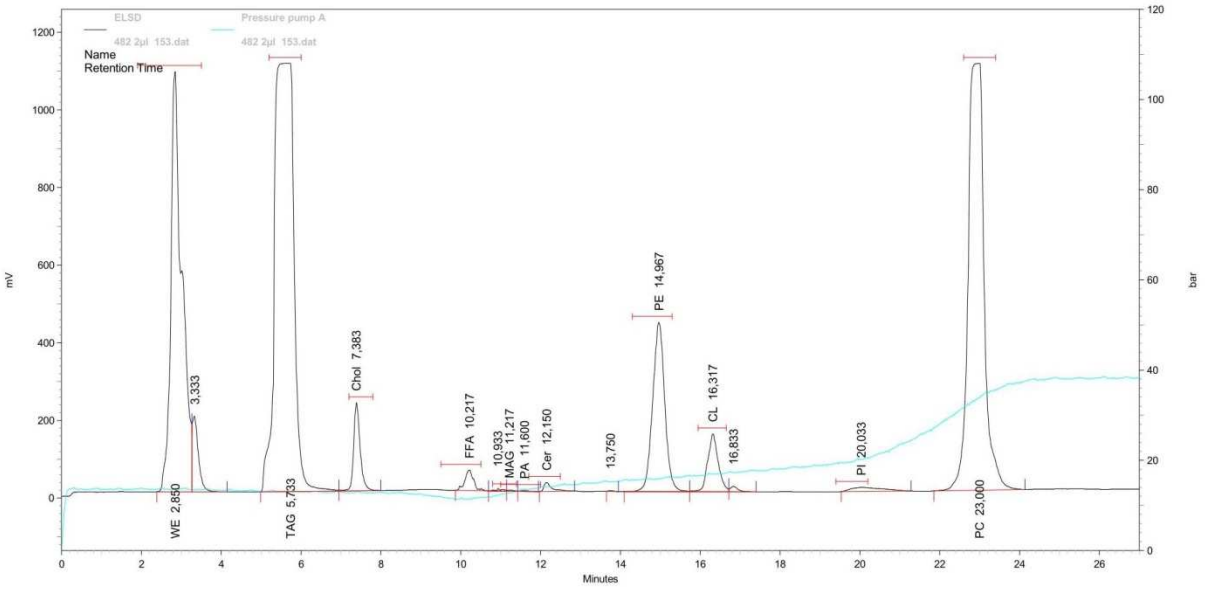

Figure 11: Exemplary HPLC chromatogramm of lipid extract of *T. hansoni* (fish no. 11). Cer: ceramide; Chol: cholesterol; CL: cardiolipin; FFA: free fatty acids; MAG: monoacylglycerol; PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; TAG: triacylglyceride; WE: wax ester

Table 10: Lipid compounds in liver tissue of *L. squamifrons* individuals of control group (2 °C) measured by HPLC and NMR spectrometry. Total lipids are stated as % of liver wet weight. All other compounds are given in % of total lipids, except UFA/all FA, PUFA/all FA and DHA/all FA, which are relative ratios measured by NMR spectroscopy.

Lipid compound	*L. squamifrons* control group (2 °C)						
	Fish no.					Mean	SD
	1	2	3	4	5		
Total lipids	1.9	2.3	1.9	3.1	4.0	2.6	0.9
TAG	17.2	23.3	13.7	5.2	10.0	13.9	6.9
PE	15.5	13.6	16.4	17.0	17.1	15.9	1.5
PC	45.1	42.5	46.7	54.3	48.6	47.5	4.4
Chol	5.9	5.1	5.5	6.0	4.8	5.5	0.5
UFA/all FA	1.89	1.72	2.01	2.03	1.83	1.90	0.13
PUFA/all FA	1.42	1.36	1.09	1.57	1.34	1.36	0.18
DHA/all FA	0.22	0.23	0.26	0.29	0.25	0.25	0.03

Table 11: Lipid compounds in liver tissue of *L. squamifrons* individuals of temperature group measured by HPLC and NMR spectrometry. Experimental temperatures are displayed in brackets next to fish no. Total lipids are stated as % of liver wet weight. All other compounds are given in % of total lipids, except UFA/all FA, PUFA/all FA and DHA/all FA, which are relative ratios measured by NMR spectroscopy.

Lipid compound	*L. squamifrons* temperature group				
	Fish no.				
	6 (5 °C)	7 (5 °C)	8 (6 °C)	9 (7 °C)	10 (7 °C)
Total lipids	1.1	3.1	2.8	2.2	2.5
TAG	9.4	35.0	29.0	22.5	14.2
PE	11.1	10.1	9.2	10.1	14.3
PC	44.8	31.9	31.2	37.1	42.3
Chol	8.0	4.9	6.8	7.0	6.1
UFA/all FA	1.99	1.60	1.86	1.63	1.74
PUFA/all FA	1.18	1.15	1.40	1.20	1.47
DHA/all FA	0.39	0.16	0.25	0.17	0.24

Table 12: Lipid compounds in liver tissue of *T. hansoni* individuals of control group (0 °C) measured by HPLC and NMR spectrometry. Total lipids are stated in % of liver wet weight. All other compounds are given in % of total lipids, except UFA/all FA, PUFA/all FA and DHA/all FA, which are relative ratios measured by NMR spectroscopy.

Lipid compound	*T. hansoni* control group (0 °C)							
	Fish no.						Mean	SD
	1	2	3	4	5	6		
Total lipids	4.3	2.3	4.8	1.7	4.4	2.4	3.3	1.3
TAG	48.8	24.9	70.5	14.1	61.1	38.5	43.0	21.4
PE	7.4	15.4	4.1	17.4	6.0	12.0	10.4	5.4
PC	23.5	42.0	11.8	53.7	16.5	31.5	29.8	15.9
Chol	2.8	5.4	2.1	7.6	2.8	5.0	4.3	2.1
UFA/all FA	1.86	2.24	1.83	2.04	2.06	1.82	1.98	0.17
PUFA/all FA	1.43	1.70	1.39	1.57	1.81	1.47	1.56	0.16
DHA/all FA	0.23	0.27	0.25	0.26	0.35	0.28	0.27	0.04

Table 13: Lipid compounds in liver tissue of *T. hansoni* individuals of temperature group measured by HPLC and NMR spectrometry. Experimental temperatures are displayed in brackets next to fish no. Total lipids are stated in % of liver wet weight. All other compounds are given in % of total lipids, except UFA/all FA, PUFA/all FA and DHA/all FA, which are relative ratios measured by NMR spectroscopy.

Lipid compound	*T. hansoni* temperature group				
	Fish no.				
	7 (4 °C)	8 (4 °C)	9 (5 °C)	10 (5 °C)	11 (6 °C)
Total lipids	4.8	7.4	4.5	4.5	5.9
TAG	70.1	70.0	83.9	63.5	68.4
PE	5.3	4.0	1.8	5.7	4.8
PC	17.6	15.2	5.0	21.4	18.3
Chol	2.0	3.2	2.7	2.6	2.7
UFA/all FA	1.03	1.29	1.79	0.90	1.49
PUFA/all FA	0.58	0.95	1.23	0.59	1.02
DHA/all FA	0.11	0.14	0.20	0.10	0.16

Figure 12 shows total lipid levels in % of liver wet weight of *T. hansoni* and *L. squamifrons* in relation to temperature. In *T. hansoni* lipid level for control group of 3.3 ±1.3 % of liver tissue was much lower than liver lipid levels between 4 and 6 °C, which ranged between 4.5 % and 7.5 %. In *L. squamifrons,* total lipid content varied between 2.6 ±0.9 % of liver tissue at 2 °C to a mean of 2.4 ±0.1 % at 7 °C. Generally higher lipid contents were measured in liver tissue of *T. hansoni* compared to *L. squamifrons*. However, interspecies differences at habitat temperature were not significant.

Figure 13 shows triacylglaceride (TAG) levels in % of total liver lipids in relation to temperature. Significantly higher TAG levels were detected in liver of *T. hansoni* (43.0 ±21.4 %) compared to *L. squamifrons* (113.9 ±6.9 %) at respective habitat temperature ($p = 0.0179$). TAG levels in liver tissue of *T. hansoni* were lower in the control group (2 °C) than values measured between 4 and 6 °C from 63.5 to 83.9 % of total lipids. In *L. squamifrons,* TAG levels in liver seemed independent of temperature, ranging between 5.2 % and 23.3 % of total lipids.

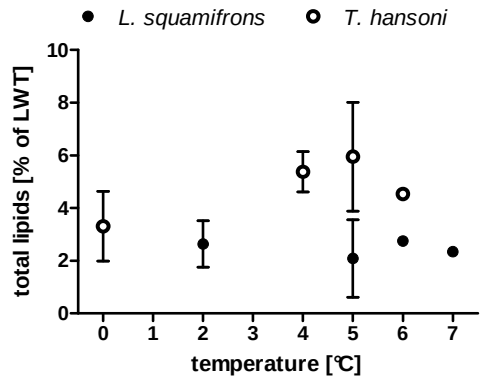

Figure 12: Temperature dependent total lipids levels in liver tissue of *L. squamifrons* and *T. hansoni* measured by HPLC. Values are means, black bars show SD. (*L. squamifrons*: 2 °C n=5, 5 °C n=2, 6 °C n=1, 7 °C n=2; *T. hansoni*: 0 °C n=6, 4 °C n=2, 5 °C n=2, 6 °C n=1)

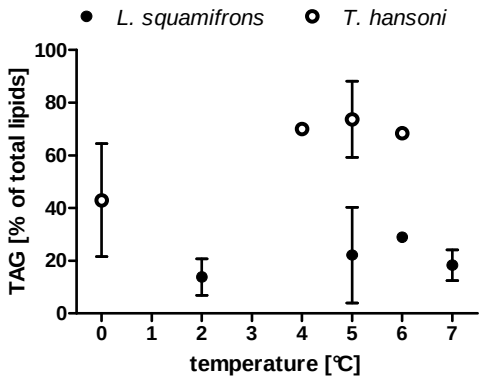

Figure 13: Temperature dependent TAG levels in liver tissue of *L. squamifrons* and *T. hansoni* measured by HPLC. Values are means, black bars show SD. (*L. squamifrons*: 2 °C n=5, 5 °C n=2, 6 °C n=1, 7 °C n=2; *T. hansoni*: 0 °C n=6, 4 °C n=2, 5 °C n=2, 6 °C n=1)

Phosphatidlyethanolamine (PE) levels seemed to decrease with increasing temperature in liver tissue of both species. In *L. squamifrons*, PE at 2 °C of 15.9 ±1.5 % was significantly higher than values measured at 5 °C with a mean of 10.6 ±0.7 % of total liver lipids (p = 0.0432) (figure 14). In *T. hansoni* PE level decreased insignificantly from 10.4 ±5.4 % at 0 °C to 4.0 % of total liver lipids at 6 °C (Figure 15). Generally, PE levels seemed to be higher in *L. squamifrons* at habitat temperature compared to *T. hansoni,* but differences were not significant.

Phosphatidylcholine (PC) levels in liver of both species are presented in figure 15. Similarly as PE, PC levels decreased with temperature in tissue of both species. Results for *L. squamifrons* decreased from 47.5 ±4.4 % at 2 °C to 39.7 ±3.7 % at 7 °C, while values for *T. hansoni* fell from 29.8 ±15.9 at 0 °C to 18.3 % of total lipids at 6 °C. Again, PC values at habitat temperature were significantly higher in liver tissue of *L. squamifrons* compared to *T. hansoni* (p = 0.0480).

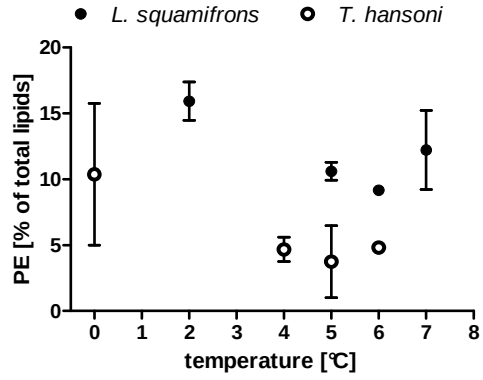

Figure 14: Temperature dependent PE levels in liver tissue of *L. squamifrons* and *T. hansoni* measured by HPLC. Values are means, black bars show SD. (*L. squamifrons*: 2 °C n=5, 5 °C n=2, 6 °C n=1, 7 °C n=2; *T. hansoni*: 0 °C n=6, 4 °C n=2, 5 °C n=2, 6 °C n=1)

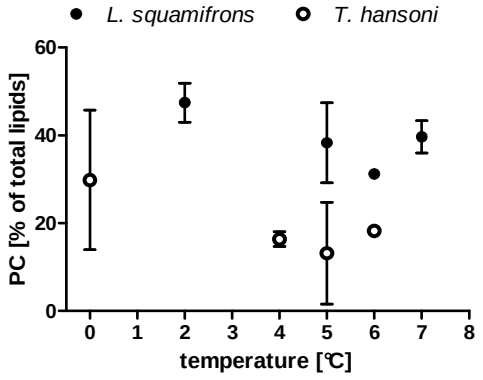

Figure 15: Temperature dependent PC levels in liver tissue of *L. squamifrons* and *T. hansoni* measured by HPLC. Values are means, black bars show SD. (*L. squamifrons*: 2 °C n=5, 5 °C n=2, 6 °C n=1, 7 °C n=2; *T. hansoni*: 0 °C n=6, 4 °C n=2, 5 °C n=2, 6 °C n=1)

The proportions of unsaturated fatty acids to all fatty acids (UFA/all FA) and polyunsaturated fatty acids to all fatty acids(PUFA/all FA) are shown in figure 16 and 17. According to NMR measurements, UFA/all FA ratios ranged between a mean of 1.90 ±0.13 (2 °C) and 1.68 ±0.08 (7 °C), while PUFA/all FA ratios ranged between a mean of 1.36 ±0.18 (2 °C) and 1.34 ±0.19 (7 °C) in liver tissue of *L. squamifrons* (Figure 16 & Figure 17). Ratios of UFA likewise PUFA to all FA were highest in liver tissue of the control group of *T. hansoni* (1.98 ±0.17 and 1.56 ±0.16 at 0 °C), varied between 0.9 and

1.79 for UFA/all FA and between 0.58 to 1.23 for PUFA/all FA between 4 and 6 °C. Ratios of UFA/all FA showed significant differences between 0 and 4 °C (p = 0.0161), while differences for PUFA/all FA were significant between 0 and 4 likewise 5 °C in tissue of *T. hansoni* (p = 0.0071). Similarly, UFA/all FA and PUFA/all FA ratios possibly indicated a slightly increasing trend in *T. hansoni* between 4 and 6 °C, which was not significant.

Interspecies comparison of UFA/all FA and PUFA/all FA ratios showed slightly higher ratios for liver tissue of *T. hansoni* (1.98 ±0.17 and 1.56 ±0.16 at 0 °C), compared to *L. squamifrons*(1.90 ±0.13 and 1.36 ±0.18 at 2 °C) at habitat temperature. Nevertheless, an insignificant trend to higher ratios of UFA and PUFA to all FA in *L. squamifrons* compared to *T. hansoni* at temperatures above habitat temperature (figure 16 & 17, cf. table 10-13) was observed.

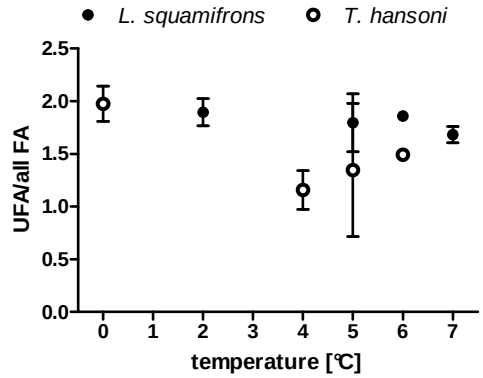

Figure 16: Temperature dependent ratios of UFA/all FA in liver tissue of *L. squamifrons* and *T. hansoni* measured by NMR spectroscopy. Values are means, black bars show SD. (*L. squamifrons*: 2 °C n=5, 5 °C n=2, 6 °C n=1, 7 °C n=2; *T. hansoni*: 0 °C n=6, 4 °C n=2, 5 °C n=2, 6 °C n=1)

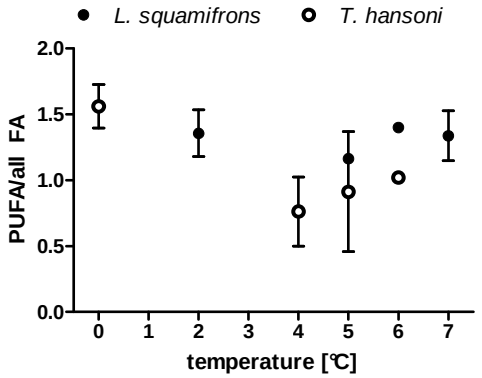

Figure 17: Temperature dependent ratios of PUFA/all FA in liver tissue of *L. squamifrons* and *T. hansoni* measured by NMR spectroscopy. Values are means, black bars show SD. (*L. squamifrons*: 2 °C n=5, 5 °C n=2, 6 °C n=1, 7 °C n=2; *T. hansoni*: 0 °C n=6, 4 °C n=2, 5 °C n=2, 6 °C n=1)

NMR measurements showed that the ratio of docsahexaenoic acid (DHA), a highly unsaturated PUFA, to all FA coincided with figure 16 and 17, decreasing with temperature from 0.25 ±0.03 (2 °C) to 0.20 ±0.05 (7 °C) in liver tissue of *L. squamifrons* and from 0.27 ±0.04 (0 °C) to 0.16 (6 °C) in tissue of *T. hansoni* (figure 18). However, only in *T. hansoni* significant higher values were detected between 0 and 4 °C (0.12 ±0.02) likewise 5 °C (0.15 ±0.07) (P = 0.0041, figure 18).

Cholesterol (chol) levels in liver tissue of the two fish species are displayed in figure 19. Values obtained for *L. squamifrons* ranged between 5.5 ±0.5 at 2 °C to 6.5 ±0.6 % of total liver lipids at 7 °C, while data of *T. hansoni* ranged from 4.3 ±2.1 at 0 °C to 3.2 % of total liver lipids at 6 °C. However, values for any of the two species were not significantly different.

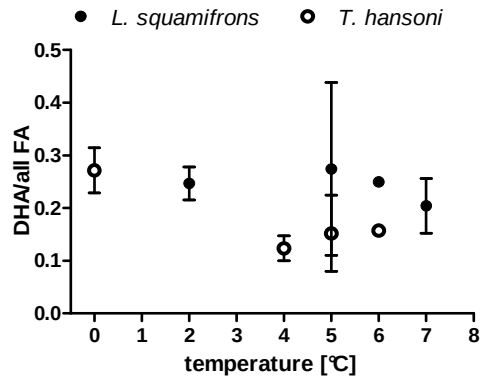

Figure 18: Temperature dependent ratios of DHA/all FA in liver tissue of *L. squamifrons* (closed circles) and *T. hansoni* (open circles) measured by NMR spectroscopy. Values are means, black bars show SD. (*L. squamifrons*: 2 °C n=5, 5 °C n=2, 6 °C n=1, 7 °C n=2; *T. hansoni*: 0 °C n=6, 4 °C n=2, 5 °C n=2, 6 °C n=1)

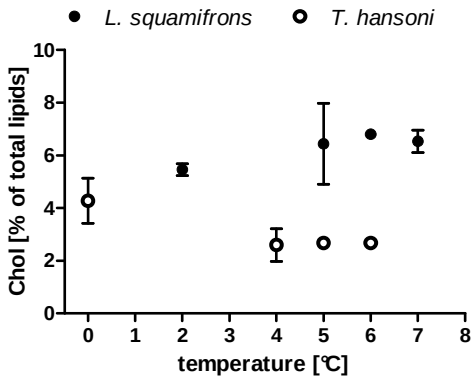

Figure 19: Temperature dependent cholesterol levels in liver tissue of *L. squamifrons* (closed circles) and *T. hansoni* (open circles) measured by HPLC. Values are means, black bars show SD. (*L. squamifrons*: 2 °C n=5, 5 °C n=2, 6 °C n=1, 7 °C n=2; *T. hansoni*: 0 °C n=6, 4 °C n=2, 5 °C n=2, 6 °C n=1)

3.2. Water soluble compounds

Extraction of water soluble compounds

For the extraction of water soluble compounds, PCA extraction and the extraction according to Folch were compared. Both extraction procedures showed comparable results without significant differences. However, comparing handling and time efficiency of both methods, the use of Folch's extraction proofed to be faster and more convenient while producing results with smaller standard deviation than those obtained by PCA extraction. Results of peaks of different metabolic compounds such as adenosine triphosphate (ATP), phosphocreatine, glycogen, succinate, acetate were compared to allow profound statistics. As examples, results obtained by both methods for succinate and acetate are shown in figure 20.

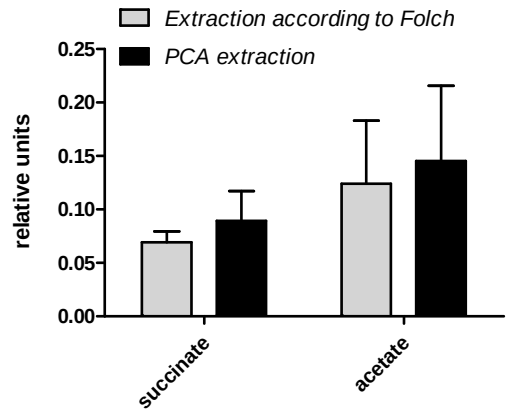

Figure 20: Content of water soluble compounds in liver tissue of *L. squamifrons* (control group) obtained by extraction according to Folch and PCA extraction (each n=5). Values are stated in relative units. Bars show mean values ±SD.

Table 14: Results of PCA extracts of liver tissue of control group of *L. squamifrons* measured by NMR spectrometry. Values are stated in relative units.

Compound	*L. squamifrons* control group (2 °C)						
	Fish no.					Mean	SD
	1	2	3	4	5		
Succinate	0.072	0.062	0.061	0.053	0.199	0.089	0.062
Acetate	0.071	0.079	0.066	0.426	0.085	0.145	0.157

Table 15: Results of extracts obtained according to Folch of liver tissue of control group of *L. squamifrons* measured by NMR spectrometry. Values are stated in relative units.

Compound	*L. squamifrons* control group (2 °C)						
	Fish no.					Mean	SD
	1	2	3	4	5		
Succinate	0.064	0.051	0.061	0.060	0.110	0.069	0.023
Acetate	0.074	0.060	0.077	0.358	0.051	0.124	0.131

Temperature dependent water soluble compounds

Figure 21 shows an example of an NMR spectrum measured in water soluble extracts of *T. hansoni*. Detailed results of water soluble metabolites for *L. squamifrons* are shown in table 16 and 17, while table 18 and 19 display values observed for *T. hansoni*.

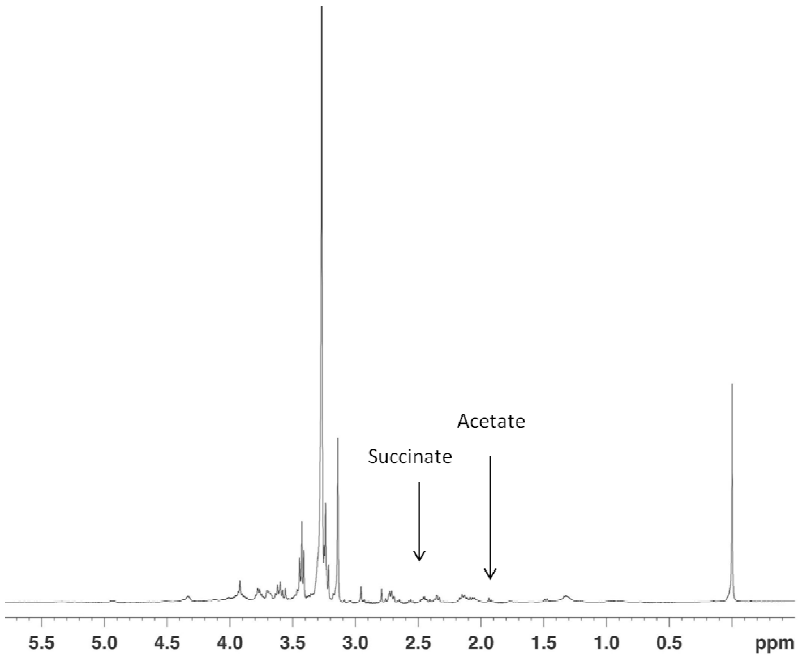

Figure 21: Exemplary NMR spectrum of water soluble extract of *T. hansoni* (fish no. 10).

Table 16: Water soluble compounds in liver tissue of *L. squamifrons* individuals of control group measured by NMR spectroscopy. Values are stated in relative units.

Compound	*L. squamifrons* control group (2 °C)						
	Fish no.					Mean	SD
	1	2	3	4	5		
Succinate	0.064	0.051	0.061	0.060	0.110	0.069	0.023
Acetate	0.074	0.060	0.077	0.358	0.051	0.124	0.131

Table 17: Water soluble compounds in liver tissue of *L. squamifrons* individuals of temperature group measured by NMR spectroscopy. Experimental temperatures are displayed in brackets next to fish no. Values are stated in relative units.

Compound	*L. squamifrons* temperature group							
	Fish no.							
	6 (5 °C)	7 (5 °C)	8 (6 °C)	9 (7 °C)	10 (7 °C)	11 (7 °C)	12 (7 °C)	13 (9 °C)
Succinate	0.063	0.056	0.073	0.064	0.064	0.158	0.066	0.061
Acetate	0.014	0.069	0.013	0.005	0.312	0.116	0.035	0.070

Table 18: Water soluble compounds in liver tissue of *T. hansoni* individuals of control group measured by NMR spectroscopy. Values are stated in relative units; n.d.: signal below detection limit

Compound	*T. hansoni* control group (0 °C)							
	Fish no.						Mean	SD
	1	2	3	4	5	6		
Succinate	0.057	0.011	0.018	n.d.	0.022	0.026	0.027	0.020
Acetate	0.189	0.090	0.007	0.008	0.091	0.183	0.077	0.075

Table 19: Water soluble compounds in liver tissue of *T. hansoni* individuals of temperature group measured by NMR spectroscopy. Experimental temperatures are displayed in brackets next to fish no. Values are stated in relative units; n.d.: signal below detection limit

Compound	*T. hansoni* temperature group				
	Fish no.				
	7 (4 °C)	8 (4 °C)	9 (5 °C)	10 (5 °C)	11 (6 °C)
Succinate	0.032	n.d.	0.011	0.031	0.011
Acetate	0.186	0.053	0.092	0.060	0.042

Temperature dependent liver succinate levels of both species are shown in figure 22. For *L. squamifrons*, succinate levels ranged between of 0.069 ±0.023 relative units at habitat temperature and 0.061 rel. units measured at 9 °C in liver tissue. In tissue of *T. hansoni,* succinate values at habitat temperature of 0.022 ±0.021 rel. units were determined, while succinate levels increased between 4 and 6 °C from 0.016 ±0.022 up to a mean of 0.026 rel. units. On the whole, the succinate level in liver of *L. squamifrons* was significantly higher compared to levels in *T. hansoni* at habitat temperature of 2 and 0 °C respectively ($p = 0.0053$).

Figure 23 displays temperature dependent liver acetate levels. Acetate content in liver tissue of *L. squamifrons* ranged between 0.013 and 0.358 relative units. In *T. hansoni*, liver acetate levels seemed constantly between 0.007 and 0.186 rel. units between 0 and 5 °C. Only the value measured in the 6 °C sample indicated an increasing trend, which was not significant as being based on only one measurement.

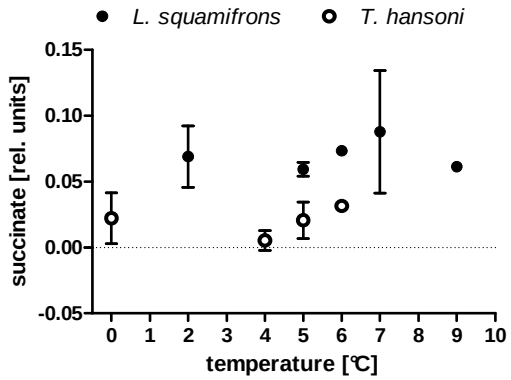

Figure 22: Temperature dependent succinate levels in liver tissue of *L. squamifrons* (closed circles) and *T. hansoni* (open circles). Values are means, black bars show SD. (*L. squamifrons*: 2 °C n=5, 5 °C n=2, 6 °C n=1, 7 °C n=4, 9 °C n=1; *T. hansoni*: 0 °C n=6, 4 °C n=2, 5 °C n=2, 6 °C n=1)

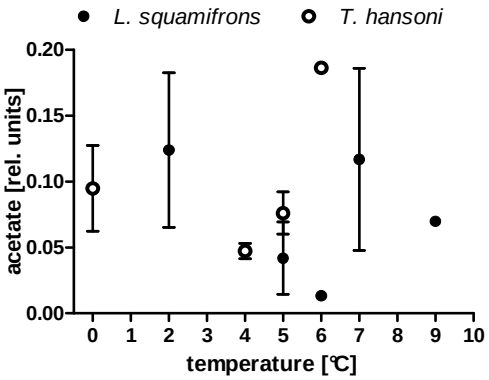

Figure 23: Temperature dependent acetate levels in liver tissue of *L. squamifrons* (closed circles) and *T. hansoni* (open circles). Values are means, black bars show SD. (*L. squamifrons*: 2 °C n=5, 5 °C n=2, 6 °C n=1, 7 °C n=4, 9 °C n=1; *T. hansoni*: 0 °C n=6, 4 °C n=2, 5 °C n=2, 6 °C n=1)

4. Discussion

4.1. Extraction procedure

Lipids

The lipid extraction procedure using methanol and chloroform is a well-established method and proofed to be very convenient. Various variations of protocols mostly based on the initial technique of Folch (1957) are used commonly. The protocol used here was modified according to Folch. The addition of salts to the upper phase was restricted to KCl, which is supposed to improve partitioning of polar phospholipids into chloroform layer by increasing polarity of the aqueous phase. Moreover, chloroform was replaced with the less toxic dichloromethane. After centrifugation the two phases were clearly to distinguish, assisting a clean phase separation and thus a clean partition of lipid and water soluble compounds.

Water soluble compounds

There are a number of different extraction techniques for biochemical analysis and metabolite profiles in NMR spectroscopy. The most common is PCA extraction for water soluble compounds. methanol/chloroform (M/C) extraction is a less widely used technique, possibly due to the more elaborate and potentially harmful work with organic solvents. However, M/C extraction can facilitate the concurrent extraction of water soluble metabolites and lipid compounds from the same sample.
In the framework of this study, the use of M/C extraction for the preparation of water soluble metabolites was tested. Comparison of PCA extraction and M/C extraction leads to comparable results for water soluble metabolites by NMR spectroscopy. However, results obtained after Folch extraction displayed smaller standard deviations than those of PCA extraction (cf. table 14 & 15). Results obtained in other studies support these observations. Defernez and Colquhoun (2003) as well as Le Belle and co-workers (2002) found M/C extraction to be less variable and more efficient than PCA extraction. Moreover, the metabolite yields from M/C extraction were found to be greater than those from PCA extraction (Le Belle et al. 2002). A smaller standard deviation indicates more precise and definite results, which simplifies comparison between experimental groups and makes it easier to state whether there are significant differences between different treatments.
Another advantage of Folch's extraction compared to PCA extraction was its simple protocol and time efficiency due to avoidable pH adjustments. While both extraction protocols are quite similar regarding the number of extraction steps and chemicals needed, only for PCA extraction a pH adjustment of the sample extract is obligatory; a time consuming task. Even after pH adjustment, spectral shifts were observed more often in PCA extracts than in Folch extracts, probably due to pH variations. Lin and co-workers (2007) reported variable peak shifting in spectra of PCA extracts, while peaks of M/C extracts consistently moved to higher chemical shifts. They suggested peak shifting in PCA extracts to be due to variable final pH, while the constant peak shift in M/C extracts is caused by the salt, inducing small but consistent changes in the solution (Lin et al. 2007). Although pH variation was not a problem in this study, the use of phosphate buffer in D_2O as solvent for dried extracts might avoid this potential error, as done in other studies (Wu et al. 2011). Generally, shifts in spectra could be caused by the used solvent, contamination or changes in measurement parameters, such as temperature.

Nevertheless, results of this study are based on a number of only 5 samples. For a comprehensive comparison of both methods, a larger sample size and different types of tissue should be tested, as tissue composition might affect result efficiency of the respective methods. For detailed methodological comparison of extraction techniques regarding efficiency and protocol variations, studies are already available in literature (Le Belle et al. 2002; Lin et al. 2007; Wu et al. 2011).
In this study the potential of the established standard M/C extraction in comparison to the established standard PCA extraction was assessed, to avoid resource wasting. In conclusion, when metabolite as well as lipids shall be extracted, M/C extraction seems to be the method of choice, as both extractions can be done in one step, saving chemicals, effort and time likewise.

4.2. Lipids

Lipid spectra and data obtained in this study seem to be the first data recorded for *L. squamifrons* as well as *T. hansoni*. However, comparison of lipid spectra in both species showed no measureable differences regarding present lipid compounds, although levels of some compounds varied between species. Regarding lipid composition and content selected compounds were taken into account and discussed below.

Comparison of chromatographic (HPLC) and spectroscopic (NMR) analysis

HPLC and NMR spectroscopy are techniques based on completely different physical processes. The used ^{1}H NMR spectroscopy takes advantage of differences in atomic interactions of hydrogen atoms bound in different functional groups, while HPLC is based on a compound's interaction with the mobile and stationary phase, namely adsorption, dispersion, ion-exchange, exclusion or affinity processes.
However, lipid analysis in the framework of this study showed comparable trends in results of both methods. Lipid class results obtained by HPLC likewise NMR spectroscopy allowed direct comparison of relative ratios of phosphatidylethanolamine/phosphatidylcholine (PE/PC) and phospholipids/triacylglycerides (POL/TAG). PE/PC ratio seemed constant in all samples disregarding temperature, but values measured by HPLC were significantly higher than those obtained by NMR in both species. This might be due to solubility problems of PE in the used solvent $CDCl_3$. In a test run, a much larger PE-signal was detected after the addition of dimethyl sulfoxide, compared to the same sample measured in $CDCl_3$ without dimethyl sulfoxide. Dimethyl sulfoxide is considered to be a very strong solvent that dissolves both polar and non-polar compounds and is miscible in a wide range of organic solvents. Thus, optimisation of the solvent for lipid measurements by NMR spectroscopy might produce results resembling those obtained by HPLC. POL/TAG ratios show a decreasing trend with rising temperature in both species with both methods likewise, thus supporting comparability of the two techniques.
As differences in standard deviations are less pronounced for POL/TAG ratios than for PE/PC ratios, HPLC seems to produce less variable results. A smaller standard deviation supports more precise and definite results, simplifying comparison between experimental groups and easing statements regarding differences between treatments. For comparison of standard deviations only control groups of both species were assessed, as only here a minimum sample size of 5 was given as a basis for analysis.

However, discussing reliability and precision of results, the lack of an internal standard in the solvent, which limits the comparability of single measurements, has to be discussed as a disadvantage in this study's NMR experiments. By using relative ratios of the different compounds, errors of lacking internal reference are avoided, as ratios are assumed to be independent of extract concentrations. Nevertheless, an internal standard would have allowed easier comparison of single compounds, rather than ratios. Ideally an internal standard is a compound very similar to the chemical species of interest, to keep effects of sample preparation on both substances similar. A common and convenient mean in NMR spectroscopy are solvents already prepared with internal standards, such as $CDCl_3$ containing tetramethylsilane (TMS).

Moreover, initial measurements of pure standards for the identification of all lipid compounds would be a better starting point for sample analysis, than data derived from literature. Although this practice is time consuming and cost intensive, it would be an ideal basis for compound identification, as deviations due to varying experimental setups and used chemical are minimized. However, due to time as well as technical limitations, it was not possible to carry out standard measurements for all lipids in this study.
Similarly to this study, other studies used chromatographic techniques for cross-validation of NMR analysis of lipid extracts and recorded both methods to be in good agreement (Le Belle et al. 2002). Preiss and co-workers (1996) analysed nitroaromatics and nitramines in ammunition waste water by NMR spectroscopy and HPLC simultaneously. They found NMR spectroscopy to give a good overview of organic compounds in a sample and graded it as a very useful mean for non-target analysis, hereby supporting motivations of this study to use NMR measurements for "non-target" or "profiling" analysis. They carried out combined experiments with gas chromatography for result validation and reported NMR measurements to be less sensitive compared to gas chromatography, confirming observations by others (Preiss et al. 1996; Aursand et al. 2008).
The clear advantage of NMR spectrometry is its profiling character. NMR spectroscopy allows non-target analysis, by measuring profiles of lipid or water soluble compounds without limitation to certain groups. Changes in metabolites levels in tissue can be tracked by NMR spectrometry, enabling further specific and targeted analysis. In this way, NMR techniques are a very valuable tool e.g. in the framework of physiological and ecological studies for the analysis of environmental effects such as temperature, ocean acidification, environmental toxics or even nutritional studies (Preiss et al. 1996; Gribbestad et al. 2005).
However, HPLC is known to be a more sensitive technique and is thought to be less cost intensive in aquisition compared to NMR spectrometry setups. The recent combination of HPLC with mass spectrometry (LC/MS), moreover promises a high degree of sensitivity regarding quantification likewise identification of unknown compounds, and additionally allows profiling approaches for the analysis of mixtures (Halket et al. 2005; Theodoridis 2008).

In conclusion, despite the general agreement between results obtained from NMR spectroscopy and HPLC analysis, there are various factors to be improved for further studies. Although the critical factors explained above should be easy to resolve, they could improve the used NMR technique significantly, making it a valuable combination with, or even alternative to chromatographic methods such as HPLC (Aursand et al. 2008). Nevertheless, for discussion of lipid composition in this study, HPLC results were used as those allowed better comparison with results of other studies.

Lipid composition

In the framework of this study, the effect of temperature on different lipid compounds as well as interspecies differences in lipid composition in liver tissue of the Antarctic fish species *L. squamifrons* and *T. hansoni* were assessed. As no literature on this issue was found, this seems to be the first data on liver lipid composition of the two species.

There was no temperature dependent development in total liver lipids observed in *L. squamifrons* between 2 and 7 °C and neither in *T. hansoni* between 4 and 6 °C. However, control group of *T. hansoni* displayed much lower total liver lipid contents compared to the temperature group of this species. The liver samples for control and temperature groups of *T. hansoni* were derived from different cruises in December 2003 and April 2011. Although differences in location of the catch are minor, differences in time i.e. season might affect tissue composition of the fish due to differences in overall condition caused e.g. by food availability and composition (Fraser et al. 1987; Sargent et al. 1999) or reproductive status (Marshall et al. 1999). As spawning of *T. hansoni* is suggested to take place between January and February (Gon & Heemstra 1990), sexual maturation is likely to have affected lipid composition in liver tissue (Henderson & Almatar 1989; Marshall et al. 1999). Reductions in liver size and energy content close to spawning have been observed e.g. in cod and are suggested to be related to concomitant gonadal growth close to spawning (Eliassen & Vahl 1982). The control group of *T. hansoni*, is likely to have been close to spawning (caught in December, spawning in January/February). Thus, liver lipid levels are suggested to be low, as energy is invested in gonadal growth and egg production. In contrast, the temperature group caught in April is supposed to have started recreation of energy stores in liver 1 to 3 months after spawning. Consequently, liver lipid levels of the control and temperature group of *T. hansoni* are suggested to be hardly comparable. Thus, results of the control group of *T. hansoni* will not be considered in the discussion in terms of temperature effects, to avoid misleading interpretations of the results.

Liver lipid content seemed not to be affected by an acute temperature increase for both species. In contrast, long-time experiments by Brodte et al. (2008), who compared temperature dependent lipid composition of Antarctic eelpout *Pachycara brachycephalum* and boreal eelpout *Zoarces viviparus*. They found total lipid content (in ash free dry mass determined by CHN analysis) to decrease with increasing temperature in liver tissue of both species (Brodte et al. 2008). The Antarctic eelpout *P. brachycephalum* shows a circum-Antarctic depth-dependent distribution and feeds mainly on krill and amphipods. The boreal eelpout *Z. viviparus* displays a very similar lifestyle as its Antarctic counterpart, but lives in temperate waters with broadly varying temperatures. In Brodte's study, animals acclimated to 0 °C were incubated at 2, 4 and 6 °C for a period of 4 months, contrasting short-term experiments with 1 °C temperature increase per 24 hours in this study. Although, investigations of the thermal responses of *P. brachycephalum* on the transcriptomic level after long-term exposure to 5 °C (Windisch et al. 2011) supported results of Brodte et al. (2008), short-time temperature exposure did not seem to affect lipid composition in the two species investigated here.

Similarly to total liver lipids, triacylglyceride (TAG) levels seemed not to be related to temperature in liver tissue of both species in this study. This agrees well with TAG level being independent of temperature in liver tissue of Antarctic eelpout (Brodte et al. 2008). TAGs play an essential role as storage compounds in Antarctic fish. During long-term exposure to elevated temperatures, decreasing storage lipids are suggested to indicate a shift to lipid catabolism (Windisch et al. 2011).

Measurements of total lipid levels revealed values of 2.6 ±0.9 % and 3.3 ±1.3 % of liver wet weight in *L. squamifrons* and *T. hansoni* at respective habitat temperatures. For complete body, a lipid content of 2,7 % of wet body weight was recorded for *L. squamifrons*, while values of 3.7 ±2.5 % were

obtained for *T. hansoni* (Lenky et al. 2011). For *Trematomus bernacchi*, a species closely related to *T. hansoni*, that inhabits a similar ecological niche, total liver lipid contents of 4.59 % of liver wet weight were recorded for specimens caught in McMurdo Sound (Ross Sea) (Clarke et al. 1984). In the Antarctic eelpout *Pachycara brachycephalum* (caught near King George Island) total lipids were shown to account for around 55 % of ash free dry mass in liver tissue (Brodte et al. 2008), resulting still in a much higher value of 31.3 % lipids in liver wet weight (by using a liver water content of 77.2 % as determined by Johnston & Battram (1993)). Differences in values obtained for *P. brachycephalum* and species in this study are potentially driven by the use of different methods (Brodte determined values by CHN analysis). Generally deviations could be due to species-specific differences, gender, age or sampling season. Regarding gender, especially female specimens are generally considered to use liver lipids as energy store for egg production (Marshall et al. 1999). Age has to be considered in terms of maturation as well as life time used for the storage of lipids. Sampling season could affect lipid levels via spawning times, but moreover food availability and composition, which is strongly dependent on season in Antarctic ecosystems (Fischer et al. 1988) could affect results.

Lipid composition is considered to be dominated by triacylglycerides in many Antarctic fish (Clarke et al. 1984). This is supported by, TAG levels of 43.0 ±21.4 % of total lipids in liver tissue of *T. hansoni* and 13.9 ±76.9 % in tissue of *L. squamifrons* at respective habitat temperatures. For *T. bernacchii*, TAG levels of 19 % of liver wet weight were recorded (Clarke et al. 1984), while TAG values of 82.5 ±4.3 % of total lipids were determined in liver tissue of *P. brachycepahlum* (at 0 °C) (Brodte et al. 2008). Values measured in this study showed very high inter-individual variability, but seem to fit well with ranges observed by others. Generally, as mentioned above, factors such as sampling time, region, food availability and composition as well as age, gender and maturation of the single individuals could impact results of different studies.

Total lipid likewise TAG levels were shown to be generally higher in liver tissue of high-Antarctic *T. hansoni* compared to sub-Antarctic *L. squamifrons*, although differences were not significant. These results agree with observations reported by Brodte et al. (2008), who found significantly higher lipid and TAG levels in liver of the Antarctic eelpout compared to temperate eelpout. Elevated TAG levels are considered to reflect enhanced levels of storage lipids and lipid turnover in Antarctic species adapted to live in the cold (Reinhardt & Van Vleet 1986; Brodte et al. 2008). Thus higher total lipid and TAG levels in high-Antarctic *T. hansoni* might be due to a higher degree of cold adaptation living in a habitat with even less temperature fluctuations than sub-Antarctic *L. squamifrons*. Hoofd and Egginton (1997) suggested intracellular lipids, having a high oxygen permeability, to play an important role in determining oxygen delivery to fish skeletal muscle. Thus, a high intracellular lipid content could enhance oxygen diffusion in the cold.

Besides, differences in total lipid levels could indicate differences in food supply or differences in prey spectra. Regarding food supply, higher total lipid levels in the high-Antarctic species would suggest better food supply for *T. hansoni*. However, this is unlikely, as high-Antarctic waters are generally known to be driven by strong seasonality in primary production (Fischer et al. 1988), which is the basis for further trophic levels. Considering prey composition, Sidell and Hazel (2002) found the Antarctic icefish *Chaenocepohalus aceratus* to display highest lipase activity compared to other Antarctic fish species and suggested this finding to be due to prey species of *C. aceratus* containing larger lipid levels compared to prey species of other fish. Both species in this study inhabit similar ecological niches and prey on similar species. However, *L. squamifrons* is suggested to include low energy containing cnidarians and thaliaceans in its diet, while diet of *T. hansoni* focuses on krill, euphausids and polychaetes containing larger lipid fractions (Gon & Heemstra 1990; Phleger et al.

1998). Despite the fish having been starved for several days before sampling, an influence of feeding habits cannot be excluded.
Hagen and co-workers (2000) reported the mode of life of a species to be reflected by a species' total lipid content. However, no relation between lifestyle and lipid content was found for benthopelagic *L. squamifrons* and demersal *T. hansoni*.

Phospholipid composition is suggested to play an important role regarding membrane functioning. Phosphatidylethanolamine (PE) adapting a more conical shape supports a lose structure of the membrane at lower temperatures, while phosphatidylcholine (PC) with a cylindrically shaped head group allows tight packing and supports membrane integrity at higher temperatures (Miller et al. 1976; Hazel 1995; Pruitt 1988). Therefore, higher levels of PC relative to PE would be expected with increasing temperature.
Results obtained by HPLC indicate a slight decrease in PE and PC levels with increasing temperature for both species. Thus, an acute temperature increase might have been sufficient to trigger changes in phospholipid levels. However, generally decreasing levels of phospholipids might be caused by rising TAG levels elevating lipid content on the whole. Moreover, enhanced membrane fluidity at warmer temperatures might decrease the importance of PE and PC to preserve membrane fluidity leading to changes in membrane composition and lower levels of phospholipids.
In this study, PE levels of 15.9 ±1.5 % of total liver lipids in *L. squamifrons* and 10.4 ±5.4 % in tissue of *T. hansoni* were measured for control groups by HPLC. PC levels ranged between 47.5 ±4.4 % for *L. squamifrons* and 29.8 ±15.9 % of total liver lipids in *T. hansoni*. These values result in total phospholipid levels of around 69.2 % (*L. squamifrons*) and 42.5 % of total lipids (*T. hansoni*), which seem high compared to values of 19.0 % obtained for liver tissue of *T. bernacchi*, 21.4 % of *Pagonthenia borchgrevinki* and 44.5 % for liver of *Dissostichus mawsoni* (Clarke et al. 1984). It seems that species, such as *L. squamifrons* (distribution 45-56 °S, *T. hansoni* (distribution 53-78 °S) and *D. mawsoni* (distribution 45-78 °S), with a broader distribution range, also in lower latitudes, show higher phospholipid levels in liver tissue compared to *T. bernacchii* (distribution 61-78 °S) or *P. borchgrevinki* (distribution 60-78 °S), with narrow distribution limits in high latitudes. It might be suggested that phospholipids support membrane integrity in environments with large temperature variations. Thus, species from lower latitudes, where ambient water temperatures vary stronger might have higher phospholipid levels than species from higher latitudes, where water temperatures are rather constant. This would agree with suggestions of Bock and co-workers (2001) who found higher levels of PE and PC in Antarctic *P. brachycephalum* compared to temperate *Z. viviparus*.

Membrane unsaturation is suggested to play an important role for maintenance of membrane integrity at cold temperatures. Especially *cis*-double bonds are considered to introduce a kink into acyl chains, leading to less compact packing of UFAS. This is supposed to offset the increase in membrane lipid order at low temperatures and enhance membrane permeability (Bell et al. 1986; Hazel 1995). In this study, development of unsaturated fatty acids/all fatty acids (UFA/all FA) as well as polyunsaturated fatty acids/all fatty acids (PUFA/all FA) ratios in liver of *L. squamifrons* seemed independent of temperature. In *T. hansoni*, both unsaturation indices displayed a significant maximum at habitat temperature, which might be influenced by different sample populations used for control and temperature groups. Ratios of UFA/all FA and PUFA/all FA might indicate a slightly increasing trend with temperature between 4 and 6 °C, which is however influenced by large interindividual differences. Brodte observed insignificant decreasing levels of unsaturated fatty acids from 86.7 % at 0 °C to 79.9 % of fatty acids at 6 °C in liver tissue of *P. brachycephalum*, while an

insignificant increasing trend from 67.6 % at 4 °C to 71.9 % of fatty acids at 18 °C was measured in liver tissue of *Z. viviparus*. PUFAs increased from 4.6 % at 0 °C to 22.9 % at 6 °C in liver of *P. brachycephalum* and from 33.5 % at 4 °C to 39.2 % of fatty acids at 18 °C in liver tissue of *Z. viviparus* (Brodte et al. 2008). Gonzalez-Cabrera et al. (1995) did not find any changes in unsaturation of membranes in liver tissue of *Trematomus bernacchi* and *Trematomus newnesi* after 35 days of acclimation to 4 °C. Thus, short-term exposure to elevated temperatures in the present study as well as in the study of Gonzalez-Cabrera et al. did not lead to changes in fatty acid unsaturation, while long-term experiments by Brodte et al. seemed to affect fatty acid unsaturation.
Ratios of unsaturated fatty acids (UFAs) and polyunsaturated fatty acids (PUFAs) to all fatty acids were determined by NMR spectroscopy in this study, which did not allow quantification. Thus, comparison with data from literature is not possible. Interspecies comparison of UFA and PUFA/all FA showed slightly higher mean values for liver tissue of *T. hansoni* than for *L. squamifrons* at habitat temperature, but higher ratios of UFA and PUFA to all FA in liver tissue of *L. squamifrons* above habitat temperature. This difference could indicate again differences in *T. hansoni* used for control and temperature group, as mentioned above. Disregarding different results between habitat and non-habitat temperatures, higher UFA and PUFA levels in species living at lower water temperature, such as *T. hansoni*, would agree with the hypothesis of UFA and PUFA to support membrane integrity. Hulbert and Else showed a high unsaturation of the membrane to be related to higher metabolic rates in various animals (Hulbert & Else 1999). As *T. hansoni* was shown to have higher metabolic rates than *L. squamifrons* (Sandersfeld project report 2011), higher UFA/all FA and PUFA/all FA ratios would agree with this theory. However, high variability of results does not allow conclusion about interspecies differences in this study.
Logue and co-workers (2000) analysed brain synaptic membranes of fish species from various thermal habitats. They found cold-adaptive increases in membrane disorder to be related to increases in the proportion of UFAs from 35 % to 60 % in phosphatidylcholine (PC) and from 55 % to 85 % in phosphatidylethanolamine (PE). They suggested the reason for this finding to be related to the maintenance of the specific phospholipids molecular shape over the complete biological range of temperatures.

According to the membrane pacemaker theory, metabolic activity and membrane related processes are influenced by the composition of the membrane bilayer, e.g. by stimulating activity of membrane proteins. Especially the highly polyunsaturated docosahexaenoic acid (DHA) is considered to play an important role for metabolism, acting as a form of pacemaker (Hulbert & Else 1999). Disregarding differences between control and temperature groups of *T. hansoni*, docosahexaenoic acid/all fatty acids (DHA/all FA) ratios were constant with rising temperature in both species. Considering the pacemaker role for DHA, parallels with metabolic rate can only be assumed in interspecies comparison, as marine fish mainly rely on highly unsaturated fatty acids from the consumed diet (Sargent et al. 1999). But no significant differences in DHA levels were found in liver tissue between the two species investigated. However, it was shown that the mitochondrial proton leak accounts for about 20 % of the metabolic rate in mammals, rats and lizards (Brand et al. 1991; Rolfe & Brown 1997). As higher densities of mitochondria were shown to be present in muscle tissue of Antarctic fish (Johnston et al. 1998; Peck 2002), differences in DHA levels might rather be expected in muscle than in liver tissue.

Another lipid compound playing an important role for metabolism is cholesterol, being crucial for trans-membrane signal transduction, thus influencing metabolic processes. Moreover, cholesterol is

known to support bilayer fluidity at low temperatures and to enhance bilayer cohesion (Evans & Needham 1987; van Meer et al. 2008). However, cholesterol levels were not affected by temperature for any of both species in this study. Similarly, for *P. brachycephalum* no changes of cholesterol/triacylglyceride ratios were observed with temperature (Brodte et al. 2008). Thus, neither a short-term temperature increase, as done in this study, nor a long-term incubation at warmer temperatures, as done in Brodte's study, seems to affect cholesterol levels in Antarctic fish. Generally, cholesterol levels for both species were slightly higher with values of 5.5 ±0.5 % for *L. squamifrons* and 4.3 ±2.1 % of total lipids in *T. hansoni*, than cholesterol levels in liver of *P. brachycephalum* of 1.7 ±0.8 %, but close to values obtained for *Z. viviparus* of 4.9 ±0.8 % of total liver lipids (Brodte et al. 2008).

4.3. Water soluble compounds

Besides lipids, the effects of temperature on the water soluble metabolites, succinate and acetate, in liver tissue of the Antarctic fish species *L. squamifrons* and *T. hansoni* were assessed and will be discussed in the following.

The performance of ectotherms is strongly influenced by ambient temperature. At the optimal temperature, an organism meets best conditions, which is displayed by low routine metabolic costs. At upper and lower critical temperatures, oxygen supply can no longer match the increasing demand. When this thermal tolerance limit is crossed, a decrease of metabolic rate at elevated temperatures correlates with the onset of anaerobic metabolism, which is indicated by the accumulation of anaerobic end products, such as succinate, formed in anoxic mitochondria (Grieshaber & Hardewig 1994; Zielinski & Pörtner 1996; Pörtner et al. 1999).

Analysis of succinate showed a rising pattern with temperature in *T. hansoni*, but not in *L. squamifrons*. Interestingly, succinate levels in *T. hansoni* measured at habitat temperature are higher than values obtained at 4 and 5 °C and increase subsequently between 4 and 6 °C. Van Dijk and co-workers found control succinate levels in Antarctic eelpout *P. brachycephalum* to be around 0.02 µmol*g^{-1}. They found a decrease in succinate levels in liver of Antarctic eelpout between 0 and 3 °C, but detected a significant rise between 9 and 12 °C. Similar development was resembled by data of *Z. viviparus*, with decreasing succinate levels between 3 and 21 °C and a sharp rise between 21 and 24 °C indicating a crossing of critical temperatures (van Dijk et al. 1999).

The liver is an organ with high oxygen demand, thus anaerobic metabolism in considered to occurs early in liver tissue (van Dijk et al. 1999). However, in the temperature window investigated, anaerobic metabolism did not rise significantly.

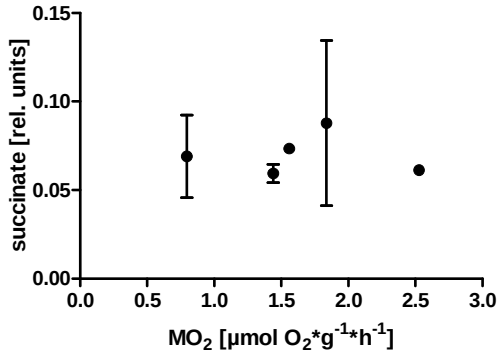

Figure 24: Succinate content in liver tissue of *L. squamifrons* in dependence of whole animal oxygen consumption (MO_2). Values are means, black bars show SD. Respiration data is derived from Sandersfeld project report (2011). (2 °C n=5, 5 °C n=2, 6 °C n=1, 7 °C n=4, 9 °C n=1)

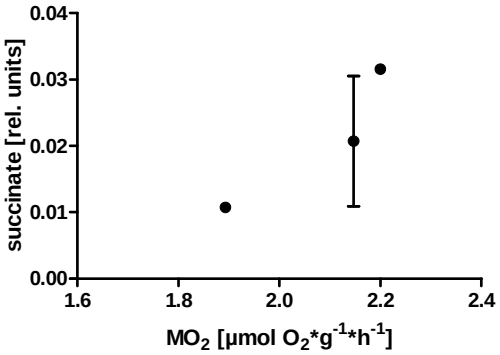

Figure 25: Succinate content in liver tissue of *T. hansoni* in dependence of whole animal oxygen consumption (MO_2). Values are means, black bars show SD. Respiration data is derived from Sandersfeld project report (2011). (*T. hansoni*4 °C n=1, 5 °C n=2, 6 °C n=1)

Relating data from this study with temperature dependent respiration rates of the same animals (Sandersfeld project report 2011), displayed different patterns for both species (figure 24 & 25). For *L. squamifrons*, liver succinate levels seemed constant, ranging between 0.059 ±0.005 and 0.088 ±0.047 relative units, with increasing oxygen consumption between 0.796 µmol $O_2*g^{-1}*h^{-1}$ and 2.528 µmol $O_2*g^{-1}*h^{-1}$ (figure 24). In contrast, liver succinate levels of *T. hansoni* showed a steep increase with respiration rates from value of 0.013 ±0.026 at a respiration rate of 1.893 µmol $O_2*g^{-1}*h^{-1}$ up to 0.032 relative units at a metabolic rate of 2.200 µmol $O_2*g^{-1}*h^{-1}$ (figure 25). As samples of control and temperature group of *T. hansoni* were derived from different cruises (cf. section 2.1), only respiration rates of animals from the temperature group were available. Consequently, the control group was not considered in figure 25. Although no significant rise in succinate levels was detected for any of the two species investigated, similarities with results of van Dijk's study suggest experimental temperatures of this study to at least be close to critical thermal thresholds for *T. hansoni*.

The liver is a central place for lipid metabolism, lipid biosynthesis as well as catabolism. Acetate is an important compound for fatty acid synthesis. Fatty acid chains are constructed by the addition of acetate units derived from acetyl-coenzyme A. Similarly in lipid catabolism, fatty acids are broken down to acetate in framework of *β*-oxidation (Zeeck et al. 1997).
In *T. hansoni* likewise *L. squamifrons*, acetate content did not seem to be dependent on temperature, agreeing with constant total lipid levels in liver tissue determined for both species.

4.4. Temperature effects on metabolism

Considering the impact of a short-term temperature increase on metabolism of the two Antarctic fish species investigated, changes in phospholipid as well as succinate levels were detected.
Analysis of temperature dependent respiration rates showed a significant increase of oxygen consumption from 4 °C upwards in *T. hansoni* as well as in *L. squamifrons* compared to respective habitat temperatures of 0 and 2 °C (Sandersfeld project report 2011; figure 26). These results suggest rising energy demand in the animals' maintenance costs with increasing temperature from 4 °C upwards. If rising energy demand cannot be covered by aerobic metabolism, anaerobic metabolism occurs, leading to an accumulation of succinate. However, temperature dependent succinate levels were constant in *L. squamifrons*, but increased in liver of *T. hansoni*. However, as differences were

not significant, this did not allow conclusion about critical temperatures. Regarding lipid compounds, stable total liver lipid and triacylglyceride (TAG) levels did not show an impact of an acute temperature increase on lipid metabolism, which was supported by constant acetate levels. Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) levels being higher at warmer temperatures indicated a role in sustaining membrane functioning at low temperatures and thus agreed with observation by others (Bock et al. 2001). Ratios of unsaturated fatty acid/all fatty acids (UFA/all FA) and polyunsaturated fatty acid/all fatty acids (PUFA/all FA) likewise docosahexaenoic acid (DHA) and cholesterol (chol) did not seem to vary as a response to acute temperature changes.

On the whole, although changes in anaerobic metabolites found here were not significant, a steep rise in succinate levels with metabolic rate suggest that experimental temperature of 6 °C was close to the upper critical temperatures of *T. hansoni,* while *L. squamifrons* might be able to tolerate temperatures above 9 °C. Upper critical temperatures in this range would be well below critical thermal maxima determined by Bilyk and DeVries of 13.09 ±0.69 and 15.38 ±1.02 °C for *T. hansoni* and *L. squamiforns*. For determination of critical thermal maxima, specimens were heated by 0.3 °C/min until a persistent loss of equilibrium was observed. The critical thermal maximum is a measure of acute tolerance, but was proven to correlate with longer term measures of tolerance (Bilyk & DeVries 2011). However, experimental procedure, rate of temperature change and time for acclimation are known to play a crucial role and can have a huge impact on obtained results (Terblanche et al. 2007; Bilyk & DeVries 2011).
For example, very different results were obtained for thermal capacity of *T. hansoni* in different studies with different experimental protocols. Robinson and Davison (2008) tested acclimatory ability of *T. hansoni* form McMurdo Sound to 4 °C water temperature and found respiration rates of 4 °C acclimated fish not to differ from that of fish acclimated to -1 °C, being measured at day 2, 10 and 16 after transfer to incubation tank. However, mean survival time of specimens was only 14 ±5 days at 4 °C. In contrast, in an experiment with animals used in this study, significant different respiration rates for *T. hansoni* were determined at 4 °C compared to 0 °C (Sandersfeld project report 2011). Experimental setups in both studies were completely different. Whereas in the study of Robinson and Davison, animals were placed from -1 to 4 °C-water directly and acclimation periods to respiration chambers are not mentioned, respiration chambers were heated by 1 °C per 24 hours accompanied by constant respiration measurements and a sufficient acclimation period was granted in this study.
A study on *Pagothenia borchgrevinki*, an Antarctic fish species living at habitat temperatures around -1.9 °C, showed that the species upregulated energy-producing metabolic processes, presumably to meet higher maintenance costs resulting from acclimation to higher temperatures. It seemed, that only when exposed to elevated temperatures for longer periods (4-5 weeks), the species was able to fully compensate for the effects of high temperatures on swimming performance (Seebacher et al. 2005). Different studies demonstrated, that often impacts of temperature can only be assessed by long-term experiments involving acclimation time, unless extreme temperature changes or exercise are applied (Farrell 2002; Gollock et al. 2006). Here, the applied protocol was chosen to allow comparison with other studies, especially in terms of the initial respiration experiments, which was not part of this study.
Moreover, origin of the investigated species might impact thermal tolerance likewise plasticity. Species such as *P. borchgrevinki* or *P. brachycephalum* were shown to have a high thermal tolerance. However, these species are also known to derive from temperate or deep sea ancestors, being immigrated to Antarctic regions (Brodte et al. 2006b; Franklin et al. 2007). Although this might not

restrict validity of results obtained in experiments with these species, it is a factor to consider and a motivation to cover a broader range of model species that occupy different functions in Antarctic ecosystems and originate from different phylogenetic backgrounds. For *L. squamifrons* and *T. hansoni*, no data hinting towards ancestors from warm water habitats, such as for *P. borchgrevinki* or *P. brachycephalum*, was found.

The assessment of thermal tolerance and plasticity is a complex issue, for which not only factors such as incubation time or experimental protocol, but also condition and habitat of the animals as well as phylogenetic background play a crucial role. However, these factors can hardly be controlled and as a consequence, such investigations have to be considered critically. In nature, various other abiotic (e.g. changes in salinity or currents) and biotic factors (e.g. changes in food web structure) might contribute to shaping an organisms response to rising temperatures, which cannot be assessed in experimental trials. However, Pörtner and Knust (2007) showed agreement of thermal limits operative in the field with lab-determined thermal tolerance. Thermal limitation in aquatic ectotherms seems to start with limited oxygen supply. Decreasing aerobic performance causes reduced growth and enhanced mortality, for single individuals likewise populations (Pörtner & Knust 2007). Besides, growth of a population depends on food supply, which in turn impacts aerobic performance and thermal sensitivity. Reduced aerobic performance beyond thermal limits enhances sensitivity to factors such as predation, starvation or disease. The thermal window of a species is shaped by trade-offs in the organismic energy budget as well as thermal adaptation processes, influencing biogeography (Perry et al. 2005), growth, performance and recruitment of a species (Pörtner 2002b; Knust & Pörtner 2007). Thus, investigations on thermal tolerance and plasticity are important contributions to assess the potential consequences of warming waters for Antarctic species.

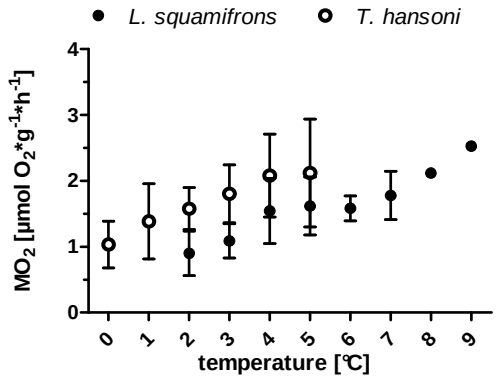

Figure 26: Temperature dependent oxygen consumption (MO_2) of *L squamifrons* (closed circles) and *T. hansoni* (open circles) (Sandersfeld project report 2011). Values are means, black bars show SD. (L. squamifrons: 2-5 °C n=5, 6 °C n=3, 8 & 9 °C n=1; T. hansoni: 0-4 °C n=5, 5 °C n=3)

Thermal tolerance of *L. squamifrons* likewise *T. hansoni* are very low compared to temperate fish, such as the boreal eelpout (cf. Van Dijk et al. 1999), but comply well with values observed for other nothotenioids (Somero & DeVries 1967; Robinson & Davison 2008; Beers & Sidell 2011; Bilyk & DeVries 2011).

Comparing thermal tolerance of sub-Antarctic and high-Antarctic species, such as, *L. squamifrons* and *T. hansoni*, one would expect a sub-Antarctic species, experiencing a more variable environmental temperature, to be more tolerant towards temperature variability than a high- Antarctic species, which constantly lives at temperatures below 0 °C. Declining width of thermal windows with decreasing habitat temperature variation has been observed for different eelpout species (Mark et

al. 2002, Van Dijk et al. 1999). O_2 consumption rates of *L. squamifrons* and *T. hansoni* agree well with these observations (figure 26). Sub-Antarctic *L. squamifrons*, experiencing a temperature range from -1 to 4.5 °C (Whitehouse 1996) in its natural environment, shows a lower slope of oxygen consumption rates with temperature than high-Antarctic *T. hansoni* (Sandersfeld project report 2011), who encounters temperatures form -1.5 to -1.8 °C in the sampling area (Fahrbach et al. 1992). Thermal tolerance of *L. squamifrons* seems to perfectly match thermal conditions in its distribution range. Individuals used in this study were caught near South Georgia at 2 °C water temperature, where annual variations are recorded only to range between -1 and 4.5 °C (Whitehouse et al. 1996). Although respiration rates of *L. squamifrons* increased significantly at 4 °C, increase of succinate seemed constant in liver and, except phospholipids, lipid compounds seemed not to be affected by temperature. Regarding respiration rates the ability of *T. hansoni* to cope with temperature changes of up to 4 °C, while living in an environment constantly below 0 °C seems to be a cosmopolitan quality in this context. However, a steep increase of liver succinate levels with rising temperature likewise oxygen consumption, indicates temperatures to be close to critical thresholds in *T. hansoni*. Possibly, a larger number of individuals might have even shown critical temperatures to lie in the investigated range. However, results obtained here for *T. hansoni* need to be considered critically in general, as acclimation effect to 0 °C cannot be excluded.

Pörtner and others suggested rearrangements of aerobic metabolism in cold adapted species to go in hand with a shift to lipid accumulation and energy storage (Crockett & Sidell 1990; Sidell & Hazel 2002; Pörtner 2002b). Considering interspecies differences, higher total lipid and TAG levels support the idea of a higher degree of metabolic cold adaptation in high-Antarctic *T. hansoni* compared to sub-Antarctic *L. squamifrons*. Moreover, comparison with data from literature indicated a possible relation between phospholipid levels in liver tissue and distribution range. Results suggested a higher phospholipid level to be associated to a broader distribution range and distribution in higher latitudes. Thus, it could be assumed, that a higher phospholipid level supports membrane integrity in species living in habitats with larger temperature variations. The suggestion of different degrees of cold adaptation agrees well with findings by Cox and Macdonald (2008) who suggested an intermediated level of cold adaptation for sub-Antarctic species, after investigating neuromuscular adaptations in sub-and high-Antarctic fish, as well as with findings of Logue et al. (2000), who suggested cold-adapted increases in membrane disorder to be related to the thermal habitat of fish species.

Temperature changes greater than applied in this study or longer incubation times might be necessary to trigger significant metabolic changes, especially in *L. squamifrons,* detectable by the methods used here. Only some of the detected changes showed to be significant and often changes in compound levels were difficult to detect due to inter-individual differences. Generally, as often in experimental studies dealing with live animals, a higher number of replicates might improve this factor, but is difficult to realize due to limited fishing possibilities in Antarctic waters.

A critical issue in this study is the fact that samples for control and temperature groups of *T. hansoni* were derived from different cruises, as differences in time (season), food availability or reproductive status might affect liver lipid composition (Fraser et al. 1987; Henderson & Almatar 1989; Marshall et al. 1999; Sargent et al. 1999). Again, available samples were limited.

In this study liver tissue was chosen due to its central role in lipid metabolism. Changes in lipid profiles are suggested to be large (Brodte et al. 2006a; Windisch et al. 2011) and accumulation of anaerobic metabolites is thought to occur early in liver tissue (van Dijk et al. 1999). However, although animals were starved for several days before sampling, an effect of ingested food on lipid

composition cannot be excluded. Farkas and co-workers (2001) used brain tissue for lipid analysis, as lipid composition is independent of ingested food and changes in composition are supposed to be related to temperature only in this organ. A comparison between lipid analysis of liver and brain tissue could help to find out whether changes in lipid composition can be detected earlier in liver tissue and whether starvation can hamper the impact of ingested food on lipid composition in liver tissue.
Moreover, for the analysis of all compounds, complete liver tissue was extracted. Thus, differentiation between storage compounds and membrane components are only based on assumptions, that e.g. phospholipids are mainly present in membranes and play a minor role in other parts of the cell and that e.g. triacylglycerides do occur mainly in form of storage compounds. However, for thorough discrimination of the different functions of lipids as well as the location in the cell where it occurs and impacts metabolism, the plasma membrane/organelle membranes and cytosolic fractions should be analysed separately. Initially, the separation of the plasma membrane was intended in this study, but as infrastructure for this method had to be established first, this goal proved to be too time consuming for the framework of this thesis.

From an ecological perspective, warming of just 1 or 2 °C above habitat temperatures is considered to be fatal for stenothermal high-Antarctic species (Pörtner et al. 1999). However, despite signs of drastic stress, as accumulation of anaerobic end products and doubling metabolic rates, even *T. hansoni* seemed to be able to cope with acute changes in temperature of 4 °C for a short time. This leaves open how long the animals might be able to sustain high temperatures for longer periods as well as impacts on locomotion, feeding and competitiveness in the natural environment, but allows comparison to similar experiments with other species and thus conclusions about thermal tolerance relative to other species.
Finally, this study showed that a short-term increase of temperature with a rate of 1 °C per day did affect succinate as well as phospholipid levels in liver tissue of *L. squamifrons* and *T. hansoni*. Results of this study complied well with current ideas about temperature dependence of lipid as well as metabolic compounds in fish. Further, recent suggestions about metabolic cold adaptation in Antarctic fish were confirmed and results indicated the existence of different degrees of cold adaptation in Antarctic fish, depending on variability of habitat temperature.

5. Conclusion

Decreasing temperature tolerance, as suggested in this study, and decreasing growth rates with increasing latitude have been found for various fish species (La Mesa & Vacchi 2001; Brodte et al. 2006a). These observations hint to temperature dependent changes in whole animal energy budget. Thus, further investigations on the temperature dependence of whole animal energy budgets are a promising tool to forward the discussion about temperature dependent shifts in distribution limits as well as implications of global warming on single species likewise ecosystems.

The impact of environmental conditions, such as temperature, on the energy metabolism of an organism is a crucial factor that can influence not only growth and reproduction, but moreover population structures and abundances. In the context of changing Antarctic ecosystems, fish as key species and link between different trophic levels play an important role. Thus, assessing the potential of key species to cope with varying environmental conditions is a challenge for polar marine research that should be taken in future. Further research might address differences in energy allocation of high- and sub-Antarctic species, to answer questions, such as what might happen to Antarctic fish when energy demand rises due to increasing temperatures, but energy-rich prey as krill is replaced by low-energy-containing thaliaceans due to retreating sea ice? And what might be possible consequences of shifts in distribution limits due to a changing environment for different species?

L. squamifrons and *T. hansoni* seem to be very suitable model organisms for the investigation of temperature impacts, as both are suggested to be key species for the monitoring of temperature related range shifts (Barnes et al. 2009) and thus should be considered for further studies.

6. References

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8. Declaration

Herewith I confirm to have written this master thesis by myself and not to have used other sources than stated before.

Bremen, February 2012 Tina Sandersfeld