Tenacity of the Antarctic limpet *Nacella concinna* after long-term acclimation to warming

Master Thesis

Presented by

Alessandra M. E. Kronschnabel

Matriculation number: 217200096

in March 2020

Marine Biology

University of Rostock

Institute of Bioscience, Department of Marine Biology, Albert-Einstein-Straße 3,

18059 Rostock, Germany

First and second superviser:

Prof. Dr. Inna Sokolova

University of Rostock, Institute of Bioscience, Department of Marine Biology

Dr. Christian Bock

Alfred-Wegener-Institute for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany

Contents

List of figures

List of tables

Index of abbreviations

°C	Degree Celsius	min	minutes
1D	One dimensional	ml	millilitre
1H	Hydrogen isotope	*N. concinna*	*Narcella concinna*
ADP	Adenosine diphosphate	NMR	nuclear magnetic resonance
AWI	Alfred-Wegener-Institut	PC	principal component
cpmg	Call- Purcell- Meiboom-Gill	PCA	principle component analysis
D20	Deuterated water	PLA-DA	partial least-squares discriminant analysis
FID	Free induction decay	ppm	parts per million
g	gram	sec	seconds
h	hours	T	tesla
Hz	hertz	TMAO	trimethylamine N-oxide
MHz	Megahertz	TSP	3-(trimethylsilyl)propionic-2,2,3,3-d4 acid, sodium salt
		UDP-glucose	Uridine diphosphate glucose

1 Zusammenfassung

Aufgrund des Klimawandels erwärmen sich die Meere überall auf der Welt, insbesondere in den Polarregionen. Tiere in der Antarktis sind stenotherm und somit spezialisiert auf einen eingegrenzten Temperaturbereich. Untersuchungen der Anpassungsfähigkeit von polaren stenothermen Tieren sind äußerst interessant. Aufgrund ihrer häufig erst späten Geschlechtsreife und langen Reproduktionszeiten ist eine schnelle Adaption fragwürdig. Die Frage ist, ob und wie schnell sich diese stenothermen Tiere in einer wärmeren Umgebung anpassen können und ob sie höhere Temperaturen überleben. Vorherige Untersuchungen zeigen limitierte Anpassungsfähigkeiten. Jedoch mit längeren Inkubationszeiten verschiebt sich die kritische Temperatur, in der Tiere noch mit Einschränkungen über einen gewissen Zeitraum überleben können, nach oben. Deshalb werden mehr und mehr Langzeitexperimente durchgeführt.

In der vorliegenden Studie wurde die Fähigkeit der Antarktischen Napfschnecke *Nacella concinna* (Strebel, 1908) am Substrat zu haften (im weiteren Verlauf „Tenacity“ genannt) an Tieren untersucht, die seit drei Jahren bei 0°C, +1.5°C und +3°C gehältert wurden. Zusätzlich wurden Metaboliten Profile im Fußmuskel der Tiere aus den einzelnen Gruppen untersucht, um Temperaturbedingte Veränderungen im Stoffwechsel zu dokumentieren.

Die Tenacity von Napfschnecken wird als Parameter eingesetzt, um die Fitness von Napfschnecken zu untersuchen. Aufgrund der „Thermal Reaction Norm“ sollte sich die Tenacity verlängern bei steigender Temperatur bis ein Maximum bei der optimalen Temperatur erreicht ist. Danach verkürzt sich die Tenacity bei weiterhin steigender Temperatur. Tiere die sich nicht akklimatisieren konnten haben eine kürzere Tenacity als Tiere die sich akklimatisieren konnten. Obwohl vorherige Versuche dies zeigen konnten, wurde in dieser Studie nach drei Jahren keine signifikante Veränderung der Tenacity-Dauer gefunden.

Metaboliten spielen eine wichtige Rolle im Metabolismus von Organismen und repräsentieren die funktionelle Antwort einer Zelle. Wenn Umweltfaktoren wie die Temperatur sich verändern, können Variationen im Metabolismus und mögliche Anpassungen an Umweltherausforderungen mittels „Metabolites Profiling“, interpretiert werden. Die ungerichtete NMR (Kernspinresonanz) –Spektroskopie an Geweben ist eine elegante Methode um Veränderungen im Metaboliten Profil zu verfolgen. In dieser Arbeit

wurde die Methode eingesetzt und Siebenunddreißig Metaboliten im Fußmuskel von *N. concinna* nachgewiesen.

In den Veränderungen der Metabolitenkonzentrationen fiel besonders auf, dass 2016 deutlich geringere relative Konzentrationen hatte als 2017 und 2019. Eine mögliche Ursache kann sein, dass die Napfschnacken durchgängig hohe Mengen Futter ab 2017 zur Verfügung hatten und vorher während der Schiffsfahrt auf der Polarstern von der Antarktischen Halbinsel zum Alfred-Wegener-Institut sich von geringen Mengen ernähren mussten.

Zwischen den Temperaturen konnten die meisten signifikant unterschiedlichen Metaboliten im akuten Versuch 2016 beobachtet werden. 2017 und 2019 sahen sich ähnlich, und nur geringe unterschiedliche Konzentrationen konnten ausgemacht werden. Jedoch gab es 2019 eine Beobachtung zwischen den +3°C Proben. Diese teilten sich in zwei Gruppen auf in der eine sehr hohe und die andere sehr niedrige relative Metabolitenkonzentrationen hatte.

Die Tiere konnten über drei Jahre bei +3°C problemlos überleben und zeigten in der Tenacity eine nicht signifikante jedoch optisch sichtbare Verbesserung. Es konnte gezeigt werden, dass die Napfschnecken sich an +3°C Wasser akklimatisieren können, wenn sie genug Nahrung zur Verfügung haben.

2 Abstract

Due to climate change seas all over the world are becoming warmer, especially in Polar Regions. Animals in the Antarctic are stenothermic and thus specialised to deal with temperature in a narrow range. Research of the ability to acclimate in polar stenothermal animals is very interesting. Because of their late maturity and long reproduction period, quick adaptation is questionable. The question is whether those stenothermic animals can acclimatise to a warmer environment and survive higher temperatures. Previous experiments show limited acclimation capacities. However, with longer incubation periods the upper critical temperature limit shifts to a higher level. The critical temperature is the temperature where animals can survive with restrictions for a specific time. Therefore, more and more long-term experiments are being conducted.

In the present study, the ability to remain attached to the substratum (called tenacity in the further course) was observed in individuals of the Antarctic limpet *Nacella concinna*

(Strebel, 1908) which were held at 0°C, +1.5°C and +3°C over three years. Additionally, metabolite profiles of the foot muscles of the limpets at the different temperatures were examined to document temperature-dependant changes in their metabolism.

Tenacity experiments are a technique to investigate the fitness of limpets. Because of the thermal reaction norm, the tenacity should be prolonged with increasing temperature until it reaches a maximum at the optimal temperature, then shorten again with a further increase in temperature. Animals which are not acclimated have a shorter tenacity duration whereas acclimated limpets have a longer tenacity duration. Although previous tests showed a positive result, in this experiment no significant differences were found in the tenacity duration after three years.

Metabolites play an important role in the metabolism of an organism and represent the functional responses of a cell. Environmental factors like temperature change, variations in the metabolism and possible acclimation to environmental challenges can be interpreted by metabolic fingerprints. The use of untargeted nuclear magnetic resonance (NMR) spectroscopy with tissue samples is an elegant method to trace changes in metabolite profiles. In this study thirty-seven different metabolites were identified by using that method.

By examining the changes of the metabolites over the years, the lower relative concentrations of the samples in 2016 compared to 2017 and 2019 stand out. A possible reason could be the amount of food. Whereas limpets in 2016 fed microalgae during the cruise on the Polarstern from the Antarctic Peninsula to the Alfred-Wegener-Institute, limpets in 2017 and 2018 fed macroalgae ad libitum.

Between the temperatures, the most significant metabolites were found in the acute trial in 2016. 2017 and 2019 looked similar and only small differences in the metabolite concentrations could be found. However, the +3°C samples in 2019 looked interestingly because the samples were divided in two groups where one has relatively lower concentrations and the other relatively higher concentrations.

The animals survived at +3°C without problems over three years. Additionally, the results showed a change in mean tenacity duration at +3°C. This study showed that limpets can acclimate to +3°C water if they feed ad libitum.

3 Introduction

3.1 Stenothermic animals

The Antarctic is an extreme habitat both on land and in the water. In contrast to the temperatures on land, the temperatures in the water are relatively stable. The surrounding waters of the Antarctic have a low temperature (-1.8°C in winter to +1°C in summer) and have exhibited almost no variation within the last 10 million years (Peck 2005a). The intertidal zone, however, can show a daily difference in temperature of 7°C (in rare occasions up to 18°C) within six hours (Waller et al. 2006a). The sublittoral zone also exhibits small changes in temperature from -1.9°C in winter to +1.8°C in summer (Clarke et al. 2008). As part of climate change an average increase of approximately 2°C in temperature is assumed in all oceans (Peck 2005b). The IPCC SRES climate scenarios for 2080 estimate even an increase for the Southern Ocean by 0.0 – 2.8°C in summer and 0.5 – 5°C in winter (Thornes 2002).

Because Antarctic animals are stenothermic (the optimal temperature range where a live without restriction is possible is very small), they can only survive a narrow range of temperatures. As an environmental factor, temperature influences organisms in all their life functions. Those influences contain changes in the rates of biochemical and physiological processes as well as the stability of biomolecules (Sokolova 2003). Temperature changes are especially crucial for aquatic ectotherms, because their body temperature depends on their habitat temperature (Sokolova 2003).

Physiological studies with Antarctic, ectothermic invertebrates show a limited adaptability within a warming environment over a longer time period (Peck und Conway 2000; Peck et al. 2004; Peck et al. 2010). Peck et al. (2004) ascertained that some organisms like the mussel *Adamussium colbecki* (E. A. Smith, 1902) in the Southern Ocean reduce their activity when the water temperature increases by 2°C. This behaviour could lead to a loss of species.

Peck (2005b) postulated three different strategies on how species could cope with changing environmental conditions. One possibility is to use internal physiological flexibility like Antarctic terrestrial species which cope with extreme temperature fluctuations of 25°C daily. However, benthic species in the Antarctic are believed to be very slow at acclimating because of their low rates of growth, development and metabolism (Peck 2005b). Most

studies with acclimation periods of less than two months showed weak or no acclimation to higher temperatures in Antarctic invertebrates (Peck et al. 2004). Therefore, it is likely not a suitable possibility for those animals (Peck 2005b). A second mechanism is to evolve new adaptations which guarantee survival in a changing world e.g expanding the range of biological capacity to encompass the new environmental range (Peck 2005a). In this case many factors play an important role. The most important factor is the time in which modifications occur in the genetic code (Peck 2005b). Because Antarctic invertebrates have a long lifespan of 40 – 50 years and often reach their sexual maturity late, the chance to evolve their genetics as quickly as climate change is happening is low (Peck 2005a). Another factor is that eggs of Antarctic animals tend to be bigger than in temperate species and the number of eggs per spawning is reduced. This could lead to less opportunities for selective pressure to produce adaptive evolutionary changes (Peck 2005a). Another strategy is to flee from the warming and migrate to higher latitudes. This is not a possibility for Antarctic animals either because the continent only extend over a few latitudinal lines (Peck 2005a).

The Antarctic limpet *Nacella concinna* (Strebel, 1908) is a typical inhabitant of the Antarctic coastal line. These animals are easy to collect and exist in a high abundance (Picken 1980) in the intertidal as well as in the subtidal zone. Several studies about the pedal mucus (Peck et al. 1993), ecology (e.g. Picken 1980; Brêthes et al. 1994; Figueiredo und Lavrado 2013) physiology (e.g. Peck 1989; Peck und Veal 2001; Hawes et al. 2010) and DNA (Hoffman et al. 2010; González-Wevar et al. 2011) of the limpet can be found. As a species that lives also in the intertidal zone it is relatively robust considering the variable disturbances in that zone e.g. desiccation due to tidal cycles, temperature fluctuations and wave exposure (Obermüller et al. 2011). Animals which live in the upper water layers are exposed to fluctuating environmental conditions in contrast to animals from deeper waters, which are protected by the surrounding water masses that function as a buffer for fast temperature changes (Obermüller et al. 2011). The impact of climate change on littoral and sublittoral species is a current object of research. In the present study limpets from the sublittoral zone were used and previous studies showed no differences between the intertidal population and the subtidal population at the Antarctic Peninsula neither genetically (Hoffman et al. 2010; González-Wevar et al. 2011) nor in their tenacity (Morley et al. 2011; Morley et al. 2012). However, physiologically differences including tolerance to freezing (Waller et al. 2006b), metabolic response to air exposure (Weihe und Abele 2008) and righting ability

(Morley et al. 2010) have been documented. The collecting parameters and the stress factors that occur regularly in the habitat of *N.concinna* make it a good model organism to examine a possible adaptation to warming in an exothermic animal.

3.2 The distribution and biology of *Nacella concinna*

3.2.1 The occurrence of *N. concinna*

N. concinna lives in rugged sub- and eulittoral zones of the Antarctic Peninsula and adjacent Islands (Figure 1). In their habitat the limpets live up to a depth of 110 m (Picken 1980). Walker (1972) described the occurrence of two different *N. concinna* populations. He ascertained that individuals longer than 2 cm reach a greater shell height in the intertidal zone than those living in the sublittoral zone. In general, observations show that animals within the higher intertidal zone have a greater shell height with a smaller shell circumference, and limpets below the mean low water level have a smaller shell height with a bigger circumference (Walker 1972; Nolan 1991; Kim 2001).

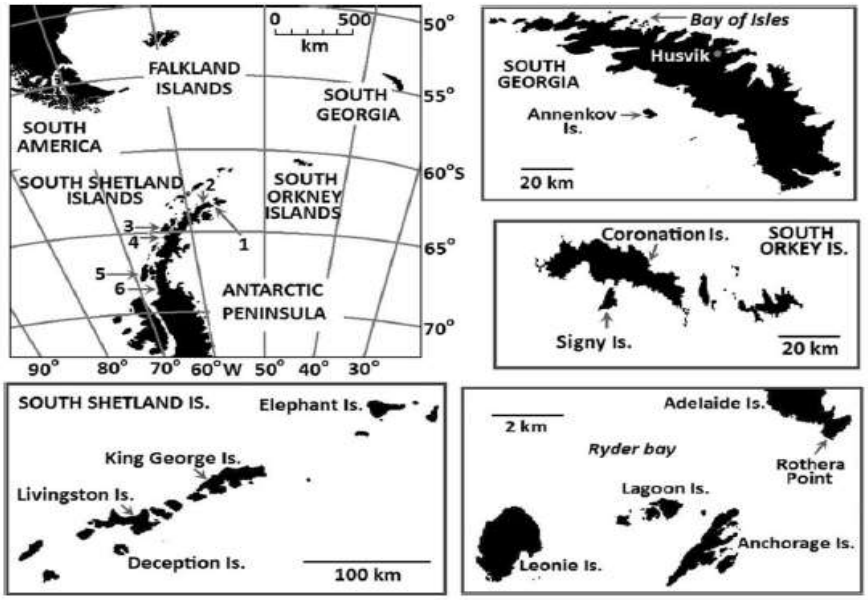

Fig 1: Shown is as an overview of the Antarctic Peninsula region and the adjacent Islands in detail (1 Esperanza Bay, 2 Covadonga Bay, 3 Anvers Is., 4 Galindez Is., 6 Stonington Is.), South Georgia, South Orkney Is., South Shetland and Ryder Bay (southern Adelaide Is.) (Suda et al. 2015).

3.2.2 Thermal tolerance and temperature effects

Water temperatures on the Antarctic Peninsula vary over the year in the sublittoral zone from -1.9°C to +1.8°C (Peck 2005a; Clarke et al. 2008). In the intertidal zone higher temperature differences occur during the low tide because the areas fall dry. In this situation the temperature ranges from -1°C to +17°C (Waller et al. 2006a). Originally scientists had thought that *N.concinna* migrates in winter to inter- and subtidal depths and comes back in summer (Walker 1972). However, Waller et al. (2006b) found some individuals in the intertidal zone during winter.

The thermal range *Nacella concinna* survives in South Georgia is estimated to be between -12.9°C (frozen in ice (Hargens and Shabica 1973)) and +15°C (Davenport 1997).

The upper thermal limit is the highest temperature that the animals survive after exposure over a few days (Peck et al. 2002). The surrounding temperature increases gradually until the animals show no reaction to external stimuli (Peck et al. 2014). The upper thermal limit of *N. concinna* was examined by Morley et al. (2011) and Peck et al. (2009; 2014). An accurate upper thermal limit could not be determined due to different results. The results varied (Table 1) depending on the length of time the animals were acclimated to a higher water temperature. After five months of acclimation the upper thermal limit increased both in 0°C and +3°C (Peck et al. 2014). The value at +3°C even became higher than at 0°C. However, after nine months of acclimation the upper thermal limit tested by Morley et al. (2011) was lower than after five months of acclimation. Still, at +2.9°C the value was higher than at -0.+3°C. Peck et al. (2010) postulated a species would be fully acclimated if the upper thermal limit had increased.

Tab. 1: The range of the upper thermal limit of *N. concinna* after different acclimation duration and temperatures.

Duration (months)	Temperature (°C)	Upper thermal limit (°C)	Sampling site and water depth	Rate of increased temperature	source
0	-1 a	+10 to +14	Antarctic Peninsula	1°C day^{-1}	(Peck et al. 2014)
1	0	+10 to +13	Antarctic Peninsula (Rothera,5-25m)	1°C day^{-1}	(Peck et al. 2009)
5	0	+14 to +17	Antarctic Peninsula	1°C day^{-1}	(Peck et al. 2014)
5	+3	+15 to +18	Antarctic Peninsula	1°C day^{-1}	(Peck et al. 2014)
9	-0.3	+6.9 ± 0.4 b	Antarctic Peninsula (Rothera, 6m)	1°C day^{-1}	(Morley et al. 2011)
9	+2.9	+8.1 ± 0.3 b	Antarctic Peninsula (Rothera, 6m)	1°C day^{-1}	(Morley et al. 2011)

a: temperature at the sampling site b: mean ± standard error

Other studies examined physiological and behavioural changes within a temperature increase. Limpets were observed to move within a range of -1.9°C and +2°C. As the temperature increases the movement speed becomes slower until +14°C is reached, at which point the limpet stops moving (Davenport 1997). At +2°C, Peck et al. (2004) observed that 50% of observed individuals of Adelaide Island lost their righting ability.

In the year 1976 Shabica examined the heart rate of *N.concinna* when exposed to different water temperatures. The measured heart rate of *N. concinna* was 1.65 beats min^{-1} between -0,8°C and 0°C (Shabica 1976). When the temperature was decreased to -2.6°C the measured heart rate was 1 beat $10min^{-1}$. After a small increase to -2.1°C the heart rate started to normalise again. At a temperature increase to +10°C the heart rate increased to 30.2 min^{-1} within one hour (Shabica 1976).

Another study by Morley et al. (2014) showed that the rasping rate (rasps sec^{-1}) rose linearly with the temperature until the rasping stopped at +12.+3°C, a temperature still below the upper thermal limit of *N.concinna*.

3.3 Long-term experiments

For a realistic prediction of how climate change will influence Antarctic organisms, long-term experiments are needed (Barnes und Peck 2008). Several approaches were tested to estimate the acclimation capacity to temperature changes of stenothermic animals (Davenport 1997; Peck et al. 2002). For example, the temperature was increased 1°C every hour, day or week and the reaction of the animals would be observed. A disadvantage of this experiment type in a laboratory is that the temperature increases very fast in a relatively short time. These extreme temperature changes within a few hours, days or weeks are unrealistic according to (Seebacher et al. 2005). The only result shown is that the animals cannot acclimate so quickly and therefore an exaggerated vulnerability can be assumed (Seebacher et al. 2005). However, because of the tide in the intertidal zone, the limpets experience regular crucial temperature changes up to +7°C (or sometimes even +17°C) a day for 6 hours and can survive (Waller et al. 2006a). Barnes and Peck (2008) have mentioned that the animals likely only survive because the temperature rose for 6 hours and then returned to 0°C. If the temperature remained high for a longer period, the Antarctic animals would suffer more (Barnes and Peck 2008; Peck et al. 2009) due to heat stress induced changes in metabolism and damaged proteins (Pörtner 2002; Sokolova et al. 2012).

Previous long-term experiments usually lasted two months (Morley et al. 2011). In 2010 Peck et al. held six different Antarctic species for 60 days in 0°C and +3°C water. Five out of six species showed no response to external stimuli in the higher temperature and thus were described as not acclimated to a higher temperature (Peck et al. 2010). One of the first longer trials was conducted by Morley et al. (2011). Their long-term experiment with *N. concinna* lasted 9 months. The limpets were held in -0.+3°C and +2.9°C water and after the nine months Morley and his colleagues observed a change in duration of tenacity. Based on these results Morley et al. (2011) suggested that Antarctic marine ectotherms require periods longer than two months to acclimate to a higher temperature. Another long-term experiment with *N.concinna* was conducted by Peck et al. (2014). After 5 months of acclimation to warmer water, Peck et al. (2014) found a shift of the upper thermal limit for *N.concinna* between 0°C (+14°C to +17°C) and +3°C (+15°C to 18°C). Therefore, long-term experiments are worthwhile to use if examining the impact of climate change on Antarctic stenothermal animals (Barnes und Peck 2008).

3.4 Objective & Hypotheses

The objective of this thesis is to investigate the impact of a warmer environment on an Antarctic stenothermal organism and whether the duration of acclimation changes the ability of a limpet to remain attached on the substratum, and the contained metabolites within the foot muscle. For this reason, both tenacity experiments and untargeted ^{1}H nuclear magnetic resonance spectroscopy were conducted over three years.

Based on previous research the following hypotheses were formulated:

- The tenacity of *N. concinna* is dependent upon temperature and acclimation time.
- With a longer period of acclimation to warmer water, the tenacity duration of the test animals will increase again.

  Limpets showed a longer tenacity after nine months of acclimation to warmer water than control limpets (Morley et al. 2011) which can be attributed as an indicator of acclimation.

- Metabolite profiles of *N. concinna* will change with acclimation temperature and duration.

  The metabolism depends upon temperature and different pathways are activated by different temperatures. If the animals can acclimate their physiology to a higher temperature after a longer period, the “stressed” metabolism should normalise again.

4 Material & Methods

4.1 Collection & maintenance of *Nacella concinna*

The limpets of the species *N. concinna* were collected by SCUBA divers at the British Antarctic Survey's Rothera Research Station (67°34'S 68°08'W) in the Antarctic autumn of 2016. They were shipped to the Alfred-Wegener-Institute in Bremerhaven and arrived in August 2016. Since then, the limpets were held in aquariums at three different temperatures: 0 ± 0.2°C, 1.5 ± 0.2°C and 3 ± 0.2°C. The aquariums were supplied with seawater from Helgoland with a salinity of ~ 33 permil and aerated with ambient air. *N.concinna* was fed with *Laminaria sp*. ad libitum. The water quality was checked regularly and the water was changed if needed.

4.2 Animal labelling

Limpets were given a small label with numbers on their shell for separation (Figure 2). For the tenacity trials a small patch of gauze was glued on the shell apex (Pattex Ultra gel) in which a fishing line was knotted. The fishing line was approximately 20 cm long. On the other end of the fishing line the label was glued. The label and the fishing line were coiled on a small wire.

Fig. 2: A labelled limpet where the label is coiled on a wire and glued on the shell apex.

4.3 Tenacity experiments

In tenacity experiments scientists scrutinize the ability of limpets to stay attached to the substratum. Older studies concentrated on the needed strength to detached limpets from the surface (Branch 1981; Davenport 1997; Flammang et al. 2002). In more recent studies the duration the limpets remained attached if a specific weight is pulling on them was more important (Morley et al. 2011; Morley et al. 2012). These experiments enable the examination of how "fit" the limpets are if different factors lead to stress e.g. increasing temperature. Additionally, this technique is non-lethal and can be used for all limpets across latitudes (Morley et al. 2011).

Tenacity tests are a good technique to measure physical response at higher temperatures because staying attached on a surface is crucial to survival whereas the occasion that a limpet loses their contact to the substratum and has to right itself up is more rarely (Barnes und Peck 2008).

4.3.1 Experimental set-up

For the tenacity measurement a frame was constructed with multiple metal rods. Two rods were fixed upright on a plate. On each rod was a second rod clamped at a 90° angle, both at the same height. Clamped between those two rods was a rod for stabilization. The two horizontal rods each held a rope and pulley system. The rope was to connect the limpets with a 125 g weight. The frame stands next to the tank of the animals (Figure 3).

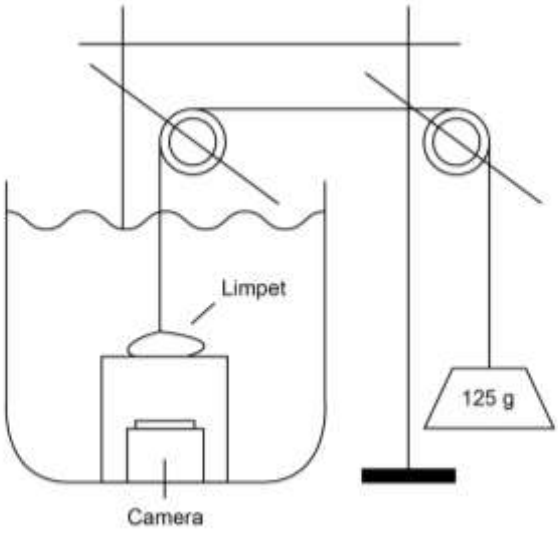

Fig. 3: Schematic drawing of the experimental set-up. The frame stands behind the tank where the limpet is set on a plate. Under the plate a camera was put to film the foot during the trials. The limpet is connected with via a wire to a 125 g weight.

Consequently, the limpets were tested in their usual water and temperature. In three different temperatures were tested; 0°C, 1.5°C and +3°C. *N .concinna* was set on a transparent plate. A ring-formed weight was fixed on the plate as a fence so the limpets could not move away from the plate. Next to the ring a centimeter scale was glued. Under the plate a GoPro Hero 5 black was put to film the trials (Figure 4). Movie frames were used to calculate the foot area using ImageJ (Fiji) by drawing around the foot with the aid of the scale next to the ring.

Fig. 4: Left: A tank where *N. concinna* animals lived. In front of the tank is the frame to see. Over the orange wheels laid the rope that connected the limpets with the weight. Right: A view inside a tank where the camera laid on the ground. Above the camera is the transparent plate with the ring-formed weight and a limpet.

4.3.2 Experimental procedure

After setting the limpet within the ring, the glued fishing line was knotted to the rope. Thereafter *N. concinna* was left alone for 15 minutes to allow the animal to re-attach its foot. Then the rope was pulled three times gently, to check if the limpet was really attached to the surface. If the limpet stayed on the plate, the weight was slowly lowered until it was fully borne by the limpet. During the experiment the limpets were always submerged by a water layer of approximately 10 cm. The time until the limpet detached was then recorded. 1 hour and 15 minutes was decided as the maximum trial time. If a limpet reached that time, the weight was taken away and the limpet was detached manually. After each trial the plate was cleaned with a paper towel and seawater to remove the mucus.

4.4 Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance spectroscopy was be used as a metabolite profiling tool to examine molecular structures, biochemical reactions, and metabolic profiling. It applies a magnetic field to nuclei and then measures the amount of energy that is necessary to put several nuclei in resonance. This is possible because nuclei have different quantities of neutrons and protons and different spins, and therefore behave like magnets. Isotopes with an odd number of neutrons and even number of protons like ^{1}H, ^{13}C, ^{15}N and ^{31}P are detectable by NMR. Due to the naturally occurring presence of ^{1}H protons in almost all organic molecules, many studies using metabolic profiling choose ^{1}H-NMR spectroscopy (Krishnan et al. 2005).

When a probe is put into the magnetic field of the NMR, the protons align with it. Then a certain radio frequency pulse is sent in to change the proton alignment by adding energy. After the radio frequency pulse stops, the protons return to their original conformation. When the protons switch from a higher energy level to a lower energy level, a free induction decay (FID) is recorded. The resulting resonances of the FID can be detected as a function of time by a receiver. With the Fourier Transformation the NMR-spectrum of the FID function can then be calculated.

An internal standard will be added to the samples as a reference for the measured magnetic properties of the protons. The position of the internal standard peak will be set to zero in the spectrum. As a result of different local chemical environments of the protons the produced peaks are characterized by their magnetic resonance frequency, intensity and fine structure (Gribbestad et al. 2005; Krishnan et al. 2005). The different magnetic resonance frequencies can be observed at several positions in the spectrum and are called a chemical shift (δ) (Fan 1996; Gribbestad et al. 2005). A metabolite can have more than one peak and those peaks can appear as a singlet, doublet, triplet or multiplet. Thus, each molecule or chemical group has its own chemical shifts and can be identified.

4.4.1 Tissue sampling

Foot tissue samples were taken for the untargeted metabolite profiling. The whole foot was removed from the limpet with a scalpel. The cut had to be made as quickly and cleanly as possible. Preferably only muscle tissue was cut without destroying the organs within the visceral hump. The foot tissue was laid on aluminium foil and freeze-clamped in liquid nitrogen. Afterwards the limpet was cut through the head to kill the animal immediately. The frozen tissue was stored in a - 80°C freezer until it was processed further.

4.4.2 Methanol-chloroform extraction

The frozen foot muscle tissue was prepared with a methanol-chloroform extraction for untargeted metabolite profiling based on NMR spectroscopy as described by Wu et al (2008). For that liquid nitrogen, ice, methanol, chloroform, Mili-Q water, and deuterated water (D_2O) were needed. Tissue samples were kept on ice during the whole preparation to prevent thawing.

Approximately 45 mg of muscle tissue was weighed and transferred into a homogenization tube. 400 µl of methanol and 125 µl Mili-Q water were added and the homogenization tubes were kept on ice until they were mixed in a Precellys chilled to 0 ° - 4°C (Precellys 24, Bertin Technologies, France). The Precellys is a benchtop instrument for

grinding, lysis and homogenization of biological samples and is ideal for the homogenization of animal tissues, even difficult samples like muscle tissue because of its high speed and special movement. The Precellys was cooled down with an external nitrogen thermostat (Cryolys, Bertin Technologies, France). Then the Precellys was programmed to 2 cycles of 20 seconds at 6000rpm. After a one minute pause the process was repeated four times to receive an optimal homogenization of the hard muscle tissue. After, 400 µl ice cold chloroform and 400 µl ice cold Mili-Q were added. Then the tubes were vortexed for 15 seconds and incubated on ice for 10 minutes. To retrieve the metabolites for NMR spectroscopy, the homogenization tubes were centrifuged for 10 minutes at 3000 rcf at 4°C. After centrifugation three phases were visible. The upper layer was the methanol layer with the polar metabolites. This phase was transferred to a new Eppendorf tube and dried by a vacuum centrifuge at room temperature overnight. The lower phase was the chloroform layer with lipids and the thin layer in between were cell debris and proteins. The chloroform layer was transferred into a brown glass vial and dried in a fume hood at room temperature. The glass vial containing lipids could be suspended with deuterated chloroform for testing with NMR spectroscopy as well. The solid phase of cell debris and proteins was discarded.

4.4.3 ^{1}H-NMR spectroscopy measurements

For the ^{1}H-NMR spectroscopy measurements the dried polar metabolites from the upper layer were suspended with 45 µl D_2O (99,9%) which contained 0.05 wt.% of 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid, sodium salt (TSP) as an internal standard marker (Sigma Aldrich, St. Louis, USA). The TSP functioned as a chemical shift reference and D_2O provided a deuterium lock. The refilled Eppendorf tubes were vortexed until the dried metabolite pellet was dissolved completely. Afterwards 40 µl of the solvent was transferred with a 100 µl Hamilton syringe into a NMR capillary (1,7mm TXI B1/S 2 1H 13C 15 N). The solution inside the NMR capillary should be bubble free and then the NMR capillary was closed airtight with Parafilm.

The 1H-NMR spectroscopy was carried out at 400 MHz using a Bruker spectrometer 9.4T (Avance III HD 400 WB, Bruker-BioSpin GmbH, Germany) at room temperature. The sequence used was Carr-Purcell_Meiboom-Gill (cpmg) with the following parameters: flip

angle 90°, acquisition time 4.01 s, relaxation delay 4 s, sweep width 8803 Hz, 32 scans with 4 dummy scans.

For processing the spectra and identifying the metabolites the Chenomx NMR Suite 8.4 software (Chenomx inc., 2018) was used (see next paragraph).

4.4.4 Spectra processing with Chenomx

The first step was to process the spectra in Chenomx Processor by multiplying the Fourier-transformed spectra with an exponential weighing function corresponding to a line broadening of 0.5 Hz. Then the spectra were manually corrected for phase and baseline, and referenced to the TSP at 0 ppm. The TSP concentration was 3.3 mM.

The adjusted spectra were analysed using Chenomx Profiler (Figure 5). The chemical shifts of the NMR signals in relation to the internal standard were aligned to the NMR signals of specific metabolites depicted from an internal database within the Chenomx profiler. In this way, the correct metabolites could be identified. Missing metabolites inside the database were manually added in Chenomx Compound Builder. For example Strombine and Alanopine were measured at a known concentration in the NMR and so the resulting peaks within those spectra build together in Chenomx Compound Builder and are saved in the Chenomx library. Furthermore, the concentration of the assigned metabolites was calculated by the Chenomx software through the concentration of the internal standard TSP.

After profiling of all identified metabolites, an Excel-table was extracted. Those Excel-tables were altered to upload them to MetaboAnalyst 4.0 (https://www.metaboanalyst.ca/) for statistical analysis (see 4.5 Statistical analysis).

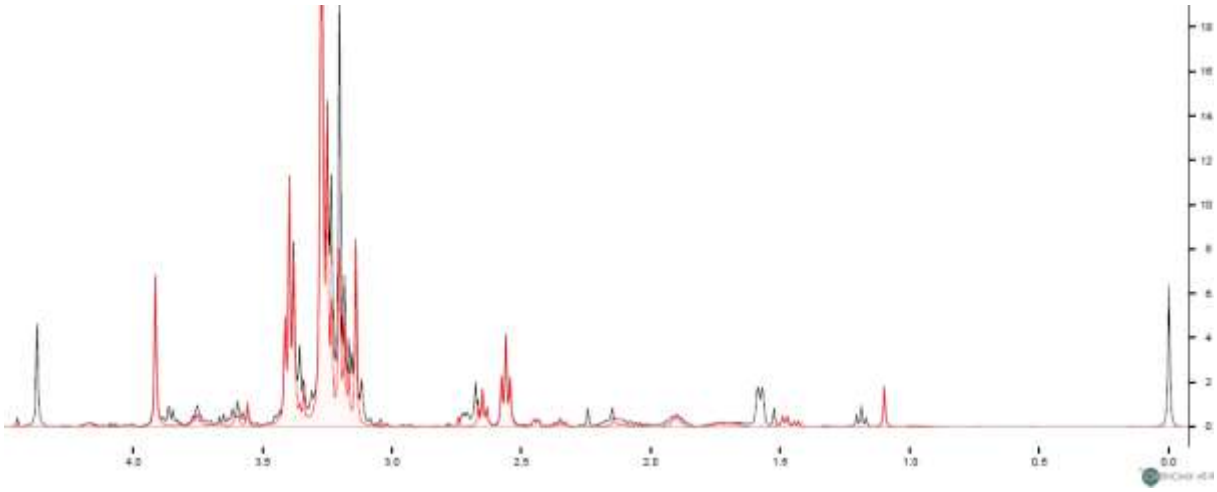

Fig. 5: An extract of a processed spectrum in Chenomx Profiler. The signal of internal TSP standard is set to 0.0 ppm. The black line belongs to the processed spectrum. The red line shows fitted peaks of possible metabolites.

4.5 Statistical analysis

The statistical analysis was conducted with the online website MetaboAnalyst 4.0 and the statistical program R (www.r-project.org/) using RStuduio (https://rstudio.com/).

MetaboAnalyst is an online web server designed to process and analyse NMR-based metabolite profiling data, amongst others. The data processing is coded with R.

The .csv files with metabolite concentrations were uploaded to MetaboAnalyst 4.0. The first step is the data integrity check. Missing values were detected and by default replaced by a small value. In the next step the data was normalized by log or cube root transformation and data scaling. The resulting graphs in MetaboAnalyst 4.0 were identified as normally distributed by visual inspection. Afterwards, different univariate (t-test, Volcano Plot, and ANOVA) and multivariate (Principal Component Analysis, partial least-squares discriminant analysis) analyses were conducted. Principal Component Analysis (PCA) is often used to identify differences between two or more groups. Therefore, it represents hundreds of variables (spectral data points) in relatively few numbers (PC scores). The advantage is that the PC scores can be plotted and make it visually easy to see similarities and differences between the samples (Picone et al. 2011). Partial least-squares discriminant analysis (PLS-DA) is used to predict the class membership (Y) by using multivariate regression techniques that extract that information via linear combination of original variables (X). Other than the

PCA, the PLS-DA considers the correlation between dependent and independent variables. The tested groups become more separated than in the PCA because the PLS-DA obtains a maximum separation among classes by rotating the PCA components (Rebelein et al. 2018). Dendograms or heatmaps were created in MetaboAnalyst 4.0 as hierarchical clustering. Heatmaps and dendograms are a visual aid to cluster different samples. For all dendograms and heatmaps, the Euclidean distance was used for the similarity measurement and ward.D (Ward's minimum variance method) for clustering algorithm (clustering to minimize the sum of squares of any two clusters).

The residual/remaining data was processed in RStudio. In the beginning the data was checked for normal distribution by using Shapiro Wilk test. If the data was not normally distributed, it was normalized by log or cube root transformation. For testing the homogeneity of variance a Bartlett test was conducted. Possible differences of the test groups were evaluated by using ANOVA and its post-hoc test TukeyHSD. For all statistical analysis the p-value threshold was > 0.05.

5 Results

5.1 Duration of tenacity

5.1.1 Duration of tenacity between temperatures in 2016 and 2019

The parametric as well as the non-parametric tests used to test the tenacity between the years showed no significant difference. Therefore, there is no difference between the duration tenacity tests in 2016 and 2019 (Kruskal Wallis, $p = 0.12$; ANOVA, $p = 0.06$). Furthermore, there is also no significance between duration of tenacity and temperature (Kruskal Wallis, $p = 0.1$; ANOVA, $p = 0.12$). In Figure 6 is a box-plot with the duration tenacity and the temperatures between the years. Shown is the median with its first and third quantile as well as the minimum and maximum duration tenacity. In 2016 the minimum and maximum and the median in 0°C and +3°C are similar. The result for the 1.5°C animals differs strongly. In this acute trial the 1.5°C animals stayed attached at minimum as long as 75 % of all animals in 0°C and +3°C. At maximum in 1.5 °C the 4500 sec border was reached what is

determined as sufficient and the test was ceased manually. In 2019 the 0°C boxplot has the shortest duration tenacity. Only one animal remained attached for 4500 sec. The other two temperatures have the same minimum and maximum values, but the median and its quantile differ conspicuous. Whereas 75% of tested animals in +3°C hold 2500 sec, the same amount of animals hold 4022 sec in 1.5°C.

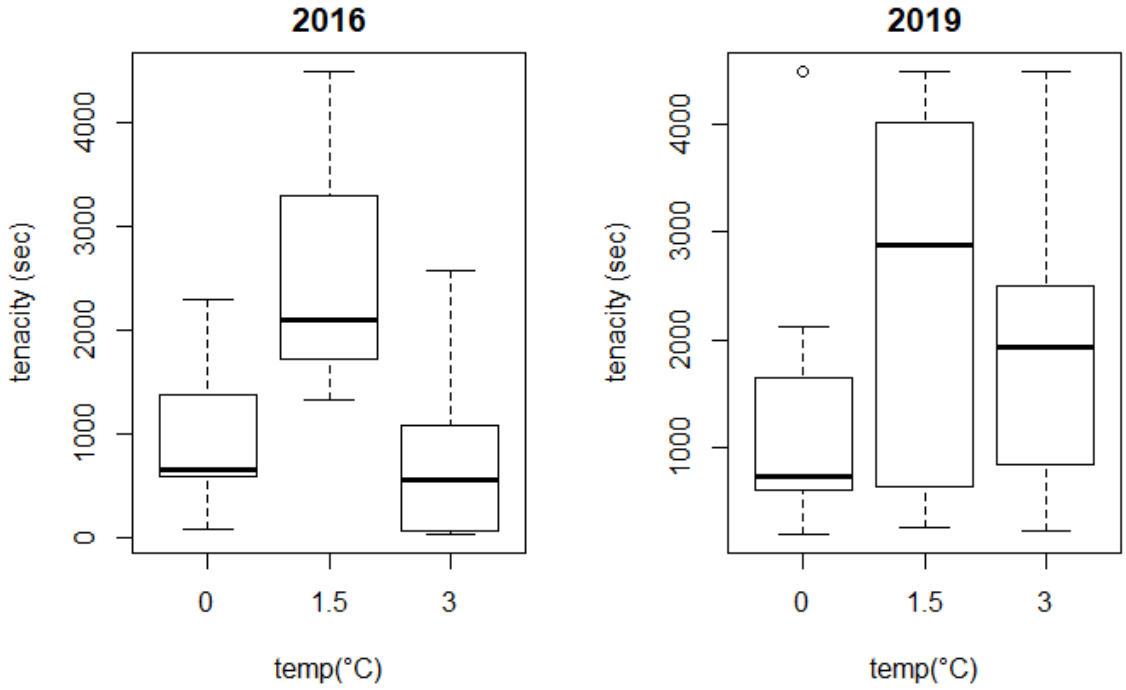

Fig. 6: The measured tenacity in seconds between the three different temperatures 0°C, 1.5°C and +3°C in the years 2016 and 2019, respectively.

5.2. Foot area of *Nacella concinna*

5.2.1 Foot area compared to duration of tenacity

As size of the animals might influence their tenacity, a comparison of foot area and duration tenacity was conducted. The linear regression between foot area and duration of tenacity shows no correlation (R^2 = -0.058, p = 0.57) (Figure 7). However, the foot area was only measured in 2019. For comparison of foot area and duration tenacity for 2016 and 2019, a linear regression between measured foot area and computed shell area was performed to see if there is a correlation between foot area and shell area. Figure 8 shows the log-transformed data in a linear regression. Because of the existent correlation (R^2 =

0.878, p = > 0.01) between foot area and shell area, a linear regression between shell area and duration tenacity for both years was used to see if there is a correlation between shell area and duration of tenacity. The cube root transformed data of tenacity and shell area is shown in Figure 9. In this linear regression is no significant correlation between the shell area and tenacity duration (R2 = 0.033, p = 0.141).

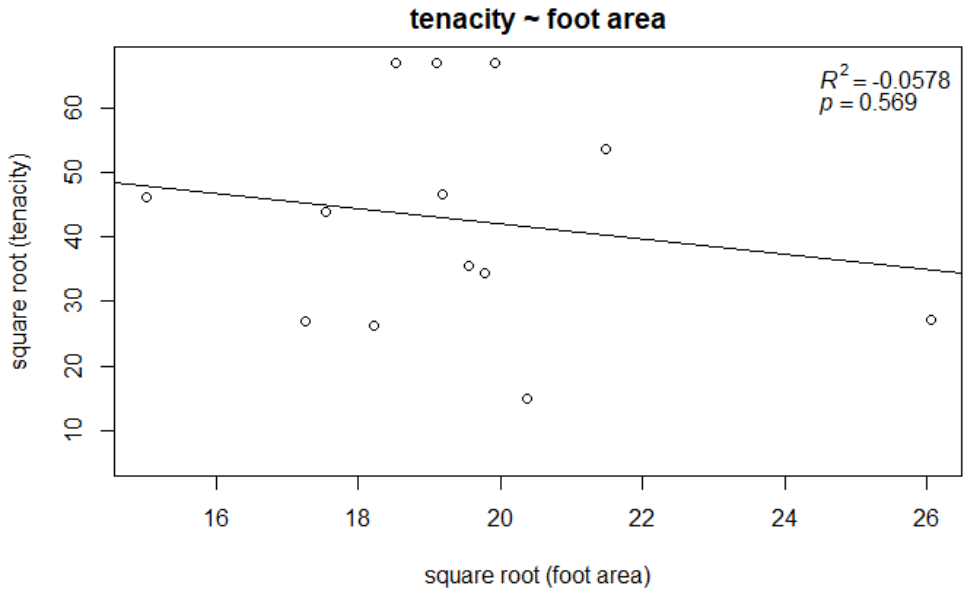

Fig. 7: The cube root transformed tenacity (y-axis) against the cube root transformed foot area (x-axis) with 2019 samples. The sample points are randomly dispersed. There is no correlation between the shown data.

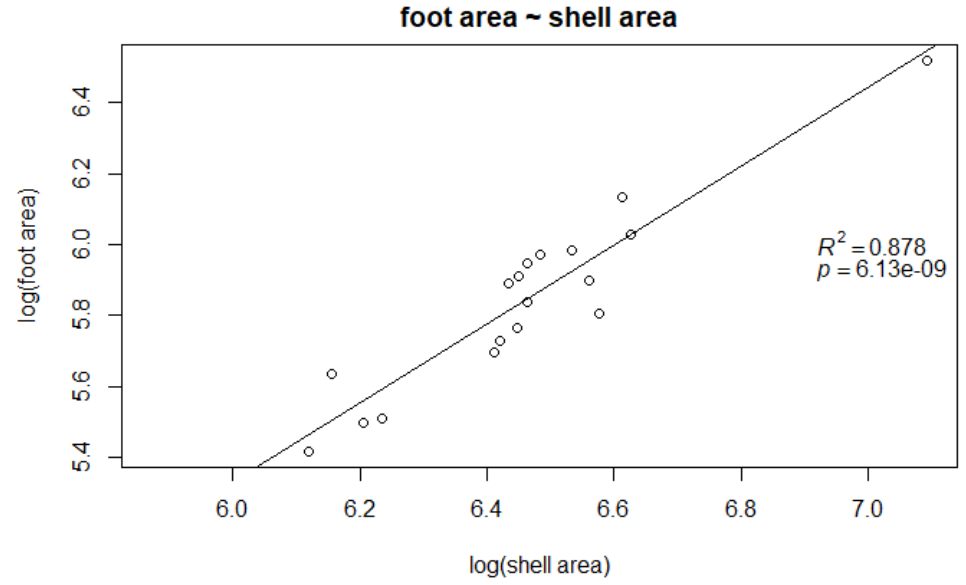

Fig. 8: The log-transformed areas compared to each other. On the x-axis is the shell area, on the y-axis the foot area. With an R^2 = 0.88 there is a high correlation between these areas. Furthermore, the p-value >> 0.05 shows a significant correlation.

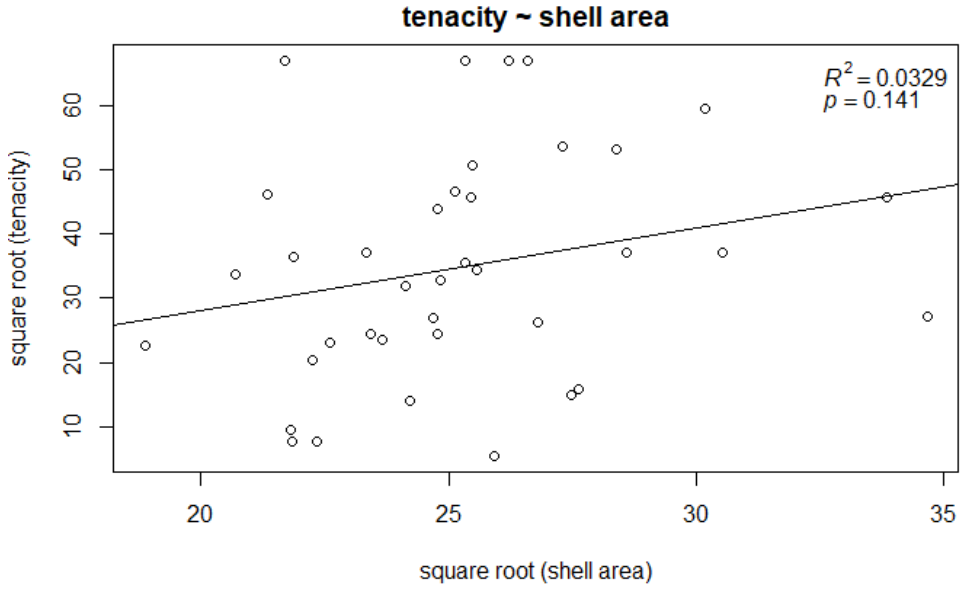

Fig. 9: The cube root transformed tenacity (y-axis) against the cube root transformed shell area (x-axis) for 2016 and 2019 samples. The sample points are randomly dispersed. There is no correlation between the shown data.

5.3 Metabolite profiling data analysis

NMR spectra of this study show the metabolites profiling of muscle tissue extracts. The different metabolites measured in the foot muscle tissue are represented by several peaks with their characteristic chemical shift.

Thirty-seven metabolites within the foot muscle tissue could be identified (see Appendix Table I) from different classes like free amino acids (e.g. valine, leucine, isoleucine, etc.), organic osmolytes (e.g. homarine, betaine, taurine, etc.), and organic acids (e.g. acetate, lactate, succinate etc).

The various metabolites lay in three different regions within an NMR spectrum. Those regions are named aliphatic (0.00 – 2.5 ppm), hydroxylic (2.5 – 6 ppm) and aromatic (6 – 9 ppm) region (Picone et al. 2011). In the aliphatic region are mostly signals of organic acids, side-chains of amino acids or lipids. Lipids, however, are almost not present in aqueous extracts. For measuring lipids the chloroform layer of the methanol-chloroform extraction is better suited. In the hydroxylic region are mainly peaks belonging to alcohols and poly-alcohols like sugars. Furthermore, alpha amino acids and unsaturated compounds are there. The third region, the aromatic region, comprises signals of (poly) phenols and other aromatics made up of purine derivatives such as ATP and its catabolites (Picone et al. 2011).

Figure 10 presents a typical ^{1}H-NMR spectrum from 0 ppm to 5 ppm of a limpet foot muscle tissue under control conditions (0°C). The pattern of the peaks did not differ in spectra of +3°C samples. For better illustration of the smaller peaks different regions were cut out and vertical scales were increased (Figure 10).

As in other gastropods, the most prominent signals in all spectra were organic osmolytes homarine, betaine and taurine (Lu et al. 2016; Tripp-Valdez et al. 2017) in the area between 2.64 ppm and 4.35 ppm. Furthermore, one peak of unknown identity (unassigned 1, 1.1 ppm) was conspicuous in many spectra.

In the aliphatic region following free amino acids could be identified: alanine, β-alanine, glutamate, glutamine, isoleucine, leucine, methionine and valine. Additionally, organic acids included in this region were acetate, lactate and succinate.

The signals within the hydroxylic region were difficult to identify because many signals which had partly the same chemical shift overlapped. Those signals belonged to alpha amino acids, alcohols, polyols and saccharides. Nonetheless, various metabolites were found because of their distinctive pattern. This included metabolites for lipid metabolism such as choline, O-phosphocholine and sn-glycero-3-phosphocholine, the organic osmolyte betaine, the amino acid glycine and the sugars glucose and UDP-glucose.

Assigned peaks within the aromatic region belonged to adenosinedipohsphate (ADP), trigonelline and hormarine.

Besides of the unassigned peak, multiple signals at 1.2 ppm, 1.6 ppm and 2.7 ppm stayed unknown. First, the unknown peak was considered to be a compound of the pedal mucus. However, different metabolite profiling investigations found also an unknown peak at 1.1 ppm in bivalves molluscs tissue (Tikunov et al. 2010; Aru et al. 2017).

In all samples the same composition of metabolites was found independent of the water temperature. Only the absolute and relative concentrations of the compounds differed.

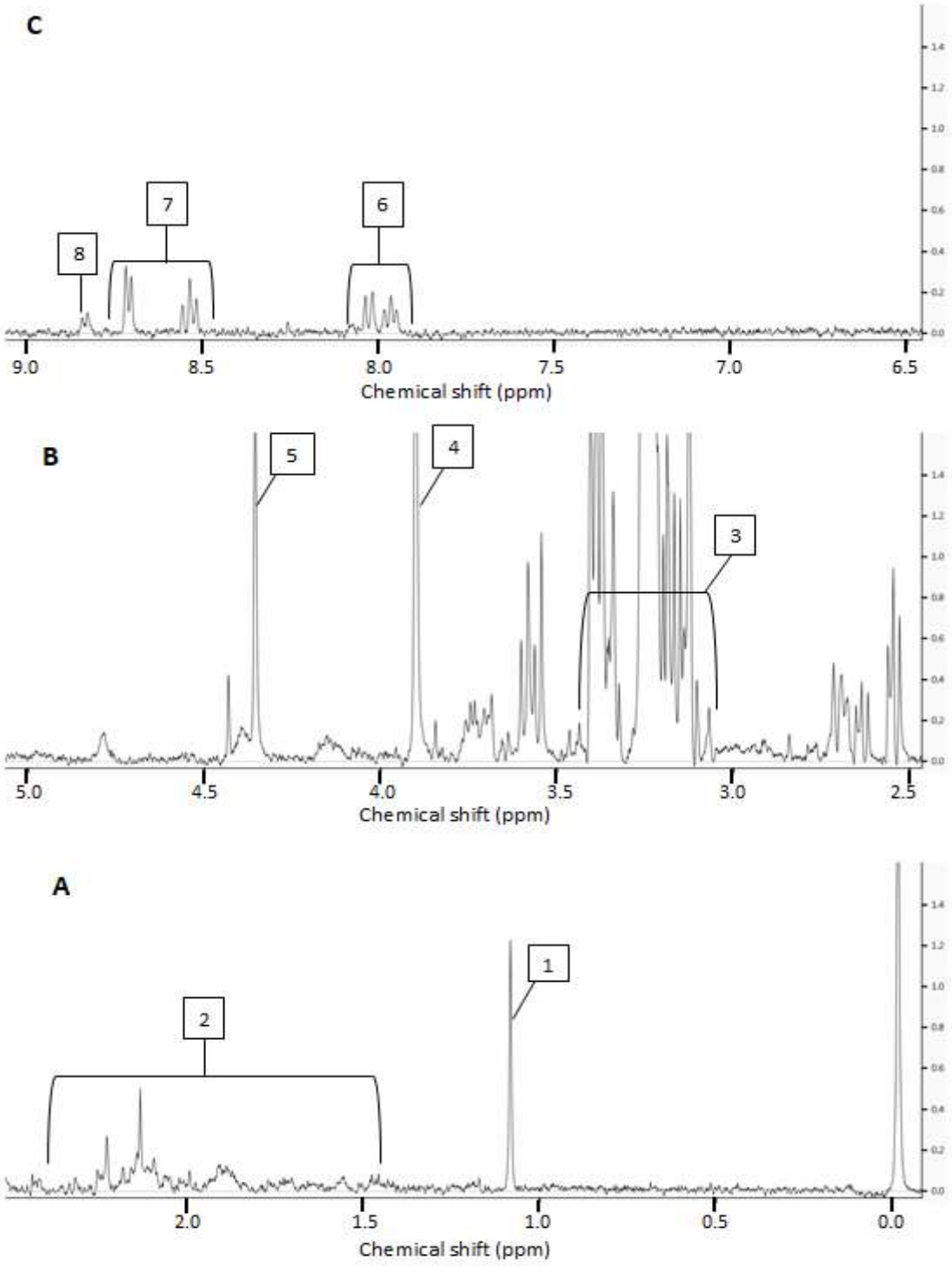

Fig. 10: A typical 1H NMR spectrum of a muscle tissue extract of a control limpet at 0°C divided into three parts for better illustration of smaller peaks.
A: Shown is the aliphatic region from 0.0 ppm to 2.4 ppm. At 0.0 is the TSP standard marker. Peaks: 1) unassigned 1, 2) many different amino acids, and organic acids. **B:** Shown is the hydroxylic region from 2.5 ppm to 5.0 ppm. Peaks: 3) amongst others carnitine, betaine, taurine, 4) betaine, 5) homarine. **C:** Shown is the aromatic region 6.5 ppm to 9.0 ppm. Peaks: 6) homarine and trigonelline, 7)homarine, 8) trigonelline.

5.3.1 Metabolite profiling data analysis for 2016

To investigate if animals with similar metabolite concentrations cluster, a Principal Component Analysis (PCA) plot was generated (Figure 11). Most of the samples within each group gather together. However, there are two samples further away from the residual samples in the control group. In the 0°C group the outliers are sample Sam4_0 and Sam6_add_0.

Moreover, for examining potential differences in metabolite concentrations between both groups a partial least square discriminant analysis (PLS-DA) was conducted. The two groups are in the PLS-DA scores plot fully separated but not far away from each other (Figure12).

To ascertain which metabolite concentrations changed significantly between the two groups in the year 2016, a t-test was conducted. Six out of thirty-seven metabolites were identified by t-test and volcano plot which is significantly different between the control animals in 0°C and those in +3°C (Table 2).

Tab. 2: Important metabolites identified by t-test and volcano plot between both groups in 2016.

	Compounds	**Concentration change**	**p-value**
1	Homarine	0°C < +3°C	0.002
2	Leucine	0°C > +3°C	0.004
3	Sarcosine	0°C < +3°C	0.004
4	Carnitine	0°C > +3°C	0.004
5	Methionine	0°C < +3°C	0.01
6	Trimethylamine	0°C > +3°C	0.02

For further visual differences hierarchical clustering was used. In Figure 13 is a dendogram showing similar clustering between 0°C and +3°C water temperature as the PCA scores plot before. The same outliers as in the PCA are separated from the residual samples. The remaining samples are almost only grouped together with the samples from the same temperature.

In Figure 14 is a heatmap with the two groups. The red and green rectangles on top indicate 0°C and +3°C water temperature, respectively. Each row stands for one metabolite

and the colours dark blue to light blue to light red to dark red show the relative concentration. The metabolites are ordered in classes. The first twelve rows are amino acids, the next seven rows from betaine to trimethylamine N-oxide are osmolytes, the next six rows are metabolites that play a role in energy metabolism, then succinate that belongs to the Krebs cycle, then three phospholipid related compounds and the residual metabolites are miscellaneous with partly more than one function. In this result the samples samcon_3_0 from 0°C water and con23_3 from +3°C are outliers in many metabolite concentrations.

The relative concentrations of almost all amino acids in the 0°C samples are higher than in the +3°C samples. Only methionine ($p = 0.01$) has in the warmer samples a higher concentration than in the colder samples. β-alanine and L-arginine are more or less even in both temperatures. The difference of leucine is even significant tested by the t-test ($p < 0.01$). In the organic osmolytes the concentration between the temperatures changed from a lower concentration at 0°C to a higher concentration at +3°C for four out of seven metabolites. In the other three cases is it the opposite. The relative concentration for the energy related compounds are diverse. No obvious trend is observable. The succinate concentration is lower in the +3°C samples as well as the concentration of phospholipid related compounds. The metabolites classed as miscellaneous have a higher concertation in the +3°C samples but the concentration of carnitine is lower. Both outliers within the heatmap are excluded from the concentration change observation.

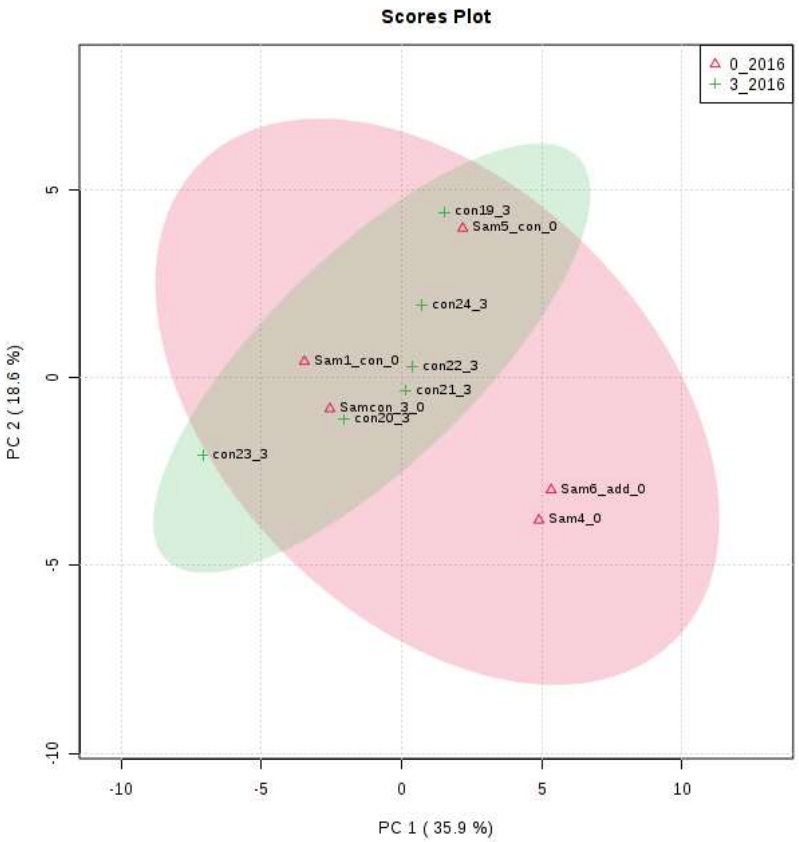

Fig. 11: PCA Scores 2D Plot for the limpets from 2016. Red triangles are animals held in 0°C water and green crosses are animals in +3°C water. This scores plot is between PC1 and PC2. The explained variances are shown in brackets. In total 54.5 % of variance is explained by PC1 and PC2. The ellipses correspond to a confidence interval of 95% for each group.

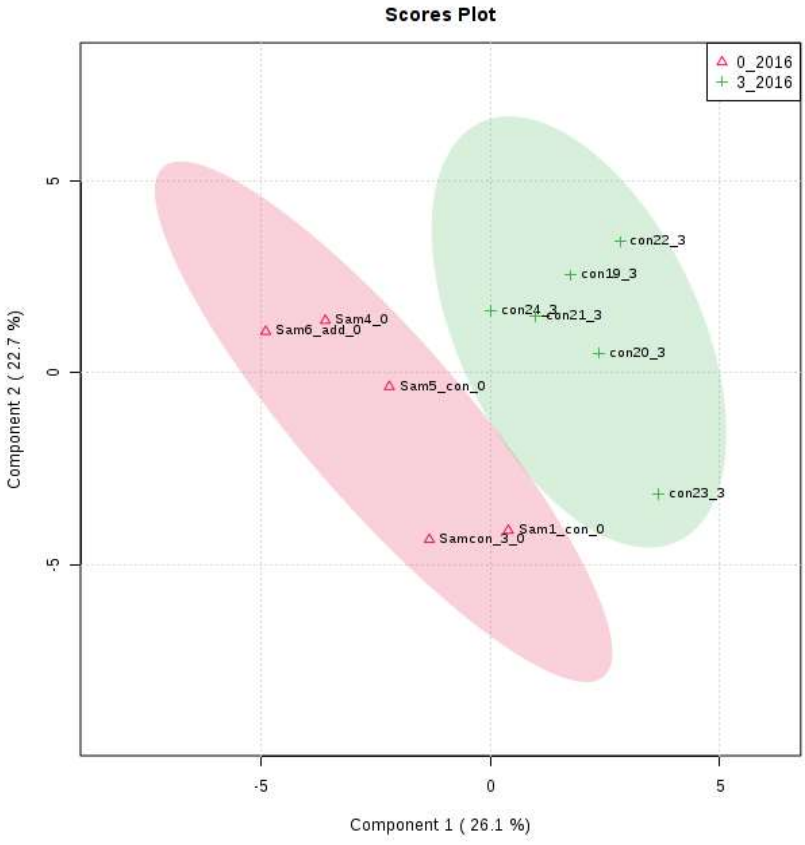

Fig. 12: PLS-DA 2D Scores Plot for the limpets from 2016. Red triangles are animals held in 0°C water and green crosses are animals in +3°C water. This scores plot is between PC1 and PC2. The explained variances are shown in brackets. In total 48.8 % of variance is explained by PC1 and PC2. The ellipses correspond to a confidence interval of 95% for each group.

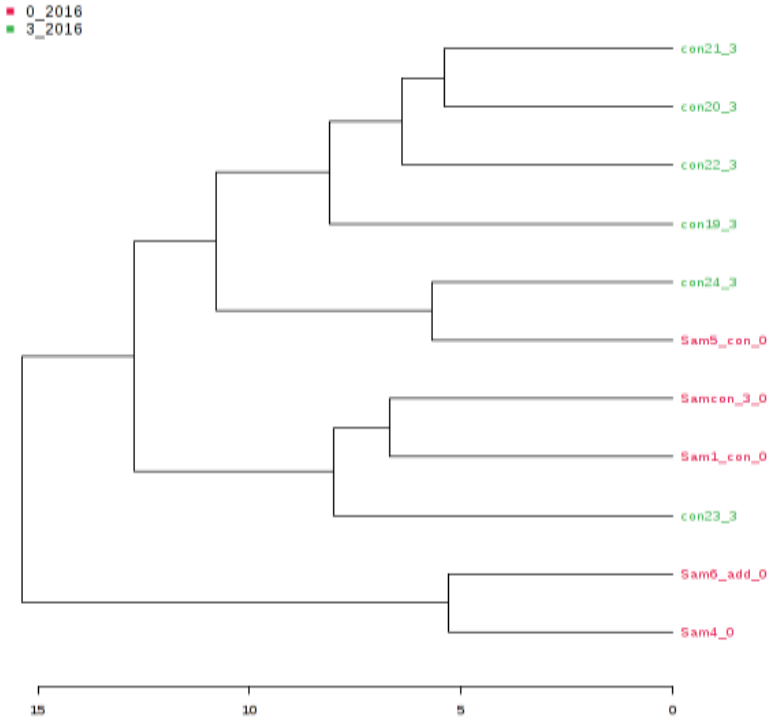

Fig. 13: Dendogram showing clustering result. Red sample names are animals in 0°C water and green sample names are animals in +3°C water.

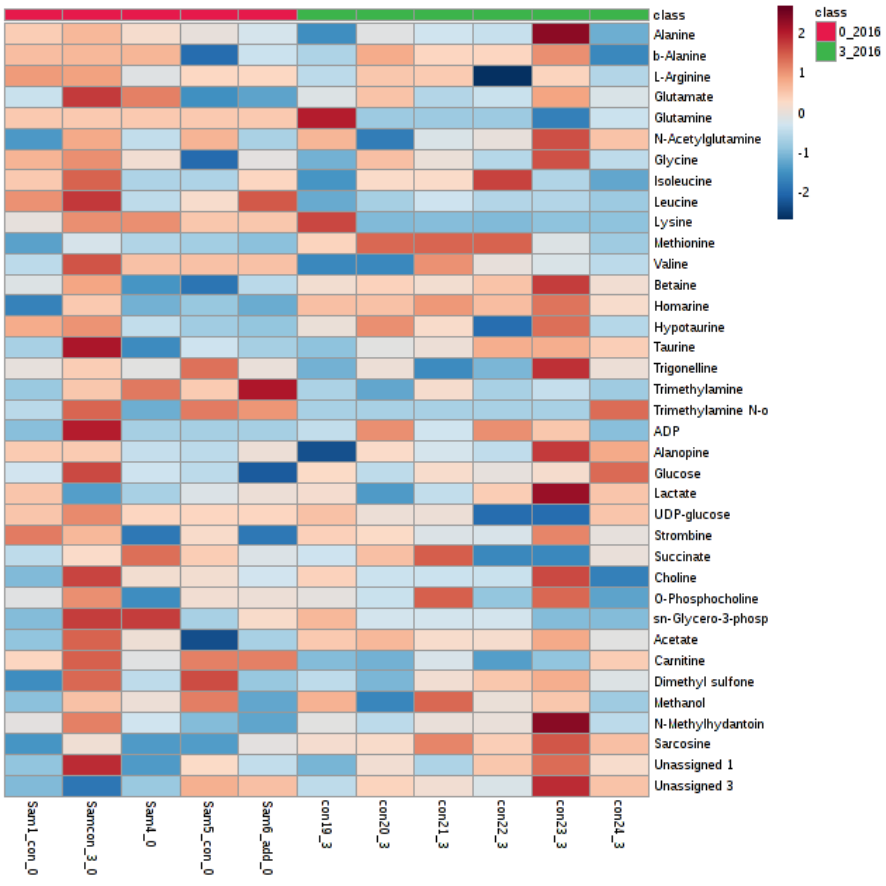

Fig. 14: Heatmap with limpets from 2016 held in 0°C (red rectangles on top) and +3°C (green rectangles on top) water.

5.3.2 Metabolite profiling data analysis for 2017

The samples from 2017 didn't differentiate much between both groups. The ellipses in the PCA score plot lay above each other. Three samples, 3_0 from the control group, 5_3 and 8_3 from the +3°C group, were further away from the centrum than the residual samples (Figure 15).

A partial least square discriminant analysis (PLS-DA) was conducted for examining potential differences in metabolite concentrations between both groups. The two groups in the PLS-DA scores plot are not fully separated from each other (Figure 16).

The t-test found three significant metabolites where the amount of metabolites changed between 0°C and +3°C in 2017 (Table 3).

Tab. 3: Important metabolites identified by volcano plot between 0°C and +3°C in 2017.

	Compounds	**Concentration change**	**p-value**
1	Trimethylamine N-oxide (TMAO)	0°C < +3°C	0.017
2	Lysine	0°C < +3°C	0.018
3	β-Alanine	0°C > +3°C	0.040

For further visual differences hierarchical clustering was used again. The dendogram in Figure 17 shows two main groups where samples of both temperatures are mixed together.

In Figure 18 is a heatmap with the two groups. The red and green rectangles on top indicate 0°C and +3°C water temperature, respectively. Each row stands for one metabolite and the colours dark blue to light blue to light red to dark red show the relative concentration. The metabolites are in the same order like in the previous heatmap. There is no distinguishable pattern between the two groups. Individual metabolites like glutamate and N-Acetylglutamine have a relatively lower concentration at +3°C than at 0°C. Only sample 4_4 and 5_3 have many metabolites in different concentrations than the remaining samples.

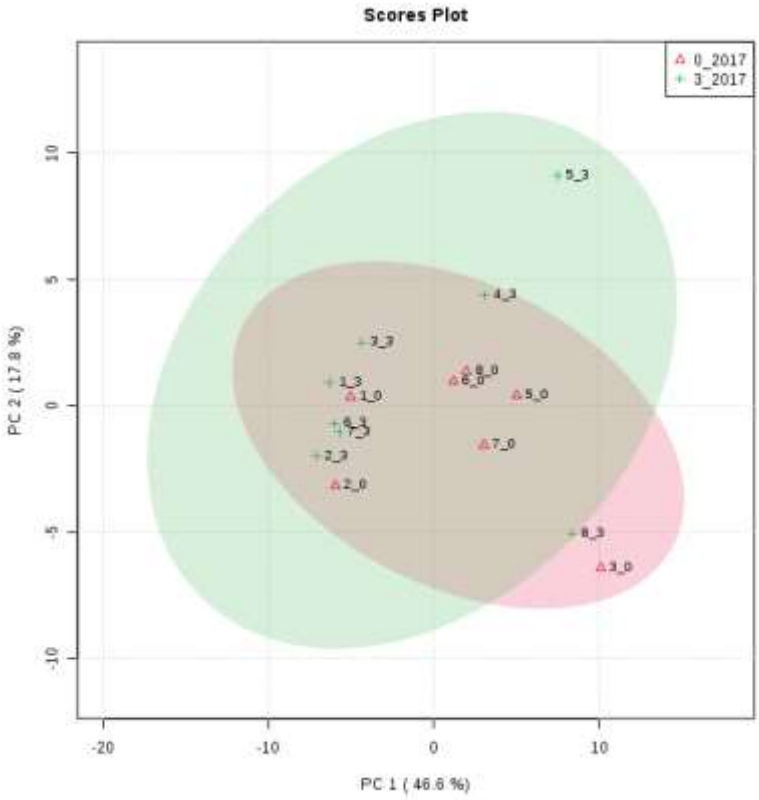

Fig. 15: PCA Scores 2D Plot for the limpets from 2017. Red dots are animals held in 0°C water and green dots are animals in +3°C water. This scores plot is between PC1 and PC2. The explained variances are shown in brackets. In total 64.4 % of the variance is explained by PC1 and PC2. The ellipses correspond to a confidence interval of 95% for each group.

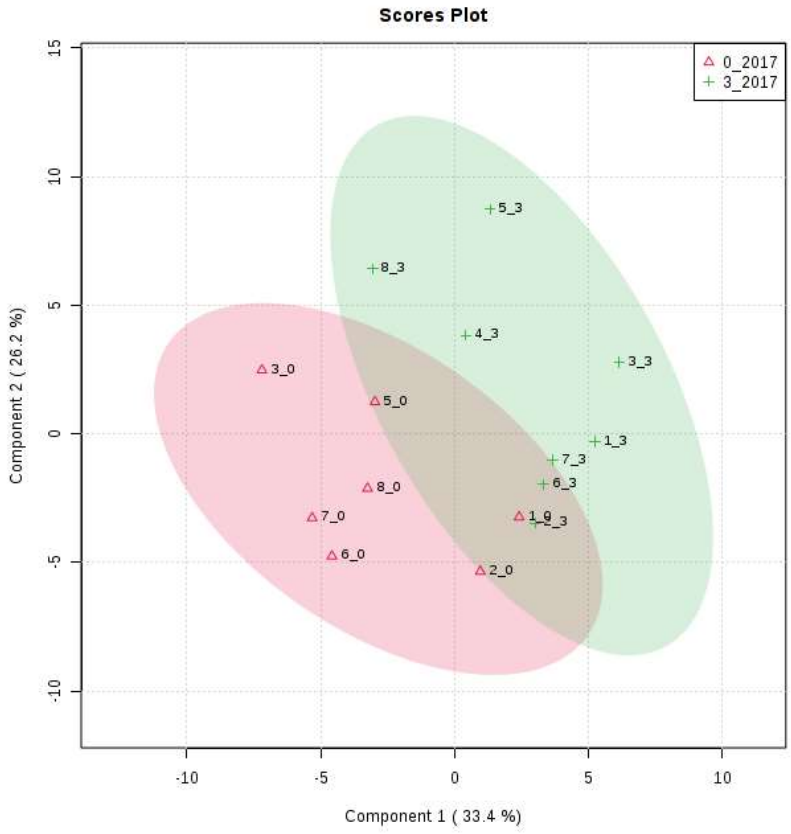

Fig. 16: PLS-DA 2D Scores Plot for the limpets from 2017. Red triangles are animals held in 0°C water and green crosses are animals in +3°C water. This scores plot is between PC1 and PC2. The explained variances are shown in brackets. In total 59.6 % of variance is explained by PC1 and PC2. The ellipses correspond to a confidence interval of 95% for each group.

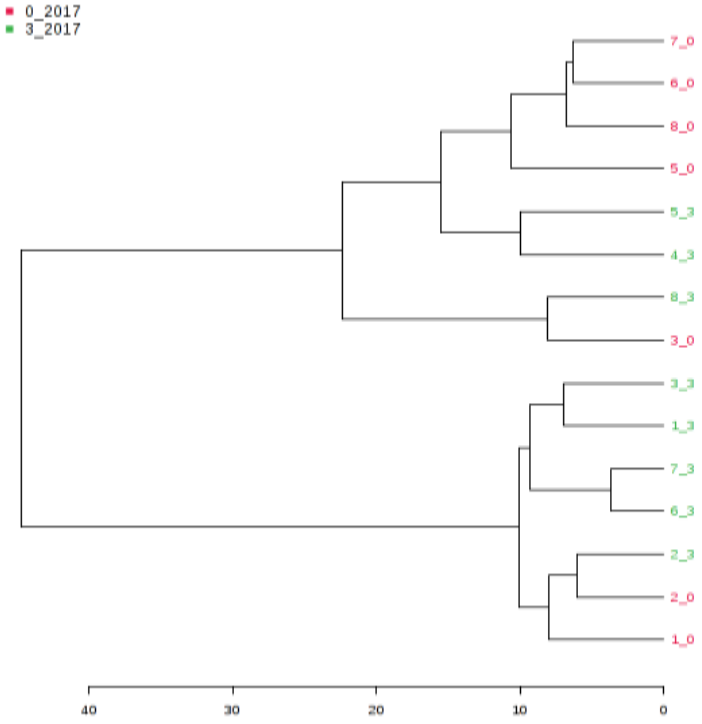

Fig. 17: Dendogram from 2017. Red sample names are animals in 0°C water and green sample names are animals in +3°C water. There is no full separation between the different temperatures.

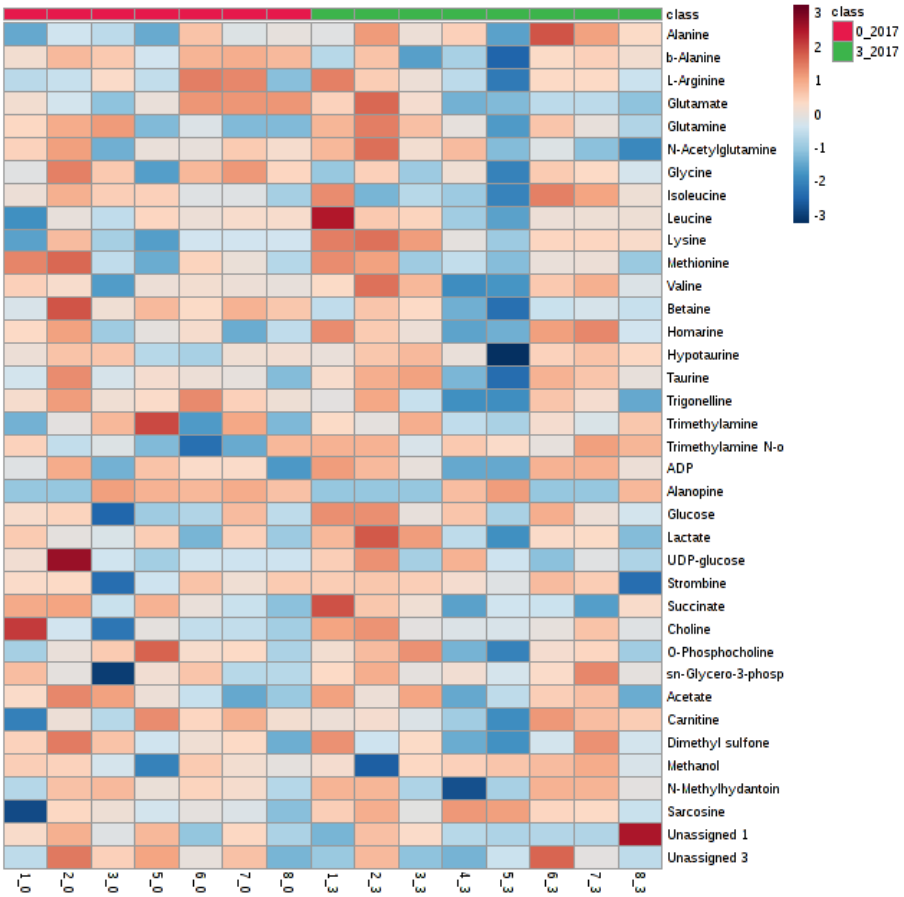

Fig. 18: Heatmap with results from 2017. Red rectangles on top indicate 0°C water and green rectangles on top indicate +3°C water. No real pattern between 0°C and +3°C exists. The sample 5_3 from the +3°C group has outstanding metabolite concentrations in many cases.

5.3.3 Metabolite profiling data analysis for 2019

In the PCA scores plot for the data from 2019 the 0°C samples have a smaller ellipse that is located within the green ellipse of the +3°C samples (Figure 19).

Figure 20 shows a PLS-DA for the different temperatures in 2019. Both groups are fully separated. The red ellipse of the 0°C samples is smaller than the green ellipse.

The t-test could identify three metabolites with different concentrations between the temperatures in the year 2019 (Table 4).

Tab. 4: Important metabolites identified by volcano plot between 0°C and +3°C in 2019.

	Compounds	Concentration change	p-value
1	N-Acetylglutamine	0°C > +3°C	0.007
2	Strombine	0°C < +3°C	0.015
3	β-Alanine	0°C > +3°C	0.030

In the dendogram for 2019 are the samples of 0°C grouped together but surrounded by two groups of +3°C samples (Figure 21).

In Figure 22 is the heatmap of the relative metabolite concentrations of the different temperatures in 2019 shown. All metabolites are in the same order from amino acids to organic osmolytes to miscellaneous. Like in the dendogram, the +3°C samples are divided into two groups. One group with relatively higher concentrations in almost all metabolites compared to one group with lower concentrations. The relative concentration of the 0°C samples are mixed between different metabolites. However, for one metabolite almost all samples at 0°C have a steady concentration distribution. The amino acids concentration at 0°C is either lower or higher than in the two +3°C groups. The same applies for the osmolytes. Metabolites for the energy metabolism look in 0°C samples almost like the second group of the +3°C samples with lower concentrations. The same is true for the metabolites classed as miscellaneous.

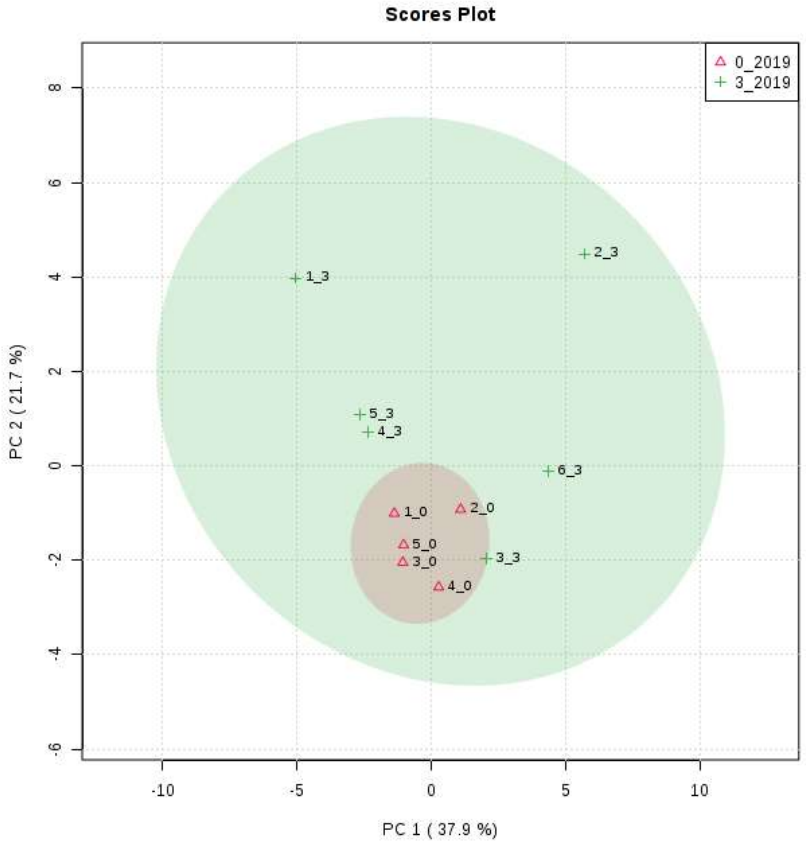

Fig. 19: PCA Scores 2D Plot for the limpets from 2019. Red dots are animals held in 0°C water and green dots are animals in +3°C water. This scores plot is between PC1 and PC2. The explained variances are shown in brackets. In total 59.6 % of the variance is explained by PC1 and PC2. The ellipses correspond to a confidence interval of 95% for each group.

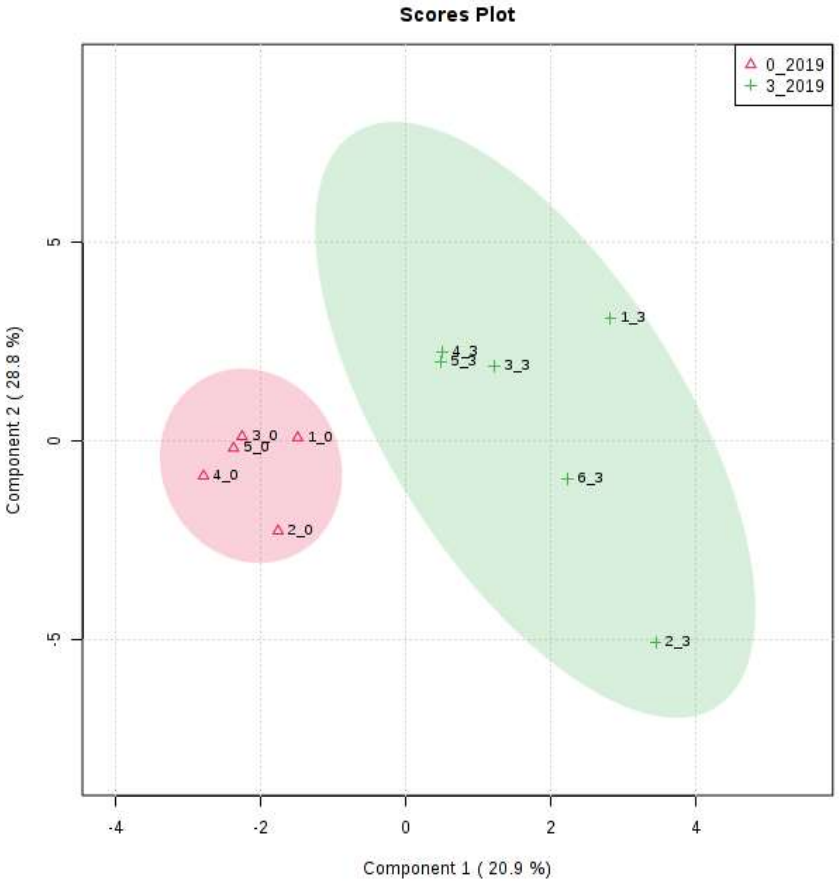

Fig. 20: PLS-DA 2D Scores Plot for the limpets from 2019. Red triangles are animals held in 0°C water and green crosses are animals in +3°C water. This scores plot is between PC1 and PC2. The explained variances are shown in brackets. In total 49.7 % of variance is explained by PC1 and PC2. The ellipses correspond to a confidence interval of 95% for each group.

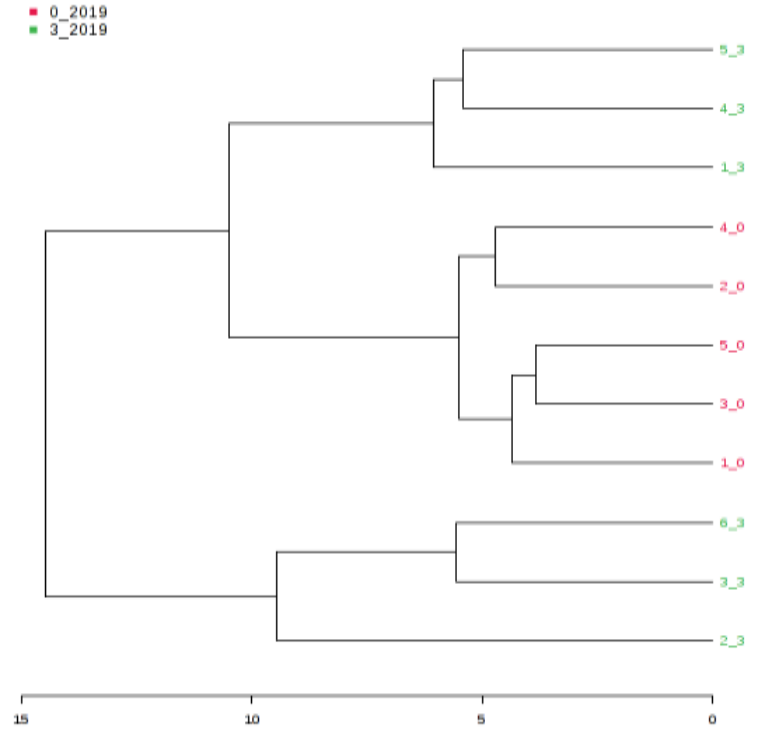

Fig. 21: Dendogram from 2019. Red sample names are animals in 0°C water and green sample names are animals in +3°C water. The 0°C samples are grouped together. The +3°C samples are split into two halves.

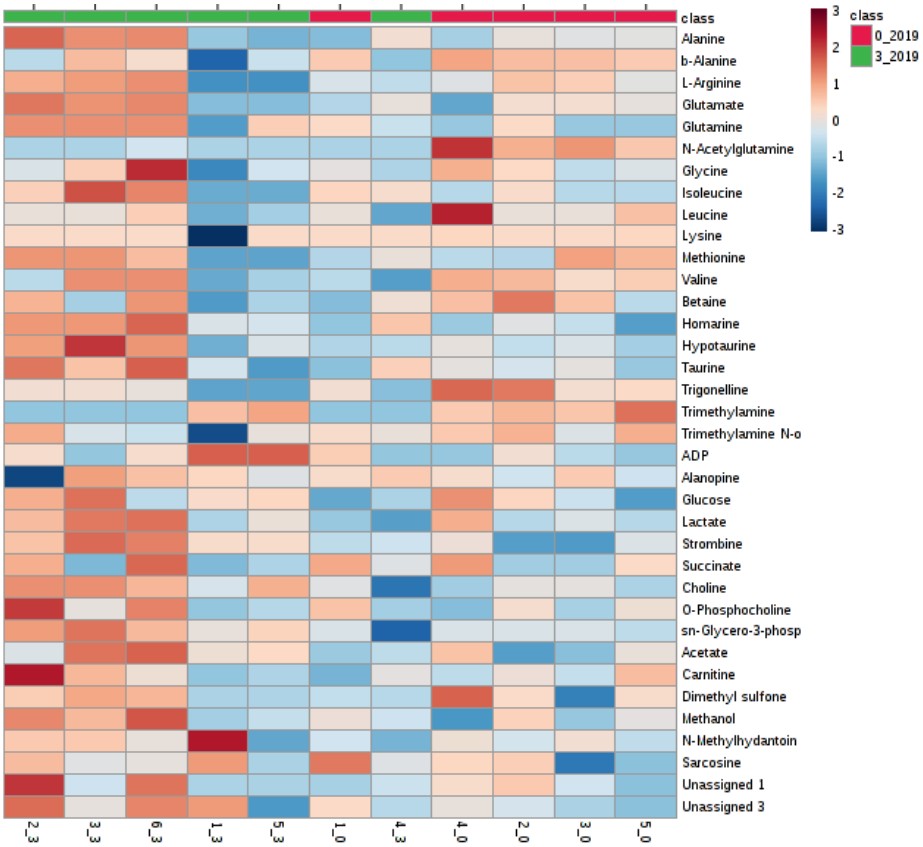

Fig. 22: Heatmap with results from 2019. Red rectangles on top indicate 0°C water and green rectangles on top indicate +3°C water. The samples in 0°C have similar concentrations. The samples in +3°C are separated into two groups.

5.3.4 Change of metabolite concentrations over the years in 0°C trial

Metabolite concentration changes over the years were estimated using One-way ANOVA. Twenty-five metabolites which changed are listed in table II with the results of the post-hoc analysis with Fischer's LSD that shows exactly where the differences are (Appendix, Table II).

The PCA in Figure 23 shows the scores plot between the three 0°C groups in 2016, 2017 and 2019. 2016 is mostly separated from 2017 and 2019, whereas the ellipses of 2017 and 2019 are overlapping.

The PLS-DA for the control data over the years is shown in Figure 24. All three groups are fully separated from each other. The green of 2017 is much bigger than the other two ellipses.

The hierarchical cluster is shown again as a dendogram and heatmap. Both figures have a similar pattern for the samples between the years. In the dendorgram the samples from 2016 seem to differentiate from the other two years, but one sample from 2016 is located between green samples of 2017. The animals from 2019 are split into two halves (Figure 25).

The heatmap shows a lower relative concentration for the 2016 data compared the other two years. The order of the metabolites is the same like before in previous heatmaps. 2017 and 2018 is no distinguishable pattern visible (Figure 26). Most relative metabolite concentrations are higher in 2017 and 2019 than in 2016. The concentration of Trimethylamine did not change vary a lot over the years. The concentration of UDP-glucose decreased after 2016. The concentrations of N-methylhydantoin, Sarcosine, unassigned 1 and unassigned 3 increased in 2017 and decreased in 2019 again. However, the change for sarcosine was not that big than in the others. The sample Samcon_3_0 from 2016 and 2_0.17 are noticeably different than the residual samples in their years. A few samples in all years have exceptionally high or low concentrations in particular metabolites like the β-alanine concentration of sample Sam5_con_0 in 2016, the trimethylamine concentration of 5_0.17 in 2017 and the leucine concentration on 4_0.19 in 2019.

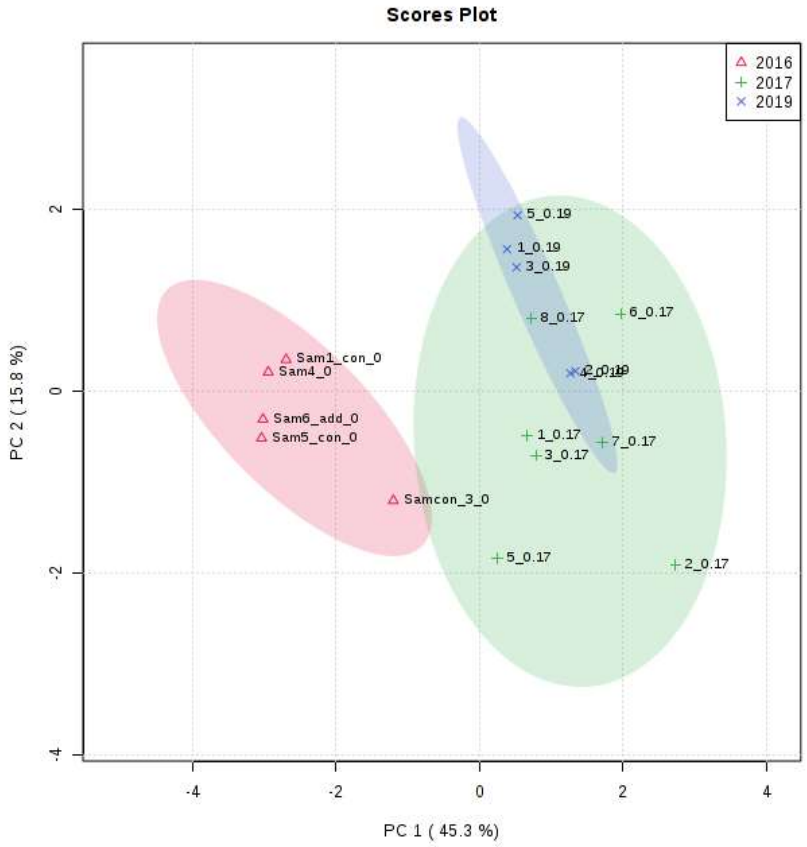

Fig. 23: PCA 2D Scores Plot for the 0°C limpets over the years. Red triangles are animals from 2016, green crosses are animals from 2017, and blue x's are animals from 2019. The red ellipse is mostly distinguishable from the other two. This scores plot is between PC1 and PC2. The explained variances are shown in brackets. In total, 61.1 % of the variance is explained by PC1 and PC2. The ellipses correspond to a confidence interval of 95% for each group.

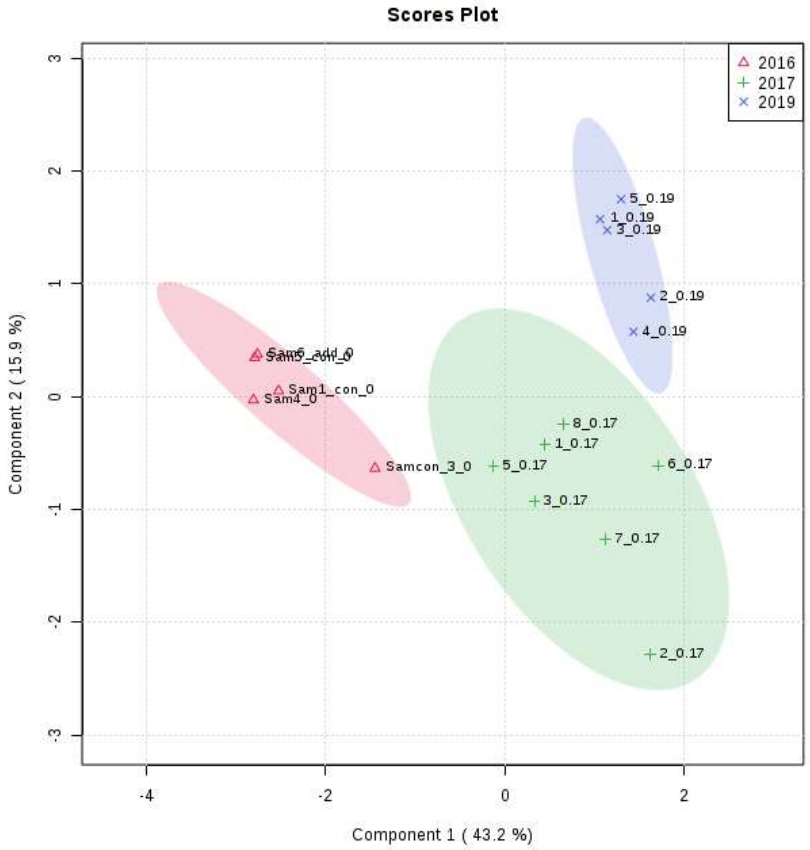

Fig. 24: PLS-DA 2D Scores Plot for the 0°C limpets over the years. Red triangles are animals from 2016, green crosses are animals from 2017, and blue x's are animals from 2019. This scores plot is between PC1 and PC2. The explained variances are shown in brackets. In total 59.1 % of variance is explained by PC1 and PC2. The ellipses correspond to a confidence interval of 95% for each group.

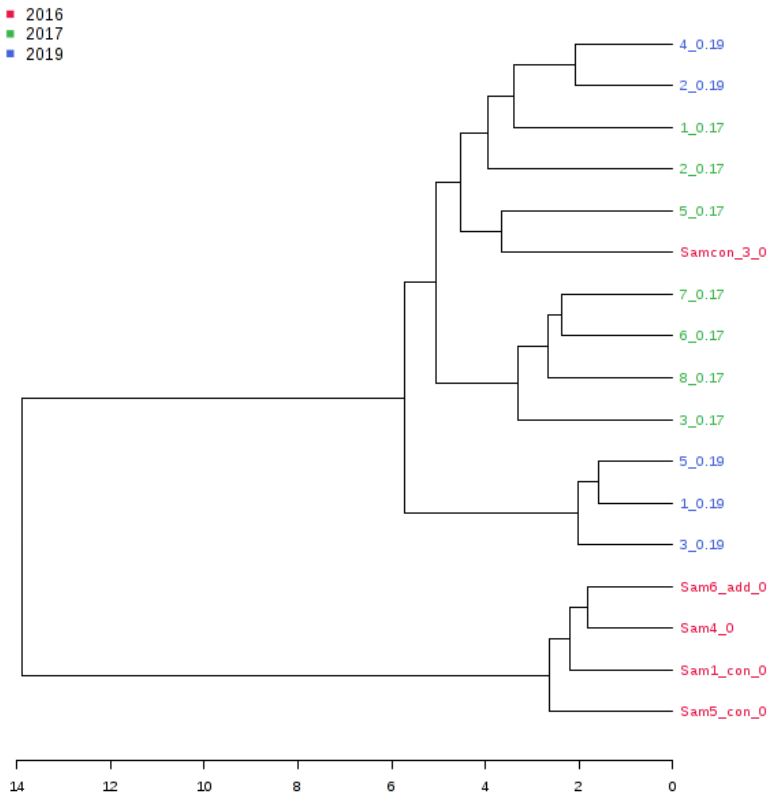

Fig. 25: Dendogram from 0°C over the years. Red sample names are animals from 2016, green sample names are animals from 2017 and blue sample names are animals from 2019. With one exception all samples of 2016 are grouped together. The residual samples are divided into three groups but grouped together against 2016. The ellipses correspond to a confidence interval of 95% for each group.

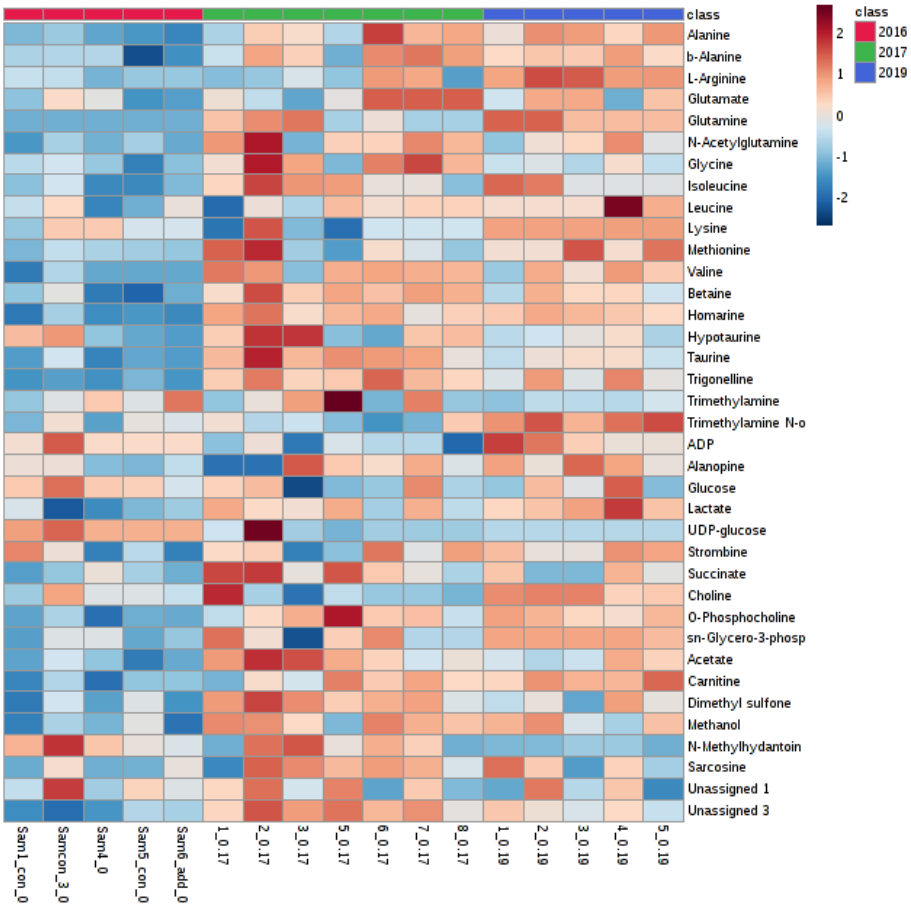

Fig. 26: Heatmap from 0°C over the years. The rectangles on top indicate the different years (red = 2016, green = 2017, blue = 2019). 2016 shows a different pattern from 2017 and 2019. 2017 has a few samples with prominent higher concentrations.

5.3.5 Change of metabolite concentrations over the years in +3°C trial

The same procedure as for the 0°C trial was proceeded for the +3°C trial. At first, distinctions between 2016, 2017 and 2019 were analysed with One-way ANOVA and the post-hoc Fisher's LSD (Appendix, table III). Twenty-two important metabolites were found.

Second, a PCA was conducted. The 2016 samples (red triangles) lay next to each other. Their ellipse is small. The samples from 2017 (green crosses) and 2019 (blue x's) are more scattered. The centrums of the green and blue ellipses overlap each other (Figure 27).

For a possibly more separated pattern, a PLS-DA was again conducted (Figure 28). The red ellipse with samples from 2016 is fully separated from the other two years. The green (2017) ellipse and blue (2019) ellipse lay party above each other.

Fourth, the hierarchical clustering was implemented and shown in a dendogram and a heatmap (Figure 29 & 30). In the dendogram the red samples from 2016 are grouped together. The data from 2017 and 2019 are mixed into two groups surrounding the 2016 samples.

The heatmap shows that samples in 2016 have a lower concentration in almost all metabolites and is clearly separated from the other two years. There is no difference in the pattern of 2017 and 2019. However, there are a few samples that have particular metabolites in exceptionally high concentrations like sample 1_3.17 in Leucine in 2017 and sample 2_3.17 in N-acetylglutamine in 2017. The concentrations of N-acetylglutamine and sarcosine are highest in 2017. The concentration of UDP-glucose decreased over the years. Furthermore, it seems that a sample has either a relatively high or low concentration in all metabolites. All metabolites are in the same order like in previous heatmaps from amino acids to phospholipid related compounds to miscellaneous.

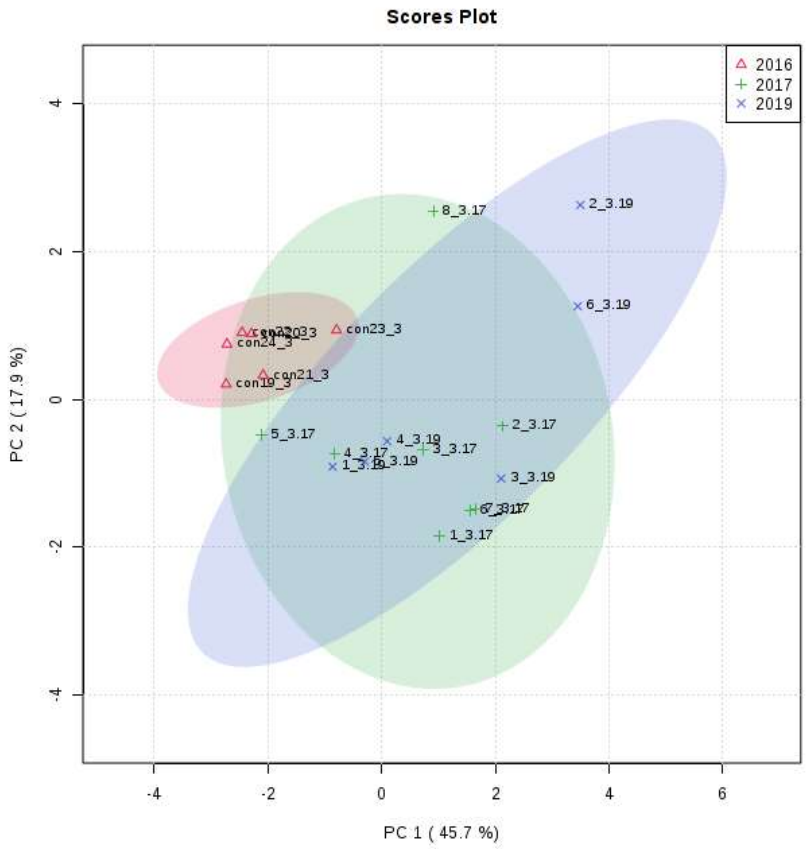

Fig. 27: PCA Scores 2D Plot for the +3°C limpets over the years. Red triangles are animals from 2016, green crosses are animals from 2017, and blue x's are animals from 2019. The red ellipse is the smallest one. No ellipse is fully separated from another. This scores plot is between PC1 and PC2. The explained variances are shown in brackets. In total 63.6 % of the variance is explained by PC1 and PC2. The ellipses correspond to a confidence interval of 95% for each group.

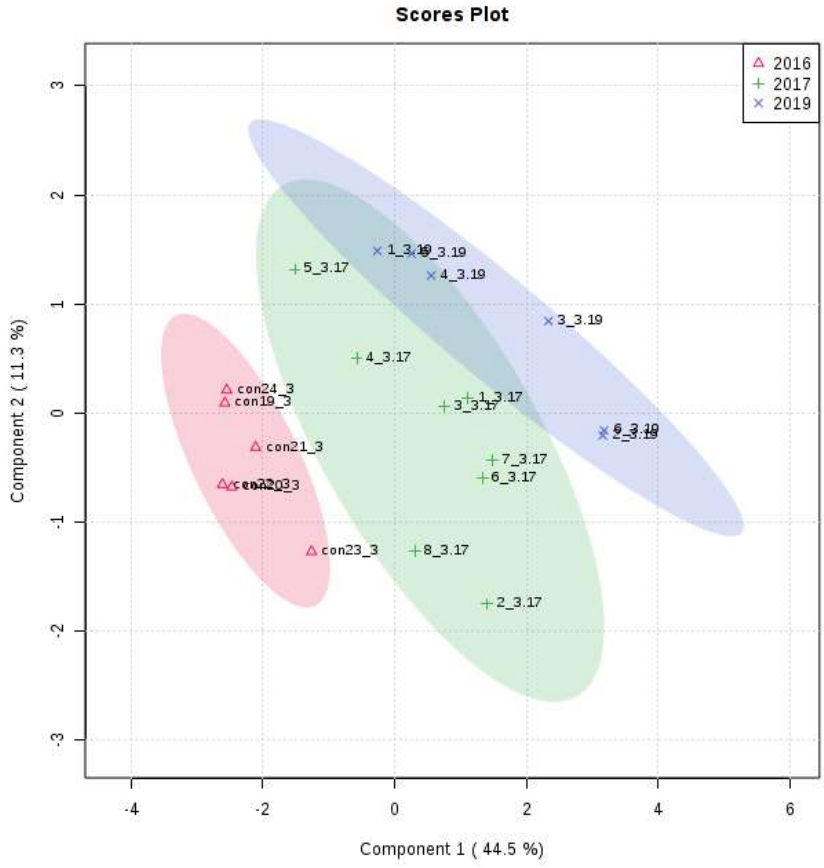

Fig. 28: PLS-DA 2D Scores Plot for the +3°C limpets over the years. Red triangles are animals from 2016, green crosses are animals from 2017, and blue x's are animals from 2019. This scores plot is between PC1 and PC2. The explained variances are shown in brackets. In total 55.8 % of variance is explained by PC1 and PC2. The ellipses correspond to a confidence interval of 95% for each group.

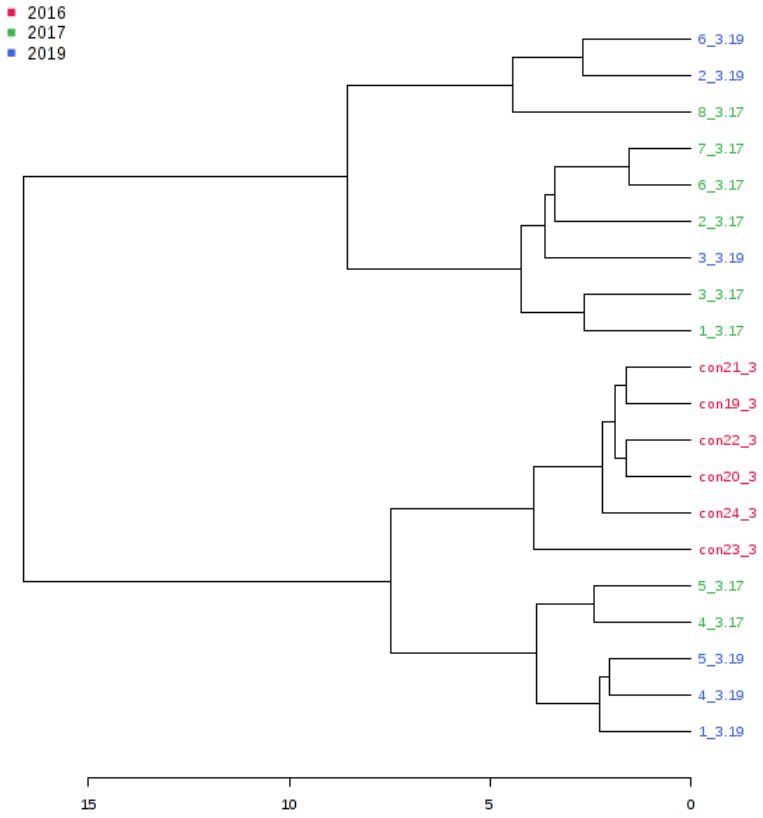

Fig. 29: Dendogram for +3°C over the years. Red sample names are animals from 2016, green sample names are animals from 2017 and blue sample names are animals from 2019. The 2016 samples are grouped together but surrounded by the 2019 and 2017 samples which are mingled.

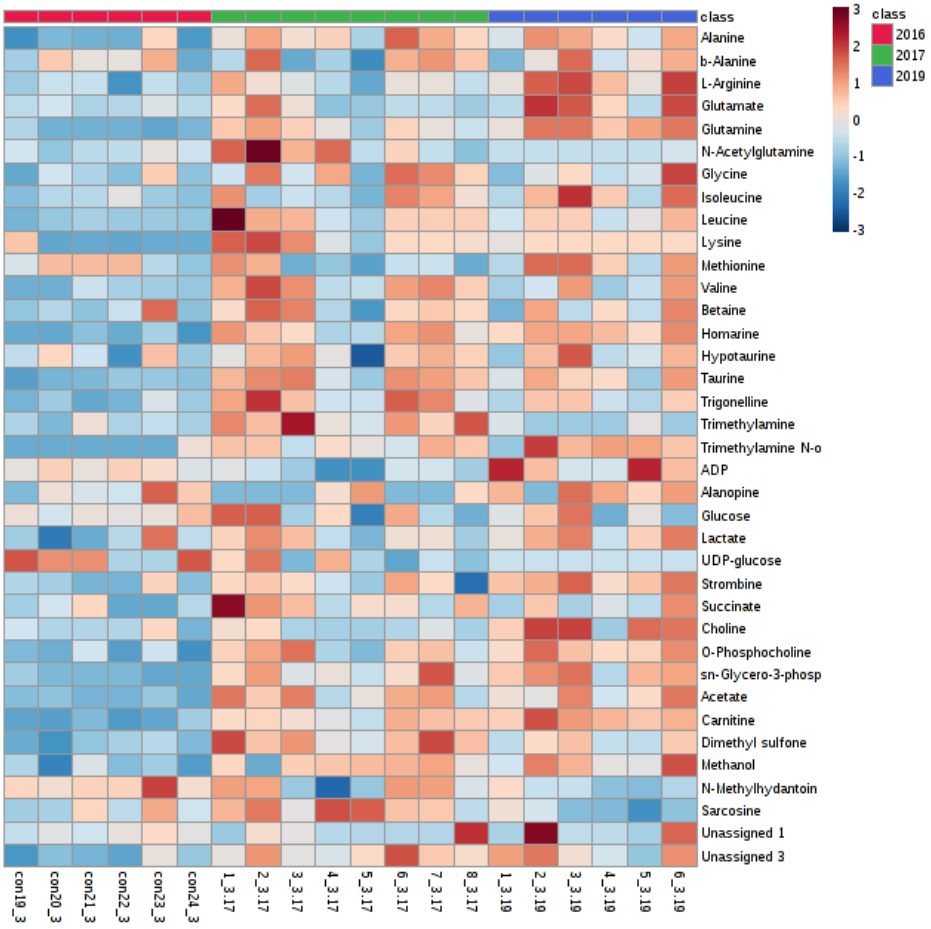

Fig. 30: Heatmap for the +3°C animals over the years. The rectangles on top indicate the different years (red = 2016, green = 2017, blue = 2019). The samples from 2016 show a different pattern from 2017 and 2019. Concentrations vary within 2017 and 2019.

6 Discussion

The ongoing climate change has many possible influences on weather conditions, ocean characteristics and organisms all over the world (Thornes 2002; Hoegh-Guldberg, 2018). Temperature is one of the most potent factors, especially for biological functions (Peck 2016). As Antarctic invertebrates are stenothermal, a warmer world could have severe impacts (Peck 2005b). This study investigated the effects of a warmer environment in an acute state and after one and three years of acclimation by testing the tenacity duration and examining metabolic patterns, with the goal to determine potential acclimation capacities in the Antarctic limpet *Nacella concinna*. There was neither a significant difference between the tenacity duration of the limpets at 0°C and at +3°C in 2016 nor in tenacity duration at +3°C after one and three years of acclimation. In all tissue samples the same metabolites were found but with different concentrations. The greatest number of significantly different metabolites was found between 0°C and +3°C in 2016. In 2017 and 2019 only three metabolites differed between 0°C and +3°C. The present results indicate a change in the metabolism.

6.1 No alteration of tenacity duration

During the acute trial in 2016, the mean duration of tenacity did not change drastically between 0°C and +3°C. The mean duration as well as the maximum duration of the +1.5°C animals was almost twice as long (Figure 6). However, only three out of six test animals in +1.5°C reattached after replacing them on the plate and could be measured. Thus, only three animals stayed attached so long (mean: 2640 sec = 44 min). A similar tenacity result at 0°C and +3°C shows that at least 50 % of the limpets could cope with a spontaneous increase in temperature. A few limpets stayed attached even longer at +3°C (max: 2580 sec = 43 min) than at 0°C (max: 2295 sec = 38 min 15 sec). This tolerance to a warmer environment might be because *N. concinna* lives both in the intertidal and subtidal zones. Animals in the intertidal zone experience temperature variances daily up to + 17°C (Waller et al. 2006a). Limpets used in this study were collected from at last 6 m depth and therefore count in the

population that lives in the subtidal zone. These animals should not be accustomed to high temperatures that occur in the intertidal zone during low tide.

Additionally, the animals were collected in the late summer of the Antarctic. The water temperature in summer at Adelaide Island is between 0°C and +1.8°C (Clarke et al. 2008) and in the subtidal at the British Antarctic Survey's Rothera Research Station is the summer temperature -1.3°C to +1.7°C (Morley et al. 2012). Therefore, the limpets could have been acclimatised to their summer conditions and showed the best performance in +1.5°C water. In 2011, Obermüller et al. showed that the upper lethal temperature and righting ability differ between *N. concinna* winter and summer individuals. The upper lethal temperature was higher for the limpets that were collected in summer and the righting ability was best at their seasonal ambient temperature. Another study by Peck and Veal (2001) showed the effects of acute starvation also differed between seasons in *N. concinna* and Bayne and Scullard (1977) have shown seasonal differences in the effects of starvation in the mussel *Mytilus edulis*. Those results were interpreted as evidence for seasonal acclimatisation to changing temperatures (Fraser et al. 2002; Obermüller et al. 2011). Morley et al. (2012) found the best tenacity duration in acute trials for N. concinna at +1°C. If the metabolisms of the tested limpets in this study were acclimatised to their summer conditions, the question remains why only half of the tested animals in +1.5°C in 2016 stayed attach and the duration of tenacity could be measured. The result at +1.5°C must be handled with caution because of the small N of 6 whereof only 3 were tested, which is not representative.

The duration of tenacity did not significantly increase after three years of acclimation in +3°C water. However, the mean tenacity duration increased from 841 sec (14 min 1 sec) in 2016 at +3°C to 1903 sec (31 min 43 sec) in 2019. Still, there was no indication that limpets acclimated for three years at a higher temperature showed a better tenacity than limpets at 0°C in 2016 (ANOVA; $p = 0.3$, TukeyHSD; $p = 0.76$) or limpets at 0°C in 2019 (ANOVA; $p = 0.3$, TukeyHSD; $p = 0.98$). Furthermore, this study found no differences in tenacity duration and temperature in 2016 (t-test; $p = 0.43$) or in 2019 (t-test; $p = 0.52$). The lack of significance cloud be due to the small sample size ($N_{2016} = 9$, $N_{2019} = 7$, each at 0°C and +3°C). However, Morley et al. (2011) found significance between temperature and tenacity both at the control temperature (-0.3°C: $F_{2, 24} = 6.0$, $p < 0.01$) as well as at the tested temperature (+2.9°C: $F_{2, 18} = 6.1$, $p = 0.01$). The individual number at -0.3°C that showed the best tenacity performance at -1.0°C was 14 whereof 9 stayed attached long enough for the tenacity test.

The number of warmer water acclimated limpets (+2.9°C) was 7 and all seven individuals could be used for the tenacity test. So both studies had N = 7 or 9. Still, the results in 2011 by Morley and his colleagues were significant, because the SE range of the mean duration of tenacity was narrower for all tested limpets (Morley et al. 2011). A difference between the tenacity tests of Morley et al. (2011) and this study is that the animals consumed different food. Where the limpets in 2011 were fed with microalgae, *N. concinna* in this experiment were fed with *Laminaria sp* (macro brown algae). More detailed discussion about possible influences by food source is in the next chapter "6.3 Identified metabolites and their concentration changes" (see below). In another tenacity experiment by Morley et al. (2012), they used a 200 g weight (instead of 125 g like in the present study) which could cause shorter tenacity duration. Furthermore, the fact that the limpets by Morley et al. (2012) where tested in the Antarctic and therefore were not shipped for several months could lead to less stressed individuals due to a shorter captivity. However, in that acute trial in 2012 the best tenacity duration for *N. concinna* was observed at +1°C, which supports the results of 2016 in this study.

Previous studies postulated a positive correlation between foot area and tenacity for limpets (Branch 1981; Smith 1991). Other studies showed a correlation between peak force required to detach animals from the substratum and contact area for different echinoderms (Flammang et al. 2002; Santos und Flammang 2007). The present study observed no significant correlation between foot area (R^2 = -0.058, p = 0.57) or shell area (R^2 = 0.033, p = 0.141) and duration of tenacity for all individuals (Figure 7 and 9). Shell area was used instead of foot area because the foot area was measured only for limpets in 2019. There was a significant correlation between foot area and shell area (R^2 = 0.878, p < 0.01) (Figure X). Limpets with larger shells also had a greater foot area. Morley et al. (2011) showed that the tenacity was significantly correlated with foot area ($F_{1, 46}$ = 6.6, p = 0.13, R^2 = 0.13).

As tenacity of limpets results from the size of the animal and therefore the foot area as well as their mucus (mucopolysaccharide secretion from the sole of the foot) (Branch 1981; Smith 1991; Morley et al. 2011), in the same ambient temperature the larger limpets should have stayed attached to the substratum longer than the smaller limpets. However, this was only the case for limpets in +3°C water (R^2 = 0.224, p = 0.037). The 0°C limpets showed no significant correlation between shell area and duration of tenacity (R^2 = -0.082, p = 0.77). Wells et al. (1998) considered that limpets in more sheltered areas should be larger than

those in exposed areas due to greater strength and therefore better tenacity. However, they found the opposite. One reason why the foot size is important is that the area which touches the substratum establishes a greater suction force if it is larger (Smith 1991). Because the foot shape is maintained by muscular activity, one suggestion is that the tenacity duration depends on the muscle energy status (Morley et al. 2011). Muscle structures and enzyme activities can be influenced by temperature and are known to alter with acclimation (Pörtner 2002) which could affect the muscle energy status (Morley et al. 2011). However, the tenacity mechanism in the foot muscle of limpets resembles the catch mechanism of bivalves (Frescura and Hodgen 1990). The catch mechanism is a persistent state of tension in molluscan smooth muscles that uses little energy (Castellani et al. 1983; Frescura and Hodgen 1990). In the limpet's foot, the musculature is a system of muscle fibre bundles containing thin actin and thick paramyosin filaments and sheathed in collagen. The collagen stiffens the foot muscle which may support the rigidity of the foot and thus enhance the tenacity (Frescura and Hodgen 1990).

The secretion of the mucus was found to differ between animals. Those who clamped tight to the substratum and held on for a longer period produced less and thicker mucus than animals that did not reattached their foot to the substratum or quickly lost contact (personal observation). Grenon und Walker (1981) described three different conditions of limpet mucus from rather vicious mixture to an inviscid liquid. Previous tenacity tests which measured the needed force to remove limpets from the substratum observed that limpets which moved shortly before the trials started needed less force to be detached (Branch 1981). Furthermore, Peck et al. (1993) noticed that limpets which were more mobile produced more mucus. The mucus is thought to assist the tenacity by acting as a viscoelastic glue or as Stefan adhesive (two layers are so close together that the viscosity of the intervening fluid abides their separation) (Smith 1991). However, Smith (1991) witnessed that the Stefan adhesive theory is incorrect and only thick mucus supports tenacity by preventing the inrush of water and therefore promotes a better suction of the foot to the substratum.

6.2 Change of metabolite concentrations over the years

Many studies have examined metabolite changes due to thermal impacts using ^{1}H nuclear magnetic resonance (NMR) spectroscopy e.g Tripp-Valdez et al. (2017), Aru et al. (2017) and Rebelein et al. (2018). ^{1}H NMR spectroscopy is an advantageous tool because it can detect a large amount of different metabolites simultaneously (Beckonert et al. 2007).

An obvious change occurred in both the control animals and warmed animals over the years. Although thought that the metabolites would not change in the 0°C animals because they were always under comparable conditions, twenty-five out of thirty-seven metabolites changed significantly between 2016, 2017 and 2019 (Appendix, Table II). These metabolites contained organic osmolytes like homarine and betaine, organic acids like acetate and lactate, and amino acids like alanine, valine and L-arginine. A possible explanation for this alteration are differences in maintenance and holding conditions in particular the different food sources the limpets were fed between 2016 (microalgae) and 2017 and 2019 (macroalgae). Clarke und Haselden (2008) explained that ^{1}H NMR spectroscopy is very sensitive and can detect possible dietary changes. However, the main metabolism fuel for *N. concinna* are proteins (Fraser et al. 2002; Obermüller et al. 2011) which exist in their natural food sources like diatoms (Shabica 1976) and other microphytobenthos (Brêthes et al. 1994) but also in *Laminaria sp* which was their only food at the AWI. The ratio of protein to lipids in *Laminaria sp.* is 5:1 (Maehre et al. 2014). Therefore, the different food source should have a minor impact.

More important is the amount of food that the limpets had at their disposal (Sokolova, personal communication). Whereas it is more costly for the limpets to graze a high quantity of microalgae, it was very convenient to feed ad libitum on *Laminaria sp.* This situation that the limpets had always more than enough food is highly unrealistic in the nature and could easily be displayed in their metabolite concentrations. Due to these circumstances a comparison between the metabolite concentrations over the years is less suitable and the following interpretation of the results will concentrate on comparisons between the different temperatures within one year. Furthermore, there will not be a discussion about every found metabolite.

6.3 Identified metabolites and their concentration changes

In 2016 four important compounds were identified by t-test and two more by volcano plot between 0°C and +3°C (Table 2). These six metabolites were homarine, trimethylamine (organic osmolyte), leucine, methionine (amino acids), sarcosine (miscellaneous, possible organic osmolyte) and carnitine (miscellaneous, amongst others energy-related compound). The PLS-DA (Figure 12) showed the two groups separated whereas the PCA (Figure 11) showed two ellipses, but at 0°C not all samples were mixed together because of outliers. Also the dendogram (Figure 13) showed no complete separation between the two temperatures and the relative concentrations in the heatmap (Figure 14) also presented an indistinguishable pattern.

The amino acids concentrations at +3°C had a lower concentration than at 0°C even though it is only significant for leucine and methionine. As mentioned before, the main fuel for the metabolism of *N. concinna* are proteins, which are built of amino acids. Therefore, the concentration of amino acids decreases when more energy is needed for the metabolism due to a higher temperature causing stress. Heat stress can lead to the damaging or denaturing of important proteins in organisms. Heat shock proteins (HSP) have the task to repair, refold or eliminate those destroyed proteins (Mayer und Bukau 2005). Different kinds of HSPs were found in *N. concinna* (Clark et al. 2008; Clark und Peck 2009). The production of HSPs as well as their work requires energy (Sokolova et al. 2012) plus amino acids.

Organic osmolytes play an important role in the process of osmotic regulation in marine mollusks (Lu et al. 2016). Previous studies showed a relation between changes in homarine concentrations and stressful conditions like warming, hypoxia, starvation and pathogen infection (Lu et al. 2016; Rosenblum et al. 2005). The homarine concentrations at +3°C increased significantly. Also the concentrations of the other osmolytes increased at +3°C compared to the samples at 0°C significantly by t-test for two organic osmolytes and sarcosine, which can function as an osmolyte (Yancey et al. 1982). The salinity did not change in the trials. However, previous research observed that high concentrations of amino acids in marine mollusks support the intracellular osmolarity (Yancey et al. 1982; Viant et al. 2003; Venter et al. 2018). Therefore, the increase of the osmolytes at +3°C could be due to the decreasing amino acid concentrations. Viant et al. (2003) suggested that homarine

concentration rises as a compensation for the reduction of the amino acids in the intracellular milieu.

Bremer (1983) wrote in his review about carnitine metabolism and function that carnitine functions as a carrier for fatty acids through the mitochondrial membrane. Thus, it has an important role in the mitochondrial fatty acid oxidation which generates energy (Bremer 1983). The carnitine concentration at +3°C is lower than at 0°C and might be caused by the higher energy-requirement.

In 2017 no metabolites were identified by t-test but the volcano plot found three significant compounds (Table 3). These three metabolites were trimethylamine N-oxide (TMAO, organic osmolyte), lysine and β-alanine (amino acids). Also the PCA (Figure 15) could not fully separate the two groups and even the PLS-DA (Figure 16) did not shown a distinct separation of 0°C and +3°C samples. This pattern appears also in the denodogram and in the heatmap (Figure 17 + 18). Thus, the animals in 2017 were similar in both temperatures. This happened most likely because the animals had more than enough food, which alleviates the impact of the warmer water (Brêthes et al. 1994; Fraser et al. 2002; Obermüller et al. 2011). The two amino acids, which were significantly different by volcano plot, seem more similar in concentration in the heatmap (Figure 18). Only the relative concentration of trimethylamine N-oxide was visibly higher in samples at +3°C than at 0°C.

Trimethylamine N-oxide (TMAO) is known as a compatible solute meaning it has no perturbing effects on cellular macromolecules and even counteracts perturbation by urea (Yancey 2005). Urea disturbs macromolecules by destabilizing their structures and it inhibits functions such as ligand binding (Yancey 2005). Clarke (1990) observed that ammonia was the predominant nitrogenous excretion in starved animals. Actively feeding limpets produced significant amounts of urea but the urea level decreased after 23 days of starvation again (Clarke et al. 1994). The excreted urea in *N. concinna* comes likely from the elimination of amino acids and/ or products of nucleic acid metabolism (Clarke et al. 1994). Because the limpets at the AWI never starved, their urea level could stay high. Therefore, the TMAO concentration could also be increased. However, the TMAO concentration is at +3°C higher than at 0°C even though limpets at both temperatures fed ad libitum. Other studies showed that *N. concinna* feeds less in winter than in summer (Fraser et al. 2002; Obermüller et al. 2011). The limpets at +3°C fed more (personal observation) and thus produced more urea. Additionally, Yancey (2005) wrote in his article about organic

osmolytes that the concentration of TMAO in an organism is higher when there is a perturbing present like urea and high temperature.

The t-test found no significant metabolites in 2019 but the volcano plot selected three compounds (Table 4). These three metabolites were N-acteylglutamine and β-alanine (amino acids), and strombine (energy-related compound). The PCA showed for the 0°C samples a much smaller ellipse and thus a smaller 95% confidence interval than for +3°C samples. The ellipse for the 0°C samples lay within the ellipse of the +3°C samples (Figure 19). However, the PLS-DA showed two fully separated ellipses. The ellipse of the +3°C samples is still many times bigger than the ellipse for the 0°C samples (Figure20). The dendogram and heatmap showed an interesting pattern (Figures 21 + 22). The +3°C samples were separated into two groups, where one group was more similar to the 0°C group. Two out of the three samples with much higher relative concentrations at +3°C were most likely more stressed while the preparation for the dissection, because the shell was accidently removed during the sampling.

The relative amino acid concentrations were lowest in one of the two +3°C groups, and highest in the other +3°C group. The 0°C samples had an intermediate concentration of amino acids. However, N-acetylglutamine was the only amino acid which had higher relative concentration at 0°C. It seems there is no research that revealed the presence of N-acetyl-glutamine in mollusks. N-acetylglutamine is a modified amino acid that produces glutamine by deacetylation (Bergana et al. 2000) and is present in human urine (Racine et al. 2004). Glutamine is an amino acid that acts as osmolyte in abalone gastropods (Venter et al. 2018). Furthermore, high glutamine concentrations were found while ammonia accumulation in mollusks was also high (Tikunov et al. 2010; Venter et al. 2018) and thus could act as a nitrogen store (Venter et al. 2018). If this was the case in this study remains the question, why limpets at 0°C produced much more ammonia than at +3°C. β-alanine is also an amino acid that functions often as an osmolyte in mollusks (Yancey et al. 1982; Tikunov et al. 2010).

Strombine is an end-product of anaerobic pathways (Kan-no et al. 1999). When the oxygen level decreases critically, anaerobic pathways switch on and supply energy. If that would be the case in this study, concentrations of lactate, alanopine and strombine, succinate and alanine were increased (Venter et al. 2018). In this study the aquariums were always fully aerated and therefore anaerobic metabolism should not have been activated.

However, the heatmaps showed different alanopine and strombine concentrations at 0°C and +3°C over the years, mostly after 2016.

6.4 Conclusions

The present study aimed to investigate the influence of a warmer environment of the tenacity duration and the metabolism of the Antarctic limpet *Nacella concinna.* Specific areas in the Antarctic like the northern Island South Georgia experienced since 1925 a temperature increase of approximately 1°C in winter and 2°C in summer in 0 – 100 m deep (Whitehouse et al. 2008). The Antarctic Peninsula is the most pronounced region in Antarctica that will be affected by global warming (Thornes 2002). The temperature during this study did influence the tenacity duration of the Antarctic limpet *Nacella concinna* like already examined in a previous study. Additionally, in the present study, the tenacity experiment showed an altered duration of tenacity with longer acclimation time to a higher temperature. After nine month of acclimation the mean tenacity duration tested by Morley et al. (2011) increased at +2.9°C higher compared to the mean duration tenacity at -0.3°C. After three years of acclimation, the mean tenacity duration at +3°C increased again.

The metabolite profiles did change with temperature and acclimation time. The difference between the temperatures within one year became less after the acute trial in 2016. Consequently, the samples at 0°C and +3°C had similar metabolite concentrations in the years 2017 and 2019 and thus the “stressed” metabolism at +3°C in 2016 acclimated after one year.

However, the amount of food that the animals had plays an important role in the metabolism. The good food resources could also helped with increasing the tenacity duration at +3°C again after three years. Because the animals did not starve at any time, they had fewer problems to deal with the warmer water and could show an overall better fitness (Obermüller et al. 2011). Based on the fact that in the other studies with *N. concinna* the limpets either starved or fed microalgae from the walls of the tanks, it is difficult to compare these data with the present results. What is clearly shown is that *Nacella concinna* can acclimate at +3°C when they have enough food.

To improve the present investigation and achieve a better understanding, the experiment conditions need to be changed. In other studies with *N. concinna* the animals will be held in a 12h:12h light:dark regime and fed with only microalgae that grows on the aquarium walls (e.g. Clark et al. 2018). A seasonal temperature change with a slow increase in each season over a few years could also be interesting to observe more realistic responses. Another suggestion is an investigation of impacts by warming of gill tissue using untargeted metabolic profiling because the gill is affected sooner than the foot muscle and is important for respiration and osmoregulation.

References

Aru, Violetta; Balling Engelsen, Søren; Savorani, Francesco; Culurgioni, Jacopo; Sarais, Giorgia; Atzori, Giulia et al. (2017): The Effect of Season on the Metabolic Profile of the European Clam Ruditapes decussatus as Studied by ^{1}H-NMR Spectroscopy. In: *Metabolites* 7 (3). DOI: 10.3390/metabo7030036.

Barnes, D. K.A.; Peck, L. S. (2008): Vulnerability of Antarctic shelf biodiversity to predicted regional warming. In: *Clim. Res.* 37 (2-3), S. 149–163. DOI: 10.3354/cr00760.

Bayne, B. L.; Scullard, C. (1977): Rates of nitrogen excretion by species of Mytilus (Bivalvia: Mollusca). In: *J. Mar. Biol. Ass.* 57 (2), S. 355–369. DOI: 10.1017/S0025315400021809.

Beckonert, Olaf; Keun, Hector C.; Ebbels, Timothy M. D.; Bundy, Jacob; Holmes, Elaine; Lindon, John C.; Nicholson, Jeremy K. (2007): Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. In: *Nat Protoc* 2 (11), S. 2692–2703. DOI: 10.1038/nprot.2007.376.

Bergana, M. M.; Holton, J. D.; Reyzer, I. L.; Snowden, M. K.; Baxter, J. H.; Pound, V. L. (2000): NMR and MS analysis of decomposition compounds produced from N-acetyl-L-glutamine at low pH. In: *Journal of agricultural and food chemistry* 48 (12), S. 6003–6010. DOI: 10.1021/jf000897j.

Branch, George (1981): The biology of limpets: Physical factors, energy flow, and ecological interactions. In: *Oceanography and Marine Biology: An Annual Review* 19.

Bremer, J. (1983): Carnitine--metabolism and functions. In: *Physiological reviews* 63 (4), S. 1420–1480. DOI: 10.1152/physrev.1983.63.4.1420.

Brêthes, J.-C.; Ferreyra, G.; La Vega, S. de (1994): Distribution, growth and reproduction of the limpet Nacella (Patinigera) concinna (Strebel 1908) in relation to potential food availability, in Esperanza Bay (Antarctic Peninsula). In: *Polar Biology* 14 (3), S. 161–170. DOI: 10.1007/BF00240521.

Castellani, Loriana; Vibert, Peter; Cohen, Carolyn; Huxley, H. E. (1983): Structure of myosin/paramyosin filaments from a molluscan smooth muscle. In: *Journal of molecular biology* 167 (4), S. 853–872. DOI: 10.1016/S0022-2836(83)80115-6.

Clark, Melody S.; Fraser, Keiron P. P.; Peck, Lloyd S. (2008): Antarctic marine molluscs do have an HSP70 heat shock response. In: *Cell Stress and Chaperones* 13 (1), S. 39–49. DOI: 10.1007/s12192-008-0014-8.

Clark, Melody S.; Peck, Lloyd S. (2009): Triggers of the HSP70 stress response: environmental responses and laboratory manipulation in an Antarctic marine invertebrate (Nacella concinna). In: *Cell Stress and Chaperones* 14 (6), S. 649–660. DOI: 10.1007/s12192-009-0117-x.

Clark, Melody S.; Thorne, Michael A. S.; King, Michelle; Hipperson, Helen; Hoffman, Joseph I.; Peck, Lloyd S. (2018): Life in the intertidal: Cellular responses, methylation and epigenetics. In: *Funct Ecology* 32 (8), S. 1982–1994. DOI: 10.1111/1365-2435.13077.

Clarke, Andrew (1990): Faecal egestion and ammonia excretion in the Antarctic limpet Nacella continna (Strebel, 1908). In: *Journal of Experimental Marine Biology and Ecology* 138 (3), S. 227–246. DOI: 10.1016/0022-0981(90)90169-D.

Clarke, Andrew; Meredith, Michael P.; Wallace, Margaret I.; Brandon, Mark A.; Thomas, David N. (2008): Seasonal and interannual variability in temperature, chlorophyll and macronutrients in northern Marguerite Bay, Antarctica. In: *Deep Sea Research Part II: Topical Studies in Oceanography* 55 (18-19), S. 1988–2006. DOI: 10.1016/j.dsr2.2008.04.035.

Clarke, Andrew; Prothero-Thomas, Elizabeth; Whitehouse, Michael J. (1994): Nitrogen excretion in the Antarctic limpet *Nacella concinna* (STREBEL, 1908). In: *Journal of Molluscan Studies* 60 (2), S. 141–147. DOI: 10.1093/mollus/60.2.141.

Clarke, Christopher J.; Haselden, John N. (2008): Metabolic profiling as a tool for understanding mechanisms of toxicity. In: *Toxicologic pathology* 36 (1), S. 140–147. DOI: 10.1177/0192623307310947.

Davenport, John (1997): Comparison of theb biology of the intertidal subantarctic limpet *Nacella concinna* and *Kerguelenella lateralis*. In: *Journal of Molluscan Studies* 63 (1), S. 39–48. DOI: 10.1093/mollus/63.1.39.

Fan, Teresa W.-M. (1996): Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. In: *Progress in Nuclear Magnetic Resonance Spectroscopy* 28 (2), S. 161–219. DOI: 10.1016/0079-6565(95)01017-3.

Figueiredo, Maria Isabel Sarvat de; Lavrado, Helena Passeri (2013): Population Structure of Nacella Concinna (Strebel, 1908) (Gastropoda Nacellidae) at Admiralty Bay, King George Island, Antarctica, in Austral Summer 2010/2011. In: *INCT-APA*, S. 119–121. DOI: 10.4322/apa.2014.107.

Flammang, Patrick; Ribesse, Jérôme; Jangoux, Michel (2002): Biomechanics of adhesion in sea cucumber cuvierian tubules (echinodermata, holothuroidea). In: *Integrative and comparative biology* 42 (6), S. 1107–1115. DOI: 10.1093/icb/42.6.1107.

Fraser, K. P.P.; Clarke, A.; Peck, L. S. (2002): Feast and famine in Antarctica: seasonal physiology in the limpet Nacella concinna. In: *Mar. Ecol. Prog. Ser.* 242, S. 169–177. DOI: 10.3354/meps242169.

Frescura, M.; Hodgsens, A. N. (1990): The fine structure of the shell muscle pf patellid prosobranch limpets. In: *Journal of Molluscan Studies* 56 (3), S. 435–447. DOI: 10.1093/mollus/56.3.435.

González-Wevar, Claudio A.; David, Bruno; Poulin, Elie (2011): Phylogeography and demographic inference in Nacella (Patinigera) concinna (Strebel, 1908) in the western Antarctic Peninsula. In: *Deep Sea Research Part II: Topical Studies in Oceanography* 58 (1-2), S. 220–229. DOI: 10.1016/j.dsr2.2010.05.026.

Grenon, J.-F.; Walker, G. (1981): The tenacity of the limpet, Patella vulgata L.: An experimental approach. In: *Journal of Experimental Marine Biology and Ecology* 54 (3), S. 277–308. DOI: 10.1016/0022-0981(81)90162-3.

Gribbestad, Ingrid S.; Aursand, Marit; Martinez, Iciar (2005): High-resolution 1H magnetic resonance spectroscopy of whole fish, fillets and extracts of farmed Atlantic salmon (Salmo salar) for quality assessment and compositional analyses. In: *Aquaculture* 250 (1-2), S. 445–457. DOI: 10.1016/j.aquaculture.2005.02.031.

Hargens, Alan R.; Shabica, Stephen V. (1973): Protection against lethal freezing temperatures by mucus in an antarctic limpet. In: *Cryobiology* 10 (4), S. 331–337. DOI: 10.1016/0011-2240(73)90052-7.

Hawes, T. C.; Worland, M. R.; Bale, J. S. (2010): Freezing in the Antarctic limpet, Nacella concinna. In: *Cryobiology* 61 (1), S. 128–132. DOI: 10.1016/j.cryobiol.2010.06.006.

Hoegh-Guldberg, O., D. Jacob, M. Taylor, M. Bindi, S. Brown, I. Camilloni, A. Diedhiou, R. Djalante, K.L. Ebi, F. Engelbrecht (2018): Impacts of 1.5ºC Global Warming on Natural and Human Systems. Global Warming of 1.5°C. An IPCC Special Report on the impacts of global warming of 1.5°C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty. [Masson-Delmotte, V., P. Zhai, H.-O. Pörtner, D. Roberts, J. Skea, P.R. Shukla, A. Pirani, W. Moufouma-Okia, C. Péan, R. Pidcock,

Hoffman, J. I.; Peck, L. S.; Hillyard, G.; Zieritz, A.; Clark, M. S. (2010): No evidence for genetic differentiation between Antarctic limpet Nacella concinna morphotypes. In: *Marine Biology* 157 (4), S. 765–778. DOI: 10.1007/s00227-009-1360-5.

Kan-no, Nobuhiro; Sato, Minoru; Yokoyama, Takehiko; Nagahisa, Eizoh (1999): Occurrence of β-alanine-specific opine dehydrogenase in the muscle of the limpet Cellana grata Gould (Archaeogastropoda). In: *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 123 (2), S. 125–136. DOI: 10.1016/S0305-0491(99)00031-0.

Kim, Dohong (2001): Seasonality of marine algae and grazers of an Antarctic rocky intertidal, with emphasis On the role of the limpet Nacella concinna Strebel (Gastropoda: Patellidae). In: *Ber. Polarforsch. Meeresforsch.* 397.

Krishnan, P.; Kruger, N. J.; Ratcliffe, R. G. (2005): Metabolite fingerprinting and profiling in plants using NMR. In: *Journal of experimental botany* 56 (410), S. 255–265. DOI: 10.1093/jxb/eri010.

Lu, Jie; Shi, Yanyan; Wang, Shuhong; Chen, Hao; Cai, Shuhui; Feng, Jianghua (2016): NMR-based metabolomic analysis of Haliotis diversicolor exposed to thermal and hypoxic stresses. In: *The Science of the total environment* 545-546, S. 280–288. DOI: 10.1016/j.scitotenv.2015.12.071.

Maehre, Hanne K.; Malde, Marian K.; Eilertsen, Karl-Erik; Elvevoll, Edel O. (2014): Characterization of protein, lipid and mineral contents in common Norwegian seaweeds and evaluation of their potential as food and feed. In: *Journal of the science of food and agriculture* 94 (15), S. 3281–3290. DOI: 10.1002/jsfa.6681.

Mayer, M. P.; Bukau, B. (2005): Hsp70 chaperones: cellular functions and molecular mechanism. In: *Cellular and molecular life sciences : CMLS* 62 (6), S. 670–684. DOI: 10.1007/s00018-004-4464-6.

Morley, S. A.; Clark, M. S.; Peck, L. S. (2010): Depth gradients in shell morphology correlate with thermal limits for activity and ice disturbance in Antarctic limpets. In: *Journal of Experimental Marine Biology and Ecology* 390 (1), S. 1–5. DOI: 10.1016/j.jembe.2010.04.040.

Morley, S. A.; Lemmon, V.; Obermüller, B. E.; Spicer, J. I.; Clark, M. S.; Peck, L. S. (2011): Duration tenacity: A method for assessing acclimatory capacity of the Antarctic limpet, Nacella concinna. In:

Journal of Experimental Marine Biology and Ecology 399 (1), S. 39–42. DOI: 10.1016/j.jembe.2011.01.013.

Morley, Simon A.; Lai, Chien-Hsiang; Clarke, Andrew; Tan, Koh Siang; Thorne, Michael A. S.; Peck, Lloyd S. (2014): Limpet feeding rate and the consistency of physiological response to temperature. In: *Journal of Comparative Physiology B* 184 (5), S. 563–570. DOI: 10.1007/s00360-014-0814-3.

Morley, Simon A.; Martin, Stephanie M.; Day, Robert W.; Ericson, Jess; Lai, Chien-Houng; Lamare, Miles et al. (2012): Thermal reaction norms and the scale of temperature variation: latitudinal vulnerability of intertidal nacellid limpets to climate change. In: *PloS one* 7 (12), e52818. DOI: 10.1371/journal.pone.0052818.

Nolan, Conor P. (1991): Size, shape and shell morphology in the Antarctic limpet *Nacella concinna* at Signy Island, South Orkney Islands. In: *Journal of Molluscan Studies* 57 (2), S. 225–238. DOI: 10.1093/mollus/57.2.225.

Obermüller, Birgit E.; Morley, Simon A.; Clark, Melody S.; Barnes, David K.A.; Peck, Lloyd S. (2011): Antarctic intertidal limpet ecophysiology: A winter–summer comparison. In: *Journal of Experimental Marine Biology and Ecology* 403 (1-2), S. 39–45. DOI: 10.1016/j.jembe.2011.04.003.

Peck, L. S. (2005a): Prospects for survival in the Southern Ocean: vulnerability of benthic species to temperature change. In: *Antarctic Science* 17 (04), S. 497. DOI: 10.1017/S0954102005002920.

Peck, Lloyd S. (1989): Temperature and basal metabolism in two Antarctic marine herbivores. In: *Journal of Experimental Marine Biology and Ecology* 127 (1), S. 1–12. DOI: 10.1016/0022-0981(89)90205-0.

Peck, Lloyd S. (2005b): Prospects for surviving climate change in Antarctic aquatic species. In: *Frontiers in zoology* 2, S. 9. DOI: 10.1186/1742-9994-2-9.

Peck, Lloyd S. (2016): A Cold Limit to Adaptation in the Sea. In: *Trends in ecology & evolution* 31 (1), S. 13–26. DOI: 10.1016/j.tree.2015.09.014.

Peck, Lloyd S.; Clark, Melody S.; Morley, Simon A.; Massey, Alison; Rossetti, Helen (2009): Animal temperature limits and ecological relevance: effects of size, activity and rates of change. In: *Funct Ecology* 23 (2), S. 248–256. DOI: 10.1111/j.1365-2435.2008.01537.x.

Peck, Lloyd S.; Conway, Lucy Z. (2000): The myth of metabolic cold adaptation: oxygen consumption in stenothermal Antarctic bivalves. In: *Geological Society, London, Special Publications* 177 (1), S. 441–450. DOI: 10.1144/GSL.SP.2000.177.01.29.

Peck, Lloyd S.; Morley, Simon A.; Clark, Melody S. (2010): Poor acclimation capacities in Antarctic marine ectotherms. In: *Marine Biology* 157 (9), S. 2051–2059. DOI: 10.1007/s00227-010-1473-x.

Peck, Lloyd S.; Morley, Simon A.; Richard, Joëlle; Clark, Melody S. (2014): Acclimation and thermal tolerance in Antarctic marine ectotherms. In: *The Journal of experimental biology* 217 (Pt 1), S. 16–22. DOI: 10.1242/jeb.089946.

Peck, Lloyd S.; Pörtner, Hans O.; Hardewig, Iris (2002): Metabolic Demand, Oxygen Supply, and Critical Temperatures in the Antarctic Bivalve Laternula elliptica. In: *Physiological and Biochemical Zoology* 75 (2), S. 123–133. DOI: 10.1086/340990.

Peck, Lloyd S.; Prothero-Thomas, Elizabeth; Hough, Nicholas (1993): Pedal mucus production by the Antarctic limpet Nacella concinna (Strebel, 1908). In: *Journal of Experimental Marine Biology and Ecology* 174 (2), S. 177–192. DOI: 10.1016/0022-0981(93)90016-H.

Peck, Lloyd S.; Veal, Rachel (2001): Feeding, metabolism and growth in the Antarctic limpet, Nacella concinna (Strebel 1908). In: *Marine Biology* 138 (3), S. 553–560. DOI: 10.1007/s002270000486.

Peck, Lloyd S.; WEBB, KAREN E.; BAILEY, DAVID M. (2004): Extreme sensitivity of biological function to temperature in Antarctic marine species. In: *Funct Ecology* 18 (5), S. 625–630. DOI: 10.1111/j.0269-8463.2004.00903.x.

Picken, G. B. (1980): The distribution, growth, and reproduction of the Antarctic limpet Nacella (Patinigera) concinna (Strebel, 1908). In: *Journal of Experimental Marine Biology and Ecology* 42 (1), S. 71–85. DOI: 10.1016/0022-0981(80)90167-7.

Picone, Gianfranco; Engelsen, Søren Balling; Savorani, Francesco; Testi, Silvia; Badiani, Anna; Capozzi, Francesco (2011): Metabolomics as a powerful tool for molecular quality assessment of the fish Sparus aurata. In: *Nutrients* 3 (2), S. 212–227. DOI: 10.3390/nu3020212.

Pörtner, H. O. (2002): Physiological basis of temperature-dependent biogeography: trade-offs in muscle design and performance in polar ectotherms. In: *Journal of Experimental Biology* 205 (15), S. 2217.

Racine, S. X.; Le Toumelin, P.; Adnet, F.; Cohen, Y.; Cupa, M.; Hantz, E.; Le Moyec, L. (2004): N-acetyl functions and acetate detected by nuclear magnetic resonance spectroscopy of urine to detect renal dysfunction following aminoglycoside and/or glycopeptide antibiotic therapy. In: *Nephron. Physiology* 97 (4), p53-7. DOI: 10.1159/000079179.

Rebelein, Anja; Pörtner, Hans-Otto; Bock, Christian (2018): Untargeted metabolic profiling reveals distinct patterns of thermal sensitivity in two related notothenioids. In: *Comparative biochemistry and physiology. Part A, Molecular & integrative physiology* 217, S. 43–54. DOI: 10.1016/j.cbpa.2017.12.012.

Rosenblum, E. S.; Viant, M. R.; Braid, B. M.; Moore, J. D.; Friedman, C. S.; Tjeerdema, R. S. (2005): Characterizing the metabolic actions of natural stresses in the California red abalone, Haliotis rufescens using 1H NMR metabolomics. In: *Metabolomics* 1 (2), S. 199–209. DOI: 10.1007/s11306-005-4428-3.

Santos, R.; Flammang, P. (2007): Intra- and interspecific variation of attachment strength in sea urchins. In: *Mar. Ecol. Prog. Ser.* 332, S. 129–142. DOI: 10.3354/meps332129.

Seebacher, Frank; Davison, William; Lowe, Cara J.; Franklin, Craig E. (2005): A falsification of the thermal specialization paradigm: compensation for elevated temperatures in Antarctic fishes. In: *Biology letters* 1 (2), S. 151–154. DOI: 10.1098/rsbl.2004.0280.

Shabica, S. V. (1976): The Natrual History of Antarctic Limpet Patinigera polaris (Hombron and Jacquinot).

Smith, Andrew M. (1991): The Role of Suction in the Adhesion of Limpets. In: *Journal of Experimental Biology* 161 (1), S. 151.

Sokolova, I. M. (2003): Metabolic plasticity and critical temperatures for aerobic scope in a eurythermal marine invertebrate (Littorina saxatilis, Gastropoda: Littorinidae) from different latitudes. In: *Journal of Experimental Biology* 206 (1), S. 195–207. DOI: 10.1242/jeb.00054.

Sokolova, Inna M.; Sukhotin, Alexey A.; Lannig, Gisela (2012): Stress Effects on Metabolism and Energy Budgets in Mollusks. In: Doris Abele, José Pablo Vázquez-Medina und Tania Zenteno-Savín (Hg.): Oxidative stress in aquatic ecosystems, Bd. 138. Chichester: Wiley-Blackwell, S. 261–280.

Suda, Cecilia N. K.; Vani, Gannabathula S.; Oliveira, Mariana Feijó de; Rodrigues, Edson; Lavrado, Helena P. (2015): The biology and ecology of the Antarctic limpet Nacella concinna. In: *Polar Biology* 38 (12), S. 1949–1969. DOI: 10.1007/s00300-015-1789-6.

Thornes, John E. (2002): IPCC, 2001: Climate change 2001: impacts, adaptation and vulnerability, Contribution of Working Group II to the Third Assessment Report of the Intergovernmental Panel on Climate Change, edited by J. J. McCarthy, O. F. Canziani, N. A. Leary, D. J. Dokken a. In: *Int. J. Climatol.* 22 (10), S. 1285–1286. DOI: 10.1002/joc.775.

Tikunov, Andrey P.; Johnson, Christopher B.; Lee, Haakil; Stoskopf, Michael K.; Macdonald, Jeffrey M. (2010): Metabolomic investigations of American oysters using H-NMR spectroscopy. In: *Marine drugs* 8 (10), S. 2578–2596. DOI: 10.3390/md8102578.

Tripp-Valdez, Miguel A.; Bock, Christian; Lucassen, Magnus; Lluch-Cota, Salvador E.; Sicard, M. Teresa; Lannig, Gisela; Pörtner, Hans O. (2017): Metabolic response and thermal tolerance of green abalone juveniles (Haliotis fulgens: Gastropoda) under acute hypoxia and hypercapnia. In: *Journal of Experimental Marine Biology and Ecology* 497, S. 11–18. DOI: 10.1016/j.jembe.2017.09.002.

Venter, Leonie; Du Loots, Toit; Mienie, Lodewyk Japie; van Jansen Rensburg, Peet J.; Mason, Shayne; Vosloo, Andre; Lindeque, Jeremie Zander (2018): Uncovering the metabolic response of abalone (Haliotis midae) to environmental hypoxia through metabolomics. In: *Metabolomics : Official journal of the Metabolomic Society* 14 (4), S. 49. DOI: 10.1007/s11306-018-1346-8.

Viant, Mark R.; Rosenblum, Eric S.; Tieerdema, Ronald S. (2003): NMR-based metabolomics: a powerful approach for characterizing the effects of environmental stressors on organism health. In: *Environmental science & technology* 37 (21), S. 4982–4989. DOI: 10.1021/es034281x.

Walker, A.J.M. (1972): Introduction to the ecology of the Antarctic limpet Patinigera polaris (Hombron and Jacquinot) at Signy Island, South Orkney Islands. In: *Br Antarct Surv Bull* 28.

Waller, C. L.; Worland, M. R.; Convey, P.; Barnes, D. K. A. (2006b): Ecophysiological strategies of Antarctic intertidal invertebrates faced with freezing stress. In: *Polar Biology* 29 (12), S. 1077–1083. DOI: 10.1007/s00300-006-0152-3.

Waller, Catherine L.; Barnes, David K. A.; Convey, Peter (2006a): Ecological contrasts across an Antarctic land–sea interface. In: *Austral Ecol* 31 (5), S. 656–666. DOI: 10.1111/j.1442-9993.2006.01618.x.

Weihe, Ellen; Abele, Doris (2008): Differences in the physiological response of inter- and subtidal Antarctic limpets Nacella concinna to aerial exposure. In: *Aquatic Biology - AQUAT BIOL* 4. DOI: 10.3354/ab00103.

Wells, Rufus M.G; McShane, Paul E.; Ling, Nicholas; Wong, Ron J.; Lee, Tricia O.C; Baldwin, John (1998): Effect of Wave Action on Muscle Composition, Metabolites and Growth Indices in the New Zealand Abalone, Paua (Haliotis iris), with Implications for Harvesting and Aquaculture. In: *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 119 (1), S. 129–136. DOI: 10.1016/S0305-0491(97)00295-2.

Whitehouse, M. J.; Meredith, M. P.; Rothery, P.; Atkinson, A.; Ward, P.; Korb, R. E. (2008): Rapid warming of the ocean around South Georgia, Southern Ocean, during the 20th century: Forcings, characteristics and implications for lower trophic levels. In: *Deep Sea Research Part I: Oceanographic Research Papers* 55 (10), S. 1218–1228. DOI: 10.1016/j.dsr.2008.06.002.

Wu, Huifeng; Southam, Andrew D.; Hines, Adam; Viant, Mark R. (2008): High-throughput tissue extraction protocol for NMR- and MS-based metabolomics. In: *Analytical biochemistry* 372 (2), S. 204–212. DOI: 10.1016/j.ab.2007.10.002.

Yancey, P. H.; Clark, M. E.; Hand, S. C.; Bowlus, R. D.; Somero, G. N. (1982): Living with water stress: evolution of osmolyte systems. In: *Science (New York, N.Y.)* 217 (4566), S. 1214–1222. DOI: 10.1126/science.7112124.

Yancey, Paul H. (2005): Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. In: *The Journal of experimental biology* 208 (Pt 15), S. 2819–2830. DOI: 10.1242/jeb.01730.

Appendix

Table I: List of compounds identified within the 1H-NMR spectrum of the foot muscle tissue extract of N. concinna and corresponding chemical shifts. (s: singlet, d: doublet, dd: double doublet, t: triplet, q: quartet, m: multiplet)

Metabolite	Chemical shift (ppm)
Amino acids	
Alanine	1.48 (d), 3.78 (m)
β-Alanine	2.56 (t), 3.18 (t)
L-Arginine	1.68 (m), 1.92 (m), 3.23 (t), 3.78 (t)
Glutamate	2.05 (m),2.13 (m), 2.35 (m), 3.76 (m)
Glutamine	2.12 (m), 2.15 (m), 2.43 (m), 2.46 (m)
N-Acetylglutamine	
Glycine	3.56 (s)
Isoleucine	0.94 (t), 0.99 (d)
Leucine	0.95 (d), 0.97 (d)
Lysine	1.45 (m), 1.69 (m), 1.89 (m), 3.1 (t), 3.74 (t)
Methionine	2.14 (s)
Valine	0.97 (d), 1.05 (d), 1.92 (m), 3.05 (d)
Organic osmolytes	
Betaine	3.25 (s), 3.87 (s)
Homarine	4.35 (s), 7.95 (dd), 8.02 (d), 8.53 (dd), 8.71 (d)
Hypotaurine	2.64 (t), 3.34 (t)
Taurine	3.26 (t), 3.43 (t)
Trigonelline	8.04 (m), 8.86 (m), 9.15 (s)
Trimethylamine	2.88 (s)
Trimethylamine-N-oxide	3.27 (s)
Energy metabolism	
ADP	4.58 (m), 6.15 (d), 8.27 (s), 8.52 (s)
Alanopine	Pure substance
Glucose	4.65 (d), 5.24 (d)
Lactate	1.33 (d), 4.11 (q)

UDP-glucose	5.98 (d), 5.99 (d), 7.94 (d)
Strombine	Pure substance
Krebs cycle intermediates	
Succinate	2.41 (s)
Phospholipid related compounds	
Choline	3.19 (s)
O-Phosphocholine	3.21 (s)
Sn-Glycero-3-phosphocholine	3.23 (s)
Miscellaneous	
Acetate	1.91 (s)
Carnitine	3.23 (s), 3.43 (m), 3.45 (m), 4.58 (m)
Dimethyl sulfone	3.16 (s)
Methanol	4.78
N-methylhydantoin	2.92
Sarcosine	2.73 (s)
Unassigned 1	
Unassigned 3	1.1 (s)

Table II: List of Important metabolites in 0°C trials over the years identified by One-way ANOVA and post-hoc analysis.

	Compounds	p-value	Fisher's LSD
1	Homarine	0.00	2017 - 2016; 2019 - 2016
2	Trigonelline	0.00	2017 - 2016; 2019 - 2016
3	Taurine	0.00	2017 - 2016; 2019 - 2016; 2017 - 2019
4	Unassigned 3	0.00	2017 - 2016; 2019 - 2016; 2017 - 2019
5	Betaine	0.00	2017 - 2016; 2019 - 2016
6	Trimethyl N-oxide	0.00	2019 - 2016; 2019 - 2017
7	Glutamine	0.00	2017 - 2016; 2019 - 2016; 2019 - 2017
8	Carntitine	0.00	2017 - 2016; 2019 - 2016
9	Alanine	0.00	2017 - 2016; 2019 - 2016
10	O-Phosphocholine	0.00	2017 - 2016; 2019 - 2016
11	Valine	0.00	2017 - 2016; 2019 - 2016
12	L-Arginine	0.00	2019 - 2016; 2019 - 2017
13	Lactate	0.00	2017 - 2016; 2019 - 2016
14	Acetate	0.00	2017 - 2016; 2019 - 2016
15	Dimethyl sulfone	0.00	2017 - 2016; 2017 - 2019
16	β-Alanine	0.00	2017 - 2016; 2019 - 2016
17	ADP	0.00	2016 - 2017; 2019 - 2017
18	Glycine	0.01	2017 - 2016; 2017 - 2019
19	Methanol	0.01	2017 - 2016; 2019 - 2016
20	N-Acetylglutamine	0.01	2017 - 2016; 2019 - 2016
21	Isoleucine	0.01	2017 - 2016; 2019 - 2016
22	Succinate	0.02	2017 - 2016
23	N-Methylhydantoin	0.02	2016 - 2019; 2017 - 2019
24	Lysine	0.03	2019 - 2017
25	UDP-glucose	0.03	2016 - 2017; 2016 - 2019

Table III: List of Important metabolites in +3°C trials over the years identified by One-way ANOVA and post-hoc analysis.

	Compounds	**p-value**	**Fisher's LSD**
1	Carnitine	0.00	2017 - 2016; 2019 - 2016; 2019 - 2017
2	Glutamine	0.00	2017 - 2016; 2019 - 2016; 2019 - 2017
3	Homarine	0.00	2017 - 2016; 2019 - 2016
4	Dimethyl sulfone	0.00	2017 - 2016; 2019 - 2016; 2017 - 2019
5	Sn-Glycero-3-phosphocholine	0.00	2017 - 2016; 2019 – 2016
6	Trimethylamine	0.00	2017 - 2016; 2017 - 2019
7	Taurine	0.00	2017 - 2016; 2019 – 2016
8	Acetate	0.00	2017 - 2016; 2019 – 2016
9	Trimethylamine N-oxide (TMAO)	0.00	2017 - 2016; 2019 – 2016
10	L-Arginine	0.00	2019 - 2016; 2019 - 2017
11	Alanine	0.00	2017 - 2016; 2019 – 2016
12	O-Phosphocholine	0.00	2017 - 2016; 2019 – 2016
13	Choline	0.00	2019 - 2016; 2019 - 2017
14	Trigonelline	0.00	2017 - 2016; 2019 – 2016
15	Sarcosine	0.00	2017 - 2016; 2017 – 2019
16	ADP	0.00	2016 - 2017; 2019 – 2017
17	Unassigned 3	0.00	2017 - 2016; 2019 – 2016
18	Methanol	0.00	2017 - 2016; 2019 – 2016
19	Lysine	0.00	2017 - 2016; 2019 – 2016
20	Valine	0.00	2017 – 2016
21	Leucine	0.01	2017 - 2016; 2019 - 2016
22	Strombine	0.01	2019 - 2016; 2019 - 2017

Acknowledgements

First of all, I want to say that I am happy that I finally finished my master thesis. And without the help of many it would have been even more exhausting. That is why I would like to thank everyone who supported me during the preparation of this study.

A big thanks goes to my supervisors **Dr. Christian Bock** and **Prof. Dr. Inna Sokolova**.

Dear Christian, I would like to express my very great appreciation to you for the fully support over the months especially at the last weekend. I had a wonderful time at the AWI and met a lot of great people. I will miss the conversations and the cake on the meetings on Thursdays, and the cake on birthdays, and the summer parties, and the "kale tours", and… No wonder why I had so little time for work.

Dear Inna, I thank you very much for supporting me to write my master thesis at the AWI and the fast responses if I had a question. I hope my interpretations are not a completely disaster for you and you will not lose all hope in me.

I would also like to extend my thanks to everyone of the integrative ecophysiology section at the AWI, especially **Anette Tillmann**, for their help in the laboratories, answering me questions, and assisting me via telephone if I worked from home.

Finally, special thanks to my partner **Nils** who provided me with unfailing support in all situations even if it was baby-sitting my dogs and cooking my meals so I had more time to write. And a special thanks to my recently met new friend **Alexis** for many laughs, helping me to sort my thoughts, fast answering if I was in a hurry, proofreading my English writing and much more. You have earned the dinner invitation.

Declaration of Authorship

I certify that the work presented here is, to the best of my knowledge and belief, original and the result of my own investigations, except as acknowledged, and has not been submitted, either in part or whole, for a degree at this or any other University. Formulations and ideas taken from other sources are cited as such.

Rostock, 02.03.2020

Place, Date Signature