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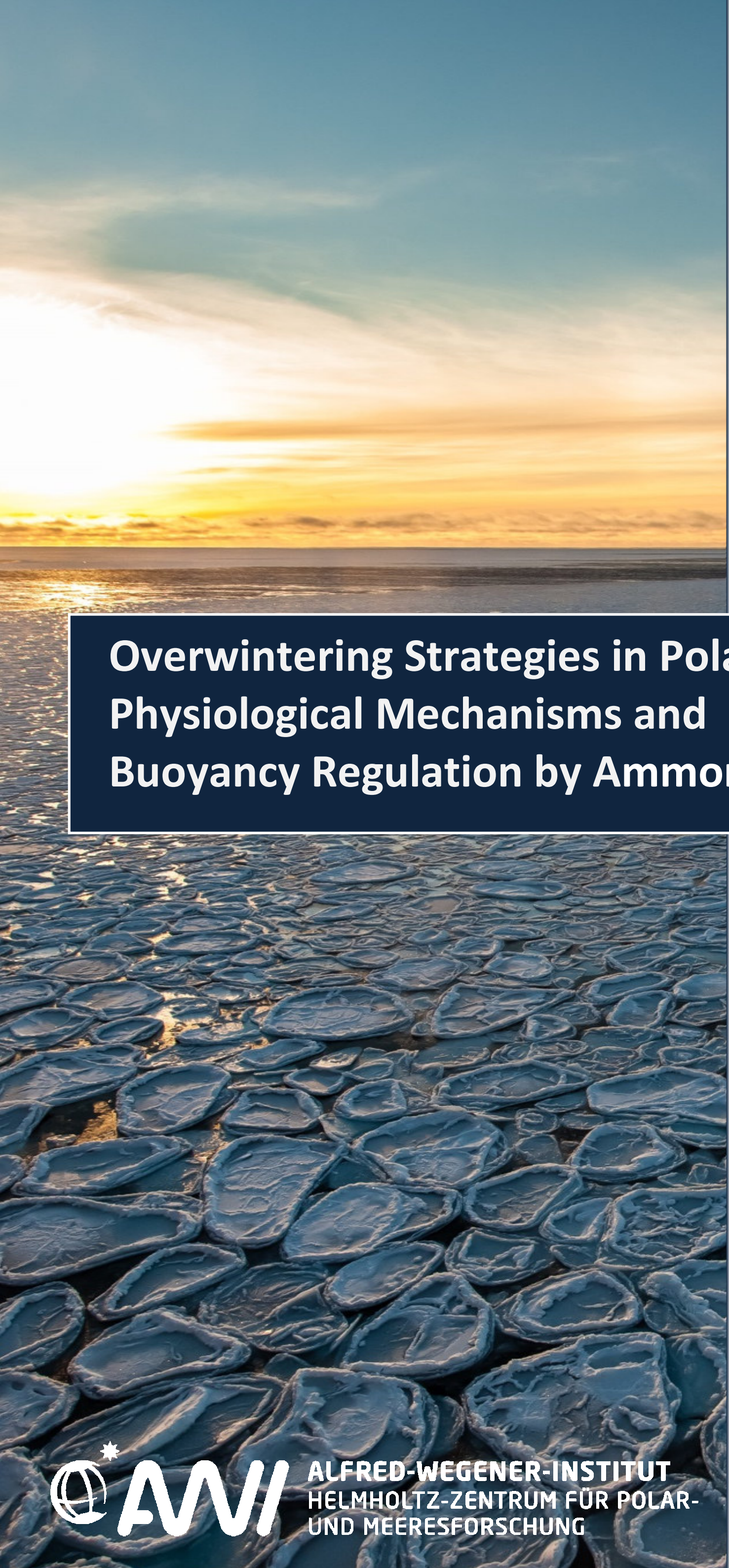

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Overwintering Strategies in Polar Copepods: Physiological Mechanisms and Buoyancy Regulation by Ammonium

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Overwintering strategies in polar copepods: Physiological mechanisms and buoyancy regulation by ammonium

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3 PUBLICATIONS

List of abbreviations

ANOVA	Analysis of Variance
APF	Antarctic Polar Front
ATP	adenosine-5'-triphosphate
AWI	Alfred Wegener Institute for Polar and Marine Research
B	Bongo net
Ca^{2+}	calcium
CI - CV	copepodite stage 1 - 5
CO_2	carbon dioxide
CTD	Conductivity-Temperature-Depth profiler
DFG	Deutsche Forschungsgemeinschaft
DM	dry mass
FA	fatty acid
Falc	fatty alcohol
h	hours
HPTS	hydroxypyrene-1,3,6-trisulfonic acid trisodium salt
K^+	potassium
KCl	potassium chloride
LC_{50}	lethal concentration causing the death of 50% of a group of test animals
Li^+	lithium
Mg^{2+}	magnesium
MS222	3-amino-benzoic acid ethyl ester
MSA	methane sulfonic acid
N	nitrogen
n	number of analysed individuals
na	not applicable
n.a.	not available
Na^+	sodium
nd (n.d.)	not determined
NH_3	ammonia
NH_4^+	ammonium
O_2	oxygen
OVM	ontogenetic vertical migration
PCA	principal component analysis
pCO2	carbon dioxide partial pressure
pH_e	extracellular pH
pH_i	intracellular pH
ppm	parts per million
psu	practical salinity unit
RV (R/V)	research vessel
sd (s.d.)	standard deviation
sec	seconds
SO_4^{2-}	sulfate
TAG	triacylglycerol
TFA	total fatty acids
TFalc	total fatty alcohols
TL	total lipid
TT	Tucker trawl
UBA	Umweltbundesamt
WE	wax ester

Summary

Copepods play a vital intermediate role in any marine pelagic food web. They channel energy from primary production to higher trophic levels and, via the biological carbon pump, substantially affect biogeochemical cycles and carbon fluxes.

The distinct seasonality of primary production is probably the most important factor influencing life-cycle adaptions of herbivorous copepod species in high-latitude ecosystems. Ontogenetic seasonal vertical migration (OVM) with a resting stage (diapause) at greater depth is known as an adaption to escape food scarcity during winter. Diapause is characterised by reduced metabolic rates and the cessation of feeding. Therefore, diapausing copepods are presumably neutrally buoyant to avoid a depletion of their restricted recources by swimming movements. However, no experimental observations or density determinations have been conducted for Antarctic copepods so far and the mechanisms triggering the on- and offset of OVM and diapause, and regulating buoyancy, are still far from understood.

Within the present study, novel hypotheses about the controlling factors of dormancy and OVM were established by testing an inter-linked role of ammonium accumulation for regulating buoyancy during diapause and an associated pH reduction for triggering metabolic depression. The accumulation of ammonium (NH_4^+) and the replacement of ions with a higher density is a known buoyancy regulation mechanism in several marine invertebrates. To keep ammonium in the less toxic ionised form, a low pH is required. Knowing that acidic pH conditions are a relevant factor inducing metabolic reduction, a low haemolymph pH might not only be a precondition for ammonium accumulation, but in addition trigger dormancy in copepods.

Buoyancy observations of anaesthetised individuals revealed that diapausing species *Calanoides acutus* was neutrally buoyant during austral winter, whereas active overwintering species *Calanus propinquus* was negatively buoyant. Diapausing and non-diapausing species differed sigificantly in their extracellular cation composition. In actively overwintering species, the cation composition of the haemolymph was similar to that of seawater. In diapausing copepods, severly elevated concentrations of up to 530 mmol L^{-1} NH_4^+ and reduced contributions of the remaining cations Na^+, Ca^{2+} and Mg^{2+} were found. Moreover, elevated ammonium levels were accompanied by acidic conditions of $pH \leq 6$. The replacement of cations with a higher molecular weight against low density ammonium decreases the overall density of the diapausing copepod and supports neutral buoyancy, while staying isoosmotic with the surrounding seawater. The low extracellular pH is necessary to form NH_4^+ and to prevent it from diffusive loss, but in addition, could play a fundamental role in the regulation of metabolic depression and thus in the control of diapause.

The present study contributes to a better understanding of the regulation mechanisms of dominant copepods` life cycles, in particular of the triggering and controlling factors of vertical migration and diapause. Since polar oceans are where climate change and global warming have the most visible and significant impacts, knowledge about physiological and metabolic adaptations of the species that inhabit it are of profound relevance for understanding and predicting effects on the whole polar marine ecosystem.

Zusammenfassung

Copepoden bilden eine entscheidende intermediäre Komponente im marinen Nahrungsnetz. Sie leiten Energie von der Primärproduktion zu höheren trophischen Stufen und spielen eine wichtige Rolle im Kohlenstoffzyklus.

Im Südlichen Ozean stellt die ausgeprägte Saisonalität der Primärproduktion den wohl bedeutsamsten Faktor für die Ausprägung von Lebensstrategien herbivorer Copepoden dar. Um dem Nahrungsmangel während der Wintermonate zu entgehen führen manche Arten ontogenetische, saisonale Vertikalwanderungen mit einem Ruhestadium in großer Tiefe, genannt Diapause durch. Während der Diapause nehmen Copepoden keine Nahrung auf und verringern ihren Stoffwechsel. Man geht davon aus, dass sie neutralen Auftrieb aufweisen, um eine Energieverschwendung durch aktives Schwimmen zu umgehen. Bisher wurden jedoch noch keine experimentellen Studien oder Beobachtungen bezüglich des Auftriebs von Antarktischen Copepoden durchgeführt. Zudem sind die Faktoren, die für die zeitliche Abfolge der Vertikalwanderung und der damit verbundenen Diapause verantwortlich sind, sowie die physiologischen Mechanismen der Auftriebsregulation, nach wie vor weitestgehend unbekannt.

Die vorliegende Studie testet eine neue Hypothese, nach der ein Ionenaustausch für die Auftriebsregulation und ein niedriger pH für die Stoffwechselreduzierung während der Diapause zuständig sind. Der Austausch von Ionen mit einer höheren Dichte und die Anreicherung von Ammonium (NH_4^+) mit einer niedrigeren Dichte, ist bereits als Auftriebsmechanismus in einer Reihe mariner Invertebraten bekannt. Um Ammonium in seiner weniger toxischen, ionisierten Form zu speichern, ist ein niedriger pH notwendig. Da niedrige pH Werte dafür bekannt sind für die Reduzierung von Stoffwechselwegen verantwortlich zu sein, könnten sie nicht nur als Voraussetzung für die Anreicherung von Ammonium, sondern auch der Stoffwechselreduzierung während der Diapause von Copepoden dienen.

Bei der Diapause-Art *Calanoides acutus* konnte ein neutraler Auftrieb während des antarktischen Winters festgestellt werden. Im Gegensatz dazu wies die aktiv überwinternde Art *Calanus propinquus* negativen Auftrieb auf. Ruhende und aktive Arten unterschieden sich signifikant in ihrer extrazellulären Ionenzusammensetzung. Die Hämolymphe der aktiven Arten

glich in ihrer Kationenzusammensetzung der von Seewasser. In inaktiven Arten wurden hingegen sehr hohe Konzentrationen von bis zu 530 mmol L^{-1} Ammonium (NH_4^+) festgestellt. Erhöhte Ammonium-Werte gingen mit reduzierten Konzentrationen der übrigen Kationen Natrium (Na^+), Calcium (Ca^{2+}) und Magnesium (Mg^{2+}) einher. Darüber hinaus lagen niedrige pH-Werte von ≤6 in der Hämolymphe vor.

Der Austausch von Kationen einer höheren Dichte gegen Ammonium mit einer niedrigeren Dichte verringert die Gesamtdichte des Individuums und unterstützt die Regulation von energiesparendem, neutralem Auftrieb während der Diapause. Der niedrige, extrazelluläre pH ist nicht nur für die Bildung von NH_4^+ erforderlich, sondern kann auch zu einer Stoffwechselreduzierung und damit zur Kontrolle der Diapause beitragen.

Die vorliegende Studie trägt zu einem besseren Verständnis der Regulation und den damit verbundenen Mechanismen von Diapause und Auftrieb dominanter Copepoden-Arten bei. Da Polargebiete besonders stark von den Auswirkungen des globalen Klimawandel betroffen sein werden, sind Studien über die Anpassungsstrategien und physiologischen Mechanismen polarer Copepoden unerlässlich, um mögliche Auswirkungen auf das gesamte Ökosystem verstehen und vorhersehen zu können.

Outline of publications

The following list provides an overview of the three first-author publications included as chapters. The thesis was imbedded in a project supported by the Deutsche Forschungsgemeinschaft (DFG) in the framework of the priority programme ‘Antarctic Research with comparative investigations in Arctic ice areas’ (grant numbers AU 175/6 and SA 1713/3) in close collaboration between Dr. Franz Josef Sartoris and Prof. Dr. Sigrid Schiel from the Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research Bremerhaven, Germany (in the following abbreviated as AWI) and PD Dr. Holger Auel from the University of Bremen. Sampling and experiments were conducted on four research expeditions on RV *Polarstern*. Further analyses were carried out in the laboratories of the University of Bremen in the Marine Zoology Department of the BreMarE Institute (Bremen Marine Ecology, Centre for Research and Education), headed by Prof. Dr. Wilhelm Hagen, and in the Integrative Ecophysiology Section headed by Prof. Dr. Hans-Otto Pörtner at AWI, Bremerhaven, Germany.

I. Schründer S., Schnack-Schiel S.B., Auel H., Sartoris F.J. (2014)

Observations of neutral buoyancy in diapausing copepods *Calanoides acutus* during austral winter

The idea of the study and the experimental design was developed together with F.J. Sartoris. Sampling and experiments were conducted by myself. I wrote the manuscript with scientific and editorial advice by all co-authors. The article is published in *Polar Biology* (2014).

Polar Biology, September 2014, Volume 37, Issue 9, pp 1369-1371

II. Schründer S., Schnack-Schiel S.B., Auel H., Sartoris F.J. (2013)

Control of diapause by acidic pH and ammonium accumulation in the haemolymph of Antarctic copepods

Field work was performed during two research expeditions by all co-authors and myself. I conducted all measurements and analyses with guidance by F.J. Sartoris. The manuscript was written by myself with scientific and editorial advice by all co-authors. The article is published in PloS ONE (2013).

PLoS ONE 8(10): e77498. doi:10.1371/journal.pone.0077498

III. Schründer S., Sartoris F.J., Schnack-Schiel S.B., Hauer C., Dürschlag J., Auel H.

Seasonal trends of haemolymph pH and cation composition of the Antarctic copepods *Calanoides acutus*, *Rhincalanus gigas* and *Calanus propinquus* and their implications for life-cycle adaptations

Sampling and field work were performed on four research expeditions with the involvement of all co-authors and myself. All pH measurements during RV *Polarstern* cruise ANTXXIX/9 were conducted by H. Auel and master student Julia Dürschlag. I shared laboratory and analytical work with support of master students Carolin Hauer and Julia Dürschlag, who used parts of the results for their M. Sc. theses:

Hauer C. (2012) Respiration and metabolic activity of Antarctic copepods - The effects of different life-cycle strategies. M.Sc. thesis, University of Bremen,

and

Dürschlag J. (2015) Structure and trophic interactions of the high-Antarctic plankton community in the Filchner Trough. M.Sc. thesis, University of Bremen.

I wrote the manuscript with advice of all co-authors.

Manuscript submitted for publication to Deep-Sea Research Part I, August 2015.

1 Scientific Background

1.1 Copepods of the Southern Ocean

Copepods play a very important intermediate role in any marine food web, channelling energy from primary production to higher trophic levels such as fishes, seabirds and whales. Their excretion products serve as nutrients for phytoplankton growth, and fecal pellets produced by copepods fuel the biogeochemical carbon cycle via the export of organic matter from the surface to the deep-sea benthic fauna (e.g. Longhurst 1991).

In the Southern Ocean, copepods numerically dominate the zooplankton community, accounting for 68-97% of the total abundance and 24-90% of the total zooplankton standing stock (e.g. Pakhomov and Froneman 2004). The copepod community is dominated by only a few endemic species, with large calanoid *Calanoides acutus, Calanus propinquus* and *Rhincalanus gigas* contributing most to total copepod biomass (e.g. Schnack-Schiel 2001; Fig. 1).

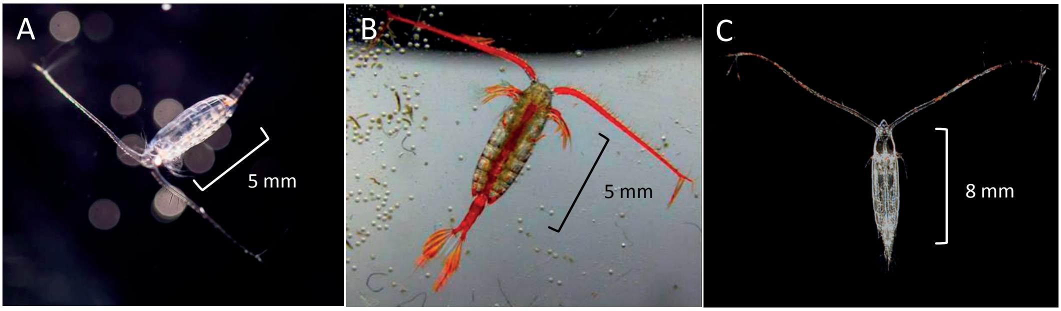

Figure 1. Dominant calanoid copepods from the Southern Ocean. *Calanoides acutus* (A, photo by M. Hoppmann, AWI, Bremerhaven), *Calanus propinquus* (B, photo by J. Huggett, Department of Environmental Affairs, Cape Town, South Africa) and *Rhincalanus gigas* (C, photo by E. Varescchi).

All three species are small-particle feeder, feeding primarily on phytoplankton and protozoans (Hopkins and Torres 1989). Predominantly herbivorous, they are strongly affected by the distinct seasonality of primary production in polar waters. Most of the inter-annual fluctuation of phytoplankton growth is based on varying intensity and daily duration of light, and on the formation and extent of sea ice, ranging from 3-4x10^6 km^2 in austral summer to 18-20x10^6 km^2 in austral winter (Eicken and Lemke 2001; Fig. 2).

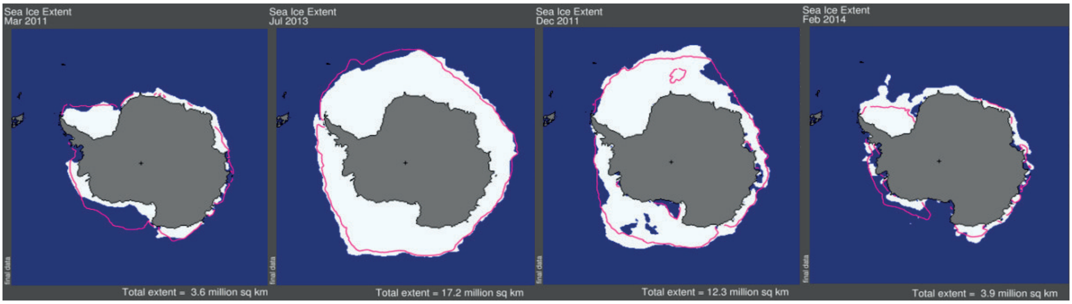

Figure 2. Monthly sea-ice extent in the Southern Ocean. Late summer/early autumn: March 2011, winter: July 2013, late spring/early summer: December 2011, summer: February 2014; derived from satellite passive-microwave observations (Fetterer et al. 2002) of the National Snow and Ice Data Center, University of Colorado, Boulder (magenta line indicates median extent for that month).

Despite relatively low mean chlorophyll concentrations (< 0.3-0.4 mg m^{-3}) over most of the Southern Ocean, very dense but brief phytoplankton blooms exceeding chlorophyll concentrations of 1.0 mg m^{-3} can be observed in three general areas: coastal/shelf waters, areas associated with the seasonal sea-ice retreat, and the vicinity of the major Southern Ocean fronts (Moore and Abbott 2000). The seasonal blooms develop rapidly following the spring melt and dispersion of sea ice and last for about 8 to 10 weeks before they decline due to grazing pressure, cell loss via sedimentation and reduced water coloumn stability (Clarke 1988). The evolutionary success of the three copepod species is based inter alia on their ability to adapt to the fluctuating food supply and to bridge periods of unfavourable food conditions during austral winter.

1.2 Overwintering strategies

The three copepod species have developed species-specific adaptations and are representatives of different overwintering strategies. First attempts to analyse their life cycles were made by Ottestad (1932, 1936), Ommanney (1936) and Andrews (1966) and discovered considerable inter-annual and intra-specific differences concerning the stage-composition and its vertical and latitudinal distribution. Seasonal vertical migration as a strategy to temporary escape from unfavourable environments was found to be a dominant feature in all three species, even though variations in timing, extent and the capability to prolong the feeding period into the winter months or overwinter actively feeding within the ice occur (e.g. Andrews 1966; Voronina 1970; Marin 1988; Atkinson 1991; Schnack-Schiel 2001). Moreover, considerable species-specific differences with regard to life-cycle adaptations can be found in lipid-biosynthesis pathways and storage pattern (e.g. Hagen et al. 1993; Kattner and Hagen 1995; Lee at al. 2006, Kattner et al. 2012), dietary preferences (e.g. Dalsgaard et al. 2003), metabolic activities (e.g. Drits et al. 1994, Pasternak and Schnack-Schiel 2001) as well as the performance and timing of seasonal/ontogenetic vertical migrations (OVM) and dormancy (e.g. Bathmann et al. 1993, Atkinson 1998).

1.2.1 Dormancy in *Calanoides acutus*

Dormancy is defined as a `state of suppressed development` (Danks 1987). It represents an energy-saving trait to bridge periods of environmental harshness (Dahms 1995). In copepods, it occurs in both freshwater and marine species of the three taxa Harpacticoida, Cyclopoida and Calanoida and can either be expressed in resting eggs, encystment or arrested development in nauplii, copepodids or adults. There is a latitudinal tendency of dormancy with most reports of copepods from higher latitudes, decreasing towards the temperate and tropical regions (Dahms 1995). Dormancy can further be divided into `quiescence` and `diapause` (Danks 1987). Quiescence (i.e. pseudo-diapause) is defined as a short-term and irregular phenomenon, which acts as a direct response to a limiting factor such as low temperature or food-scarcity without any prior acclimation (Dahms 1995). Diapause, however, is defined as a period of arrested development which is compulsory and genetically determined, but triggered by environmental factors (Danks 1987). Understanding the phenology of copepod diapause has long been an aim of biological oceanography, but the specific signals that trigger the onset, maintenance and termination and the physiological mechanisms that are responsible for metabolic depression remain poorly understood. Some studies suppose external factors such as photoperiod, temperature, chemical cues or population densities (e.g. Elgmork 1980, Einsle 1964, Spindler 1971); whereas others consider internal factors such as a "biological clock" (e.g. Miller et al. 1991, Williams-Howze and Coull 1992), or lipid amount and composition (e.g. Corkett and McLaren 1969, Pond 2012). The reasons hindering progress in addressing this issue include inter alia the inability to initiate diapause in captivity and logistical constraints of obtaining samples from overwintering depth in remote polar regions during winter. Among Antarctic copepods, the epipelagic copepod *Calanoides acutus* is the only species definitely known to undergo "true" diapause (Atkinson 1998).

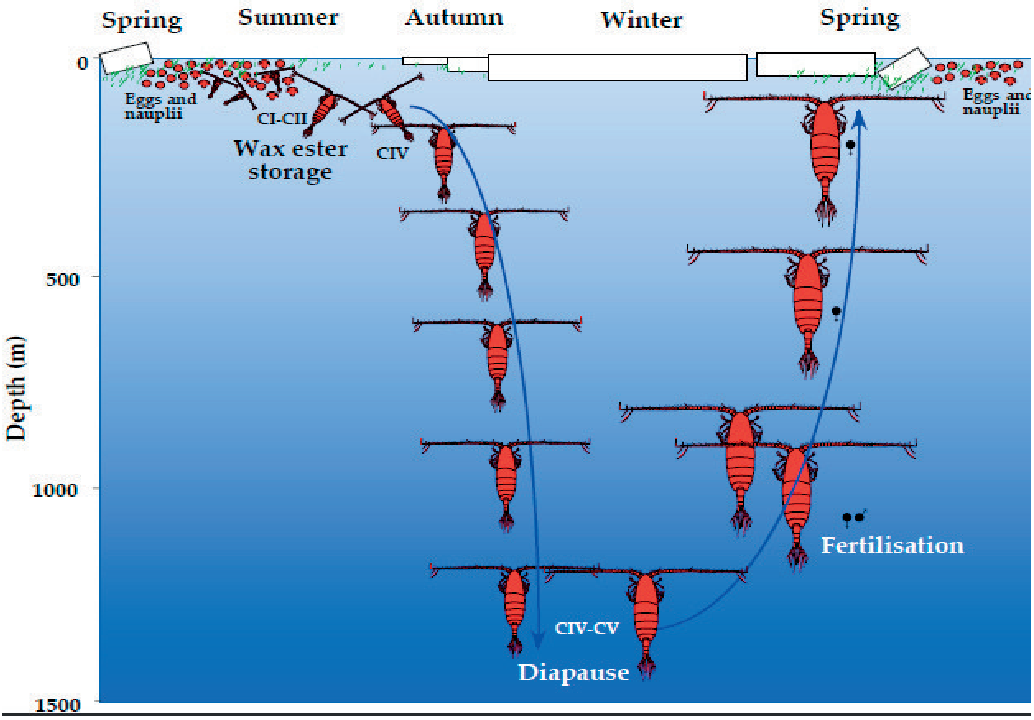

Figure 3. Schematic 1-year life cycle of *Calanoides acutus* with ontogenetic vertical migration and diapause (Picture credit: Sigrid Schiel). Offspring develops from nauplii to early copepodite stages I to III in surface waters in spring and feeds on the spring bloom, mature to stage III or IV and descends to overwinter without feeding in deep waters as stage IV and V copepodids. Before population ascends in spring, stages V moult to stage VI adults, and rise to feed in surface waters with almost all females carrying sperm sacs. Onset of spawning in November, and peak egg production in December and January (Andrews 1966, Marin 1988).

It has a clearly defined one-year life cycle and conducts extensive seasonal vertical migrations associated with a resting stage at depth (Fig. 3). The late copepodite stages CIV and CV descend to depths of below 500 m in late summer, early autumn and reside for 7-9 months in diapause, which is characterized by cessation of feeding, arrested development and reduced metabolic rates (e.g. Schnack-Schiel et al. 1991, Schnack-Schiel and Hagen 1995). Energy requirements are covered by massive lipid stores (up to 52 % of dry mass, Hagen and Auel 2001), accumulated in the previous productive season (Schnack-Schiel et al. 1991). In case of *C. acutus*, these lipid stores consist predominantly of wax esters and serve as a long-term deposit to cover basal metabolic needs during diapause as well as to fuel the restart of development, growth and reproduction in spring (Hagen and Schnack-Schiel 1996, Atkinson

1998, Lee et al. 2006). Maturating into adults and fertilisation take place in late winter, before only the fertilised females re-ascend to the surface layer to release their eggs prior to the spring phytoplankton bloom. Thus, a successful overwintering and hence reproduction in the following spring can only be achieved if diapausing copepods conserve energy for moulting, reproduction and egg development throughout winter. Just like for dormancy, the triggering factors inducing and terminating OVM and diapause in *Calanoides acutus* are still unknown.

1.2.2 Active overwintering in *Calanus propinquus*

Albeit *Calanus propinquus* is a predominantly herbivorous species, it has been observed to switch to a more omnivorous/detritivorous diet during winter, when phytoplankton concentrations in the water column are low. Similar to *C. acutus*, it has a one-year life cycle, but is able to extend its feeding, growth and reproduction periods into the winter months (e.g. Atkinson 1998). Thus, *C. propinquus* overwinters actively and parts of the population even stay near the surface during the winter months (e.g. Bathmann et al. 1993, Marin and Schnack-Schiel 1993). In contrast to the diapausing *C. acutus*, the lipid stores in *C. propinquus* consist mainly of triacylglycerols (TAG).

1.2.3 Multi-year life cycle of *Rhincalanus gigas*

Life-cycle adaptations of *R. gigas* appear to be more variable and more difficult to interpret than those of the other two species. Its geographic distribution is rather sub-Antarctic. Multiple generations are present year-round, indicating the probability of a 2-year life cycle. Main overwintering stages are CII, CIII and CIV, which continue feeding, growth and moulting in the upper water layer until autumn (e.g. Atkinson 1991). Hibernation/dormancy is likely, but probably not as intense as in *C. acutus*, or highly dependent on the latitude inhabited and the local feeding conditions, respectively (e.g. Atkinson 1991, Bathmann et al. 1993). Maturity in spring depends on water temperature starting in late November/December around South Georgia and later in colder waters further south (January in Weddell Sea) (Ommanney 1936). Wax esters are the primary lipid stores, as typical for diapausing species (Lee et al. 2006, Kattner et al. 2012). But according to feeding experiments and lipid analyses, *R. gigas* does not fit the scheme of a typical herbivore, and resembles more an omnivore with detritus and zooplankton as parts of its diet (e.g. Arashkevich 1978, Graeve et al. 1994).

1.3 The role of buoyancy regulation

The physiology of polar zooplankton is generally characterised by low oxygen consumption and growth rates compared to temperate or tropical species. Large differences in metabolic rates occur between benthic and pelagic organisms, often related to high metabolic costs for maintaining a position in the water column (Clarke and Peck 1991). Hence, energetic efficiency in staying in a relatively stable depth layer over a long period of time is of fundamental importance and buoyancy regulation plays a central role for a successful implementation of diapause. Diapausing copepods are assumed to remain motionless and neutrally buoyant throughout diapause duration, in order to avoid a depletion of energy reserves for swimming activities, which moreover could attract potential predators. However, species-specific observations of neutral buoyancy and/or density determinations for Antarctic copepods did not exist so far. Several studies have focused on the density of boreal and Arctic copepods, predominantly in relation to their lipid content and lipid class composition (e.g. Gross and Raymont 1942, Køgeler et al. 1987, Knutsen et al. 2001) and it is widely believed that lipids play a major role in buoyancy control (Visser and Jónasdóttir 1999, Campbell and Dower 2003, Irigoien 2004, Pond and Tarling 2011, Pond et al. 2012, Pond 2012). Lipids have a specific density lower than seawater and influence the overall density of an organism. However, several facts speak against the exclusive role of lipids as a buoyancy control. First of all, the seasonal pattern of lipid accumulation is out of phase with the timing of vertical migration. Copepods start to descend to overwintering depth at the end of summer, when lipid content is at its maximum, and, hence, lipid-regulated buoyancy is high. On the other hand, considerable amounts of lipids are consumed during overwintering, to fuel the re-start of development, maturation and fertilization at the end of winter when copepods are still at depth (e.g. Miller et al. 1991, Schnack-Schiel et al. 1991). Thus, at a time of minimum lipid-regulated buoyancy in spring, copepods need to ascend to release their eggs at the surface. Secondly, body lipids of copepods are generally more compressible and have a larger thermal expansion than the surrounding seawater (Yayanos et al. 1978). Therefore, pure lipid-regulated buoyancy is extremely sensitive to the biochemical composition of the individual copepod and highly depends on the relative fractions and shifts of lipids, proteins, chitins etc. which makes it rather unstable (e.g. Campbell and Dower 2003). It follows, that the maintenance of an energy-saving state of neutral buoyancy during diapause requires additional buoyancy regulation mechanisms.

1.3.1 Buoyancy regulation and potential metabolic depression by ammonium accumulation

In a recent study, high amounts of ammonium ions (NH_4^+) were detected in the haemolymph of Antarctic copepods *C. acutus* and *R. gigas* (Sartoris et al. 2010). Such high concentrations (≤500 mmol L^{-1}) are only known in a range of marine organisms that use ammonium-rich fluids for buoyancy regulation. These include pelagic deep-water shrimps, cephalopods and phytoplanktonic cells. They store fluids, in which ions of a higher molecular weight, e.g. Na^+, Mg^{2+} or SO_4^{2-}, are replaced by less dense ammonium to reduce the overall density, while still remaining isoosmotic with the surrounding seawater (e.g. Denton et al. 1969, Clarke et al. 1979, Sanders and Childress 1988, Boyd and Gradmann 2002). This mechanism has never been reported for copepods before, even though it offers a range of advantages in comparison to exclusively lipid-regulated neutral buoyancy. Ammonium is a waste product of the amino-acid/protein catabolism and is independent from ambient pressure and rapid changes with depth, as they occur during the extensive vertical migrations of diapausing copepods.

However, high concentrations of ammonia (i.e. the total of NH_3 and NH_4^+), are problematic as they are highly hazardous to most animals. In aquatic crustaceans, environmental exposure to ammonia is lethal at relatively low doses and concentrations in the haemolymph are usually below 0.8 mmol L^{-1} (e.g. Weihrauch et al. 2004). In aqueous solution, ammonia exists as either non-ionic ammonia (NH_3) or ammonium ions (NH_4^+) in a pH-dependent equilibrium, shifting towards NH_3 as pH increases. NH_3 is the most toxic form as it easily penetrates biomembranes. It follows that a low pH in ammonium- rich fluids is required to minimise the toxic effects of ammonia. Furthermore, sufficient amounts of ammonium need to be sequestered and stored to contribute to neutral buoyancy. Hence, lowering the pH in the haemolymph keeps ammonium away from cells that produce it and at the same time minimise loss via diffusion.

Low pH values, however, have profound effects on metabolic processes, as most enzyme activities or cellular pathways are highly pH-dependent. A drop of intracellular pH (pH_i) is a relevant factor reducing energy turnover and metabolic rates in several invertebrates, for instance the snail *Oreohelix* spp. (Busa and Nuccitelli 1984, Rees and Hand 1990).

Furthermore, shifts from pH_i 7.9 to 6.3 induced dormancy in the brine shrimp *Artemia* (Hand and Carpenter 1986). Such effects, however, would be beneficial for copepods in diapause, since metabolic depression is essential to save energy during the food-limited winter period. Hence, low pH conditions might not only be a precondition for ammonium accumulation, but in addition constitute a potential sought-after trigger for the induction of copepod dormancy. However, most studies so far focussed on the effects of intracellular processes. In contrast, the impacts and potential benefits of a low extracellular pH leading to a reduced aerobic energy turnover and thus metabolic depression remain to be investigated.

2 Objectives

This study aims to establish novel hypotheses about the controlling factors of dormancy and ontogenetic vertical migration in Antarctic copepod species by testing an inter-linked role of ammonium accumulation for regulating buoyancy and an associated pH reduction for triggering metabolic depression during diapause. New insights into physiological and biochemical characteristics of dominant Antarctic copepods are provided in a comprehensive data set to extend previous studies on life-cycle strategies, particularly with rare samples from the winter season and unusually deep and detailed sampling depth intervals. The present study contributes to a better understanding of regulation mechanisms for diapause and buoyancy in polar copepods and in particular of the triggering and controlling factors of vertical migration.

Hypotheses

I. During diapause, Antarctic copepods *Calanoides acutus* achieve an energy-saving state of neutral buoyancy

To date, it has merely been assumed that Antarctic copepods achieve neutral buoyancy during diapause in order to avoid a depletion of energy reserves otherwise depleted by swimming activities. However, no experimental observations or density determinations have been conducted so far. Within the present thesis, buoyancy of anaesthetised individuals of diapausing and non-diapausing species was assessed and compared visually during an expedition in austral winter (Publication I, Chapter 1).

II. Ammonium accumulation and ion replacement in the haemolymph of diapausing copepod species contribute to buoyancy regulation during diapause

Resting and non-resting copepod species were caught on three different expeditions, the haemolymph was extracted and cation compositions were measured (Publication II and III, Chapter 2 and 3). The results support the hypothesis that diapausing copepods regulate their buoyancy via altering their ionic composition. The replacement of cations with a higher molecular weight such as Na^+, Mg^{2+} and Ca^{2+} against low density NH_4^+ decreases the overall density of the diapausing copepod and supports neutral buoyancy, while staying isoosmotic with the surrounding seawater.

III. High NH_4^+ concentrations in the haemolymph of diapausing copepod species are associated with low pH_e conditions to avoid a diffusive loss and the potentially toxic effects of NH_3

In order to prove that elevated levels of ammonium in the haemolymph of diapausing species are associated with low pH_e conditions, resting and non-resting copepod species were caught on four different expeditions and the respective pH of individual haemolymph samples was measured (Publication II and III, Chapter 2 and 3). The results help testing the hypothesis of a functional link between ammonium accumulation as a buoyancy control mechanism and an associated drop in pH_e, not only as a precondition for ammonium accumulation, but in addition as a potential trigger to mediate metabolic depression during diapause.

IV. Ammonium accumulation and pH reduction is a seasonal phenomenon and varies with depth

If the hypothesis of ammonium-aided buoyancy, and at the same time, pH-induced metabolic depression is applicable, it should be a seasonal phenomenon with highest ammonium concentrations and lowest pH levels in diapausing individuals at overwintering depth, and normal pH conditions and lowest amounts of ammonium in active individuals from surface waters in spring and summer. To investigate this hypothesis, copepods of different developmental stages, sampling depths, seasons and hence, different life-cycle phases were collected and analysed, not only based on their ammonium content and pH_e, but also with regard to other physiological and biochemical characteristics such as lipid storage pattern, metabolic rates and stable isotope composition (Publication III, Chapter 3).

V. The accumulated ammonium originates either from dietary input during the productive season or from protein catabolism during diapause

The origin of the accumulated ammonium, as well as its timing and the sequence of the associated pH decrease are essential for the understanding of the physiological mechanisms behind buoyancy regulation and diapause control. Potential sources of ammonium are discussed with regard to the timing of OVM and physiological and biochemical adaptations in polar copepods (synoptic discussion, Chapter 4.5).

VI. Ion replacement and acidic pH conditions are principle controlling factors of copepod diapause also acting in Arctic species

Diapause and vertical migration are common phenomena also in several Arctic copepod species. The results of this study are compared to recent investigations concerning the extracellular cation composition and pH in Arctic diapausing species. Potential differences are discussed on a global scale (synoptic discussion, chapter 4.6).

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CHAPTER 1

OBSERVATIONS OF NEUTRAL BUOYANCY IN DIAPAUSING COPEPODS CALANOIDES ACUTUS DURING AUSTRAL WINTER

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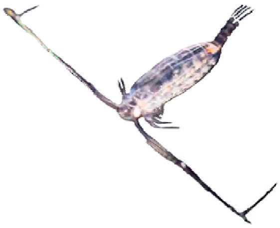

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SHORT NOTE

Observations of neutral buoyancy in diapausing copepods *Calanoides acutus* during Antarctic winter

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Abstract The herbivorous Antarctic copepod *Calanoides acutus* overwinters inactively in a resting stage (diapause) at depths below 500 m. It is assumed that during diapause *C. acutus* is neutrally buoyant in order to retain energy reserves otherwise depleted by swimming activities. However, so far, no experimental observations on its buoyancy have been reported and our knowledge of buoyancy regulation mechanisms is incomplete. In the present study, species-specific differences in buoyancy were assessed visually. Observations were made of specimens from the diapausing cohort of *C. acutus* and compared to another herbivorous copepod *Calanus propinquus*, which overwinters actively feeding in the upper water layers. Freshly caught copepods were anaesthetized in a 3-amino-benzoic acid ethyl ester (MS222) in seawater solution in order to exclude the influence of swimming movements on buoyancy control. It was shown that *C. propinquus* was negatively buoyant, whereas diapausing *C. acutus* remained neutrally buoyant. This is the first record that neutral buoyancy in diapausing copepods is maintained by the biochemical body composition without the additional need of swimming movements.

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Introduction

The two dominant Antarctic copepod species, *Calanoides acutus* and *Calanus propinquus*, have developed opposing life-cycle strategies in order to survive periods of food scarcity in austral winter. They show distinct seasonal, inter-specific, and stage-dependent differences in behavior, physiological condition, and biochemical composition. *C. acutus* descends to depths below 500 m and survives the food-limited winter season by remaining inactive during a resting stage (diapause) for several months. In contrast, *C. propinquus* is a winter-active species, remaining in the upper 500 m throughout the entire year, and switching to a more opportunistic diet during winter (e.g., Donnelly et al. 1994; Schnack-Schiel and Hagen 1995; Atkinson 1998). Both species rely on large lipid deposits as energy reserves accumulated in the previous productive season, but they accumulate different lipid classes (Schnack-Schiel et al. 1991; Kattner et al. 1994; Hagen and Auel 2001).

In *C. acutus*, neutral buoyancy during diapause would reduce a depletion of energy reserves caused by swimming activities and, hence, ensure the successful restart of development and reproduction in the following spring. In contrast, actively overwintering species are presumably negatively buoyant and need to swim to counteract sinking (Haury and Weihs 1976). Until now, these species-specific differences in buoyancy have never been observed, neither under experimental conditions, nor in situ.

The present study aims to clarify whether diapausing copepods achieve and maintain neutral buoyancy without the necessity of active swimming.

Materials and Methods

Copepods were caught on board R/V *Polarstern* during expedition ANT XXIX/6 in austral winter at station 500 (68° 0.8′ S, 6° 40.4′ W; 2 July 2013; bottom depth 4,799 m) and 507 (66° 33.7′ S, 27° 2.4′ W; 17 July 2013; bottom depth 4,880 m) in the Weddell Sea. Hydrographical data were recorded prior to the zooplankton haul using a Conductivity-Temperature-Depth (CTD) profiler (SBE 911plus). Vertically stratified samples were collected with a multiple opening/closing net equipped with five nets of 100-μm mesh size from 2,000–1,500, 1,500–1,000, 1,000–500, 500–200, and 200–0 m depth. Copepods were separated according to species, sex, and ontogenetic stage and kept in filtered seawater in a temperature-controlled refrigerator (0.4 °C) prior to the experiments. *C. propinquus* copepodite stages CV were taken from the upper water layer (200–0 m) and for diapausing *C. acutus* the main overwintering stages CIV were chosen from deeper layers (1,500–1,000 m). A graduated measuring cylinder (polymethylpentene, volume 2 l, external diameter 84 mm, and 531 mm height) was used as a settling chamber, filled with filtered seawater. Due to a pronounced thermocline in the upper 200 m where *C. propinquus* were caught, a mean experimental temperature representing conditions occurring in both water layers was chosen according to the CTD profile (0.4 °C, salinity 34.4, and calculated density 1.026 g cm^{-3}). Previous studies may have suffered from bias by dissection, preservation, freezing, and rinsing. Therefore, we deliberately avoided any form of preparation of the copepods before the experiments to exclude effects on the osmotic or biochemical composition and, thus, on the overall density of an individual. Copepods were only anaesthetized in a MS222 in seawater solution to exclude the influence of swimming movements. The exposure time to the anesthetic varied depending on the species (see "Results"). A successful anesthetization was defined as the state in which external stimuli (gentle contact/touch of the large anterior antennae) did not result in any reaction or swimming/escape activities. Successfully anesthetized individuals were gently and separately transferred into the surface layer of the measuring cylinder using a small pipette. The behavior in the water column was monitored visually and sinking speeds were estimated with a stopwatch. The length of monitoring was adapted to the duration of the anesthesia. After a certain time in filtered seawater, the anesthetizing effects subsided and individuals were checked for their condition.

Results and Conclusions

Copepods were successfully anesthetized at a concentration of 0.3 g MS222 per liter seawater at 0.4 °C. The exposure times, until narcotic effects were ascertained, varied between 4 and 5 min for *C. acutus* and up to 40 min for *C. propinquus*. After approximately 30 min in fresh and filtrated seawater, the narcotic effects of the anesthetic agent subsided in both species.

Once introduced into the settling chamber, all individuals of anesthetized winter-active *C. propinquus* started sinking. The sinking velocities differed due to the orientation of the copepod in the water column. Whereas most of the individuals sank rapidly (~ 1 cm s^{-1}) with their head first and legs folded alongside their bodies, some sank sidewise with one or both first antennae slightly spread, resulting in slower sinking velocities (~ 0.5 cm s^{-1}). After a maximum of 1.5 min, all individuals were found on the bottom of the measuring cylinder. After the narcotic effects of the anesthetic agent had subsided, all copepods started swimming again and were found distributed over the entire water column.

In contrast, all individuals of the anesthetized diapausing copepod *C. acutus* reached a point of neutral to positive buoyancy just after having them transferred to the vessel and remained floating in the surface layer, where they were located irrespective of whether the anesthetic effects had worn off.

Species-specific density determinations for Antarctic copepods do not exist so far, whereas several studies have focused on the density of boreal or Arctic copepods, predominantly in relation to their lipid content and lipid class composition (e.g., Gross and Raymont 1942; Køgeler et al. 1987; Visser and Jónasdóttir 1999; Knutsen et al. 2001). Knutsen et al. (2001) critically reviewed density measurements of pelagic copepods and showed that all of these studies comprise systematic errors. Knutsen et al. (2001) used an experimental setup and procedure similar to the present study. Sinking speeds were determined in freshly caught and anesthetized individuals of diapausing *Calanus finmarchicus*, but with one major difference to our study. Prior to determination of sinking speeds, the large anterior antennae were removed to avoid an influence on sinking velocities. Stokes' law was applied to calculate their density, with distinctly lowest densities during winter. However, the removal of the antennae should be critically viewed, since leakage of hemolymph may have contributed substantially to the overall density of the copepod and may have biased the density determination.

The fact that large proportions of low-density lipids affect the overall density and, hence, buoyancy regulation in copepods has been recognized for a long time, and several authors consider lipids as the main constituent determining buoyancy and overwintering depth (e.g., Visser and Jónasdóttir 1999; Irigoien 2004; Pond and Tarling 2011; Pond et al. 2012). However, pure lipid-based neutral buoyancy is inherently unstable and difficult to regulate as lipids are more compressible and have a larger thermal expansion than the surrounding seawater (Yayanos et al. 1978). Large compositional variability is evident for diapausing copepods and lipid levels may vary between 10 and 35 % of dry mass in overwintering copepodites CV in late winter (Hagen and Schnack-Schiel 1996). Small changes in lipid content and/or composition may have tremendous effects on the overall density of the organism and require additional buoyancy regulation mechanisms for fine-tuning (Campbell and Dower 2003; Campbell 2004).

Only recently were highly elevated levels of ammonium (NH_4^+) measured in the hemolymph of diapausing *C. acutus*, while concentrations of other cations, particularly those with a higher molecular weight (i.e., Na^+, Mg^{2+}, Ca^{2+}), were low. In contrast, none of the actively overwintering species showed elevated ammonium concentrations and their cation composition approximated that of the surrounding seawater (Sartoris et al. 2010; Schründer et al. 2013). Ammonium storage provides a potential mechanism for fine-scale buoyancy regulation. It is a waste product of protein and amino acid catabolism. Therefore, energetic costs for the accumulation of ammonium are low compared to active swimming or the accumulation of lipids. Ammonium-aided buoyancy is independent of ambient pressure, and therefore is well suited for the extensive vertical migration of copepods in autumn, as well as in assisting in the upward migration to the surface with reduced lipid levels in the following spring (Schnack-Schiel et al. 1991; Pasternak et al. 2009).

The present experimental approach suggests that diapausing *C. acutus* achieve neutral buoyancy through their biochemical body composition, without the need of swimming movements. This has a fundamental advantage as it reduces the depletion of energy reserves during diapause, when copepods do not feed. Moreover, it diminishes the risk of attracting predators, which otherwise would respond to swimming activities. To clarify how far neutral buoyancy can be maintained throughout the entire water column with varying pressure and densities represents a challenging task for future investigations.

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CHAPTER 2

CONTROL OF DIAPAUSE BY ACIDIC PH AND AMMONIUM ACCUMULATION IN THE HAEMOLYMPH OF ANTARCTIC COPEPODS

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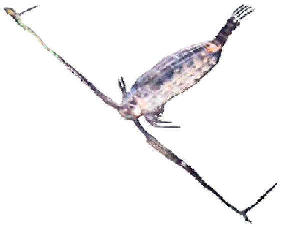

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Control of Diapause by Acidic pH and Ammonium Accumulation in the Hemolymph of Antarctic Copepods

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Abstract

Life-cycles of polar herbivorous copepods are characterised by seasonal/ontogenetic vertical migrations and diapause to survive periods of food shortage during the long winter season. However, the triggers of vertical migration and diapause are still far from being understood. In this study, we test the hypothesis that acidic pH and the accumulation of ammonium (NH_4^+) in the hemolymph contribute to the control of diapause in certain Antarctic copepod species. In a recent study, it was already hypothesized that the replacement of heavy ions by ammonium is necessary for diapausing copepods to achieve neutral buoyancy at overwintering depth. The current article extends the hypothesis of ammonium-aided buoyancy by highlighting recent findings of low pH values in the hemolymph of diapausing copepods with elevated ammonium concentrations. Since ammonia (NH_3) is toxic to most organisms, a low hemolymph pH is required to maintain ammonium in the less toxic ionized form (NH_4^+). Recognizing that low pH values are a relevant factor reducing metabolic rate in other marine invertebrates, the low pH values found in overwintering copepods might not only be a precondition for ammonium accumulation, but in addition, it may insure metabolic depression throughout diapause.

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Introduction

Herbivorous copepods are greatly affected by the distinct fluctuation of primary production in polar waters [1]. Certain copepod species have developed species-specific strategies in order to exploit peaks of phytoplankton production in spring and summer and to survive periods of food shortage during the winter season [2-4]. *Calanoides acutus* belongs to the most dominant species with regard to total zooplankton biomass in the Southern Ocean [5], and it is the only Antarctic copepod definitely known to conduct extensive seasonal ontogenetic vertical migrations to survive the food-limited winter season inactively [3]. The late copepodite stages CIV migrate to depths ≥ 500 m at the end of autumn. They further develop into copepodite stage CV at depth and pass into a dormant/resting stage termed diapause, which is characterized by a reduced metabolism in order to conserve energy throughout winter [6,7]. Energy requirements are supplied by massive internal lipid stores (up to 52% of dry mass, [8]), accumulated in the previous productive season [6]. These lipid stores also fuel the restart of development, maturation, and reproduction in spring and influence the physical density of the copepod. The maturation into adult females or males as well as mating itself takes place at the end of winter still at depth. Fertilized females re-ascend to the surface in spring to release their offspring in a time when feeding conditions are optimal [9-11]. Thus, a successful overwintering and reproduction in the following spring can only be achieved, if diapausing copepods remain neutrally buoyant in a relatively stable depth layer where they will not deplete their energy reserves through swimming activities, nor attract potential predators.

In a recent study, Sartoris et al. [12] suggested that the ammonium concentration in the hemolymph of Antarctic copepods plays a critical role for adjusting neutral buoyancy during diapause. *C. acutus* and *Rhincalanus gigas* are the only two Antarctic copepod species where highly elevated ammonium (NH_4^+) concentrations of up to 450 mmol L^{-1} have been observed in their hemolymph, whereas none of the other investigated species showed similar results. The authors hypothesized that the replacement of heavier ions (i.e. Na^+, Mg^{2+}, Ca^{2+}) by lighter ammonium is necessary to control the overall density of the copepod at overwintering depth [12]. The reduction of physical density by ion replacement and ammonium accumulation is a well-known buoyancy regulation mechanism in a variety of ammoniacal organisms such as the majority of pelagic cephalopods [13,14], deep-sea shrimp *Notostomus gibbosus* [15] and many phytoplankton cells [16], although it has never been reported for copepods before. For most organisms, ammonia (NH_3) is highly toxic and the concentration in body fluids is typically low [17]. In aqueous solution, ammonium exists as either ammonium ions (NH_4^+) or molecular ammonia (NH_3), with the toxicity strongly dependent upon the pH of the solution. As pH increases, the equilibrium shifts towards the more toxic, un-ionized ammonia (NH_3) [18-20]. Therefore, we predict a low extracellular pH (pH_e) in the hemolymph of copepods with elevated ammonium concentrations to avoid the toxicity of ammonia, similar to the ammonium-rich fluids in other ammoniacal marine invertebrates [15,21].

Four Antarctic copepod species as representatives of different life-cycle strategies were studied: *C. acutus*, *Calanus propinquus*, *R. gigas*, and *Paraeuchaeta antarctica*. Although inhabiting the same environment, they show considerable species-specific differences in terms of behavioral, physiological and lipid-biochemical properties. The epipelagic copepods *C. acutus*, *C. propinquus* and *R. gigas* are predominantly small-particle grazers, feeding on phytoplankton and protozoans [22,23]. *C. acutus* is the only diapausing species in the Southern Ocean, whereas information on the behavior of *R. gigas* is less clear. The majority of the *C. propinquus* population remains active in the upper or mid-water layers throughout the whole year, switching to a more omnivorous diet during winter [24,25]. *P. antarctica* is a carnivorous species and, therefore, capable of overwintering without a resting stage, feeding year-round on smaller copepods and other zooplankton [26,27].

The current publication extends the hypothesis of ammonium-aided buoyancy in diapausing copepods by postulating that high ammonium concentrations in the hemolymph coincide with low pH values in order to avoid toxic effects of ammonia. For that purpose, a new method providing pH measurements in small volumes (≥ 500 nL) of copepod hemolymph was developed. It is further discussed whether a pH reduction may also be considered as a possible factor that causes the initiation of metabolic depression. In order to test a direct relation between ammonium accumulation and pH regulation to diapause, diapausing and non-diapausing species were studied and compared in two different seasons.

Materials and Methods

Ethics Statement

The present study on planktonic copepods does not include protected or endangered species.

Field work and sampling in the Southern Ocean have been approved by the German Federal Environment Agency (Umweltbundesamt, UBA) as the responsible German national authority according to the national “Act Implementing the Protocol of Environmental Protection to the Antarctic Treaty”.

Sampling and sorting

Copepod sampling, pH measurements and experiments were conducted onboard R/V Polarstern on two separate cruises in early austral autumn (ANT XXVII/3, February to April 2011) and late spring (ANT XXVIII/2, December 2011). Samples were collected at a total of 20 stations south of the Antarctic Polar front (APF), except Station 210 west of South Georgia. The exact positions are shown in Figure 1.

Hydrographical data were recorded prior to any zooplankton haul using a Conductivity-Temperature-Depth (CTD) profiler (SBE 911plus). At each station, stratified depth samples were collected with a vertical haul from a maximum depth of 2000 m to the surface using multiple/opening closing nets (Multinet Maxi, mouth opening 0.5 m^2; Multinet Midi, mouth opening 0.25 m^2; 100 µm mesh size for both). Vertical hauls took a maximum of two hours. Up to nine discrete depth strata chosen according to the stratification of the water column were sampled. A flowmeter was attached to the net and used to calculate the volume of water filtered. Additional tows from a maximum depth of 300 m to the surface were conducted by a Bongo net (mouth opening 0.28 m^2, mesh size 100 µm) and Tucker Trawl (mouth opening 2.25 m^2, mesh size 1500 µm) to provide supplementary material for biochemical analyses and experiments. Immediately after capture, all copepods were removed from the cod end of the nets and eventually sorted by species, sex and developmental stage. All copepodite stages of *C. acutus*, *R. gigas*, *C. propinquus* and *P. antarctica* were kept separately in jars filled with filtered seawater in temperature-controlled refrigerators or in a cooling container at 0°C for a maximum of two hours previous to hemolymph extraction. The remaining zooplankton from each sample was fixed in 4% borax-buffered formaldehyde in seawater solution for later analyses of community structure.

Hemolymph extraction and analysis

Individual copepods were transferred to a Petri dish kept on an ice bed and dried carefully with a fuzz-free tissue to remove all remaining seawater. Hemolymph was extracted manually under a dissecting microscope using borosilicate glass capillaries, which were prepared prior to extraction with an electrode puller providing ultra-fine tips. Each hemolymph sample was diluted in 40 µL of de-ionized water and kept in a deep-freezer at -80°C. The cation composition was analyzed by ion chromatography with a DIONEX ICS 2000 at 40°C using an IonPac CS 16 column with methane sulfonic acid (MSA, 30 mmol L^{-1}) as an eluent at 0.36 mL min^{-1} flow rate. Inorganic ions such as NH_4^+, Na^+, Mg^{2+}, K^+, and Ca^{2+} were measured and peaks were identified according to retention times in comparison to a cation standard of known composition (Dionex, Six Cation Standard). Cation concentrations are presented as mmol L^{-1}.

pH_e-measurements

At least 500 nL of a hemolymph sample were used to measure pH directly onboard using a NanoDrop 3300 fluorometer (Thermo Fischer) and HPTS (8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt) as a pH indicator. After sampling a minimum hemolymph volume of 0.5

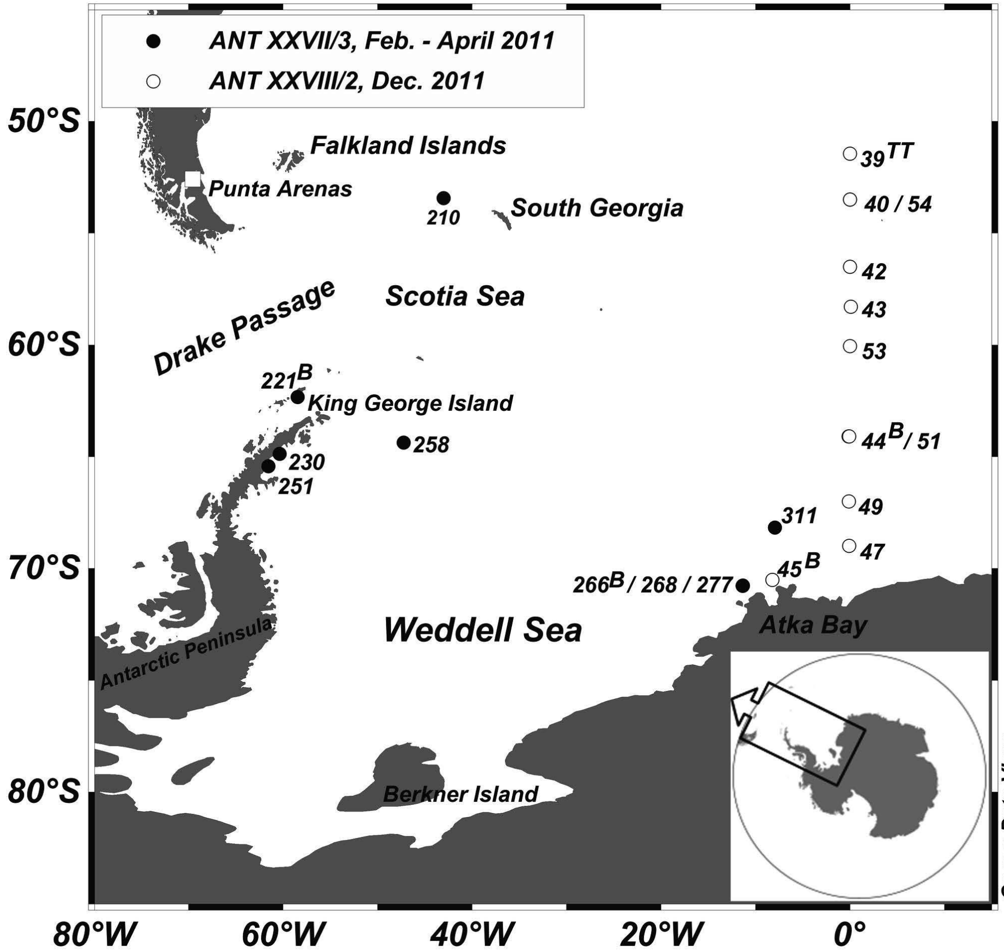

Figure 1. Study area with sampling sites. Study area with sampling sites (ANT XXVII/3: black dots; ANT XXVIII/2: open circles). Letters indicate additional tows with a Bongo net (B) or a Tucker Trawl (TT).

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µL in a pipette (0.1-2 µL), 5% per volume of a HTPS stock solution (50nM HPTS) was added into the pipette. The final HPTS concentration was about 1nM in all measurements. Fluorescence ratios were calculated by dividing the relative fluorescence resulting from 365 nm excitation by the relative fluorescence resulting from excitation at 470 nm (365:470 ratio). pH was calculated using a calibration curve with 50 mM Imidazole (Sigma-Aldrich, Steinheim, Germany) buffered seawater in the pH range from 5.0 to 8.5.

During ANT XXVII/3, pH measurements were conducted at ambient room temperature. Temperature profiles derived from the CTD were used to determine in situ temperatures and results were adjusted according to Ben-Yaakov [28] (temperature coefficient (ΔpH/°C ≈ 0.01)). During ANT XXVIII/2, pH- measurements were carried out in a temperature-controlled laboratory at in situ temperatures. Measurements resulting in units lower than pH 5.5 and above 8.0 should be interpreted with caution, since depending on the characteristics of the fluorescent dye HPTS error increases beyond these pH values.

Table 1. pH and cation composition (mmol L^{-1}) in the hemolymph of Antarctic copepod species in two different sampling periods (mean values ± s.d., with their range in parentheses), with values of seawater (cation composition derived from Prosser [51]) for comparison.

Species/		**Sampling**	**pH_e**		**Cation composition (mmol L^{-1})**					
Source	**Stage**	**Period**		**n**	**Na^+**	**NH_4^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**n**
Seawater	na	Austral Spring	8.0 ± 0.1 (7.8 - 8.3)	67	470	0	10	54	10	na
P. antarctica	CV	Austral Autumn	7.8 ± 0.3 (7.5 - 8.0)	3						nd
		Austral Spring	7.8	2	524	6	6	4	10	2
			7.6		478	6	10	45	11	
C. propinquus	CV	Austral Autumn	7.8 ± 0.2 (7.4 - 8.1)	16	488 ± 14 (468 - 499)	19 ± 3 (16 - 24)	17 ± 4 (12 - 21)	18 ± 8 (11 - 28)	9 ± 0.3 (8.7 - 9.3)	4
		Austral Spring	7.8 ± 0.4 (7.4 - 8.3)	6	498 ± 7 (491 - 504)	10 ± 4 (6 - 14)	19 ± 1 (18 - 20)	14 ± 4 (10 - 18)	9 ± 0.1 (8.8 - 9.1)	3
	F	Austral Autumn	7.8 ± 0.1 (7.6 - 7.9)	4						
		Austral Spring	7.9 ± 0.3 (7.5 - 8.5)	15	499 ± 26 (463 - 523)	15 ± 13 (6 - 48)	9 ± 3 (5 - 16)	16 ± 21 (3 - 54)	10 ± 1 (9 - 12)	9
R. gigas	CIII	Austral Autumn	6.3 ± 0.3 (5.7 - 6.7)	10	155 ± 95 (31 - 332)	376 ± 105 (176 - 512)	8 ± 4 (4 - 16)	9 ± 8 (1 - 23)	3 ± 2 (1 - 6)	10
		Austral Spring	6.1 ± 0.3 (5.7 - 6.6)	5	189 ± 48 (124 - 227)	336 ± 52 (291 - 407)	7 ± 2 (6 - 9)	11 ± 3 (7 - 15)	6 ± 2 (3 - 9)	4
	CIV	Austral Autumn	6.1 ± 0.3 (5.4 - 6.4)	8	249	264	19	13	6	1
		Austral Spring	6.3 ± 0.7 (5.1 - 7.3)	7	261 ± 58 (166 - 340)	268 ± 62 (188 - 375)	6 ± 3 (2 - 9)	9 ± 3 (2 - 12)	5 ± 1 (5 - 6)	7
	CV	Austral Autumn	6.1 ± 0.4 (5.4 - 6.8)	22	244 ± 87 (166 - 344)	285 ± 87 (184 - 356)	10 ± 7 (3 - 18)	8 ± 4 (2 - 11)	3 ± 2 (1 - 5)	5
		Austral Spring	6.1 ± 1.0 (4.8 - 7.0)	4	232 ± 115 (90 - 360)	290 ± 126 (159 - 452)	11 ± 6 (5 - 20)	13 ± 8 (3 - 26)	4 ± 2 (1 - 7)	5
	F	Austral Autumn	5.9 ± 0.4 (5.1 - 6.7)	17	198 ± 137 (55 - 427)	324 ± 155 (50 - 484)	11 ± 3 (5 - 15)	14 ± 16 (2 - 49)	3 ± 3 (0.2 - 9)	9
		Austral Spring	6.2 ± 0.5 (4.9 - 7.2)	24	246 ± 127 (15 - 465)	278 ± 131 (52 - 502)	9 ± 4 (3 - 18)	10 ± 7 (2 - 23)	5 ± 3 (1 - 11)	25
C. acutus	CIV	Austral Autumn	5.7 ± 0.2 (5.4 - 6.0)	5						nd
	CV	Austral Autumn	6.2 ± 0.6 (5.4 - 8.0)	23	181 ± 104 (28 - 427)	340 ± 119 (48 - 512)	12 ± 4 (6 - 21)	13 ± 12 (1 - 49)	4 ± 3 (0.03 - 11)	32
		Austral Spring	6.1 ± 0.6 (4.8 - 7.8)	59	224 ± 117 (30 - 514)	300 ± 126 (9 - 515)	9 ± 5 (1 - 20)	12 ± 9 (1 - 35)	5 ± 3 (1 - 10)	45
	F	Austral Spring	6.2 ± 0.6 (5.1 - 8.1)	56	248 ± 112 (56 - 400)	274 ± 121 (110 - 469)	11 ± 5 (1 - 20)	11 ± 9 (2 - 31)	6 ± 3 (2 - 11)	25

Dashed line separates non-resting (above) from resting species (below). copepodite stages 3 - 5 (CIII - CV), F females¸ n number of analyzed individuals, na not applicable, nd not determined

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Statistical analysis

All analyses were performed with R software version 2.14.2. Data were tested for normality with the Shapiro-Wilk-test. According to the distribution, a two-tailed unpaired t-test (confidence interval 95%) or a non-parametric Wilcoxon`s rank-sum test was applied to detect differences between two groups in pH_e and cation concentration. For the comparison of more than one means, either a one-way ANOVA or the non-parametric Kruskal-Vallis test was adopted. To analyze the effect of different pH values on the ammonium content in the hemolymph, a linear regression analyses was performed.

Results

Cation composition

In the hemolymph of the non-diapausing copepods *P. antarctica* and *C. propinquus*, a cation composition almost similar to the ionic composition of seawater was measured, although low levels of up to 48 mmol L^{-1} NH_4^+ occurred and Mg^{2+} values were partially reduced to a minimum of 3 mmol L^{-1} (Table 1). Highly elevated concentrations of as much as 515 mmol L^{-1} NH_4^+ and greatly reduced levels of down to 15 mmol L^{-1} Na^+ relative to an average seawater concentration of 470 mmol L^{-1} were present only in the hemolymph of *C. acutus* and *R. gigas* (Table 1, Figure 2). The Na^+ concentrations in the hemolymph were up to 97% lower than in seawater and explained most of the cation replacement. Additionally, divalent ions such as Mg^{2+} and Ca^{2+} were reduced. In *R. gigas*, the highest individual ammonium concentration was found in stage CIII between February and April (512 mmol L^{-1}, Table 1). The lowest individual ammonium concentration of all samples was determined in *C. acutus* CV (9 mmol L^{-1}, Table 1) from the surface layer (0 - 50 m) in December. This sample was also characterized by the highest pH value (pH_e 7.8, Table 1) measured for *C. acutus* CV during the corresponding sampling period. In contrast, the highest individual ammonium concentration within this species occurred in the hemolymph of CV (515 mmol L^{-1}, Table 1) from the deepest layer (2000 - 1500 m).

pH of hemolymph

In the hemolymph of the non-diapausing species *P. antarctica* and *C. propinquus*, mean pH values of 7.8 ± 0.3 were measured in both seasons (Table 1, Figure 2). Individual measurements were never lower than pH 7.4 in both species, seasons and all developmental stages (Table 1, Figure 2).

Lower pH levels only occurred in the hemolymph of diapausing *C. acutus* and *R. gigas*, and differences in the mean hemolymph pH were statistically discernible between diapausing (*C. acutus* and *R. gigas*) and non-diapausing (*P. antarctica* and *C. propinquus*) species (ANOVA, $p<0.0001$). In *R. gigas*, mean hemolymph pH ranged from 5.9 to 6.3 in both

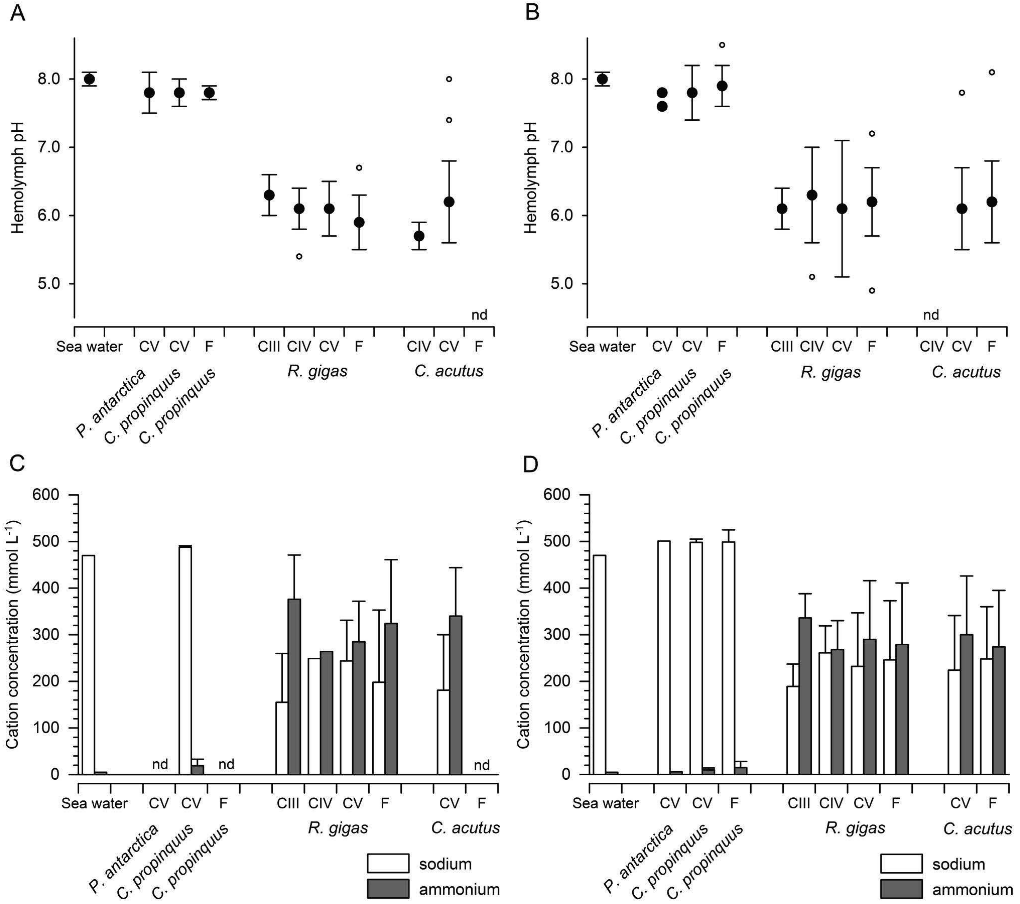

Figure 2. pH values and concentration of sodium and ammonium in seawater and hemolymph of Antarctic copepods. pH values (mean ± standard deviation; A and B, outlying results (more than 1.5 times the interquantile distance away) are represented by open circles) and concentration of sodium and ammonium (mmol L^{-1}; C and D; error bars reflect standard deviation) in seawater and in the hemolymph of Antarctic copepods from two different sampling periods (Feb. - April: A and C; December: B and D). Nd not determined, CIII – CV copepodite stages 3 - 5, F females. Number of replicates as in Table 1.

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seasons (Table 1, Figure 2). Individual measurements did not exceed pH 6.8 from February to April, while individual values higher than pH 7 were detected in December (*R. gigas* F: 7.2; CIV: 7.3, Table 1).

In *C. acutus*, mean hemolymph pH varied from 5.7 to 6.2 between February and April and 6.1 to 6.2 in December (Table 1, Figure 2). The overall highest hemolymph pH was measured in a *C. acutus* female (pH_e 8.1, Table 1) from the surface layer (100 - 0 m) in December. Overall means (without division into developmental stages) between February and April were pH 6.1 ± 0.4 in *R. gigas* (n = 57) and pH 6.1 ± 0.6 in *C. acutus* (n = 28). In December the overall mean amounted to pH 6.2 ± 0.6 for both species (*R. gigas* n = 40, *C. acutus* n = 115).

The interaction between pH and ammonium concentration in the hemolymph of *C. acutus* and *R. gigas* was tested for the expedition in December (Figure 3). The ammonium content in the hemolymph was significantly affected by acidity and increased with lower pH values in both species. The correlation was most pronounced in *C. acutus* CV ($y=-137x+1135$, $R^2=0.41$, $p<0.01$), followed by *C. acutus* females ($y=-104x$

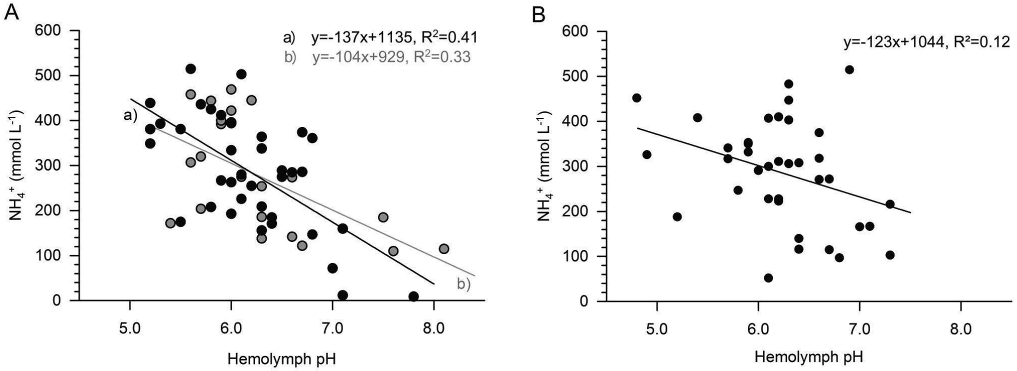

Figure 3. Relationship between pH and concentration of ammonium (NH_4^+). Relationship between pH and concentration of ammonium (NH_4^+, mmol L^{-1}) in the hemolymph of *C. acutus* (A; copepodite stage CV (a) y=-137x+1135, R^2=0.41, p<0.01; females (b) y=-104x+929, R^2=0.33, p<0.01) and *R. gigas* (B; all stages combined, y=-123x+1044, R^2=0.12, p=0.034).

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+929, R^2=0.33, p<0.01). In *R. gigas*, the correlation was less pronounced, but still discernible (all stages combined: y=-123x +1044, R^2=0.12, p=0.034) (Figure 3).

Discussion

The overall physical density of a diapausing copepod at depth must equal the density of the surrounding seawater to provide a stable position in the water column and to avoid a depletion of energy reserves for swimming activities. Proteins and the exoskeleton (1080 - 1240 kg m^{-3} in boreal-Atlantic diapausing *Calanus finmarchicus* [29]), exceed the density of seawater (≈ 1037.4 kg m^{-3} at 0°C temperature, 35 psu salinity and overwintering depth of 2000 m, calculation based on Fofonoff and Millard [30]). Thus, diapausing copepods must compensate the down-force by accumulating less dense body components that provide uplift and help maintain an overall neutral buoyancy.

An increasing number of studies have focused on the central role of low-density lipids in both regulating buoyancy and determining overwintering depths in diapausing copepods [10,29,31-35]. Only recently, special emphasis has been placed on the exact composition and degree of unsaturation of the stored wax esters. Wax esters are assumed to undergo phase transitions from liquid to solid state at high water pressures typical of overwintering depths and low temperatures. Such phase transitions could favorably increase the overall density of copepods and facilitate neutral buoyancy at depth [33-35]. However, notwithstanding the importance of lipid deposits as energy reserves, they are rather counterproductive in the course of downward migration, since copepods start to descend at the end of the productive season when lipid contents are at their maximum and hence, lipid-regulated buoyancy is high. In addition, Campbell and Dower [10] showed that lipid-based buoyancy is rather unstable and difficult to regulate, since small changes in lipid content will have dramatic consequences for buoyancy.

Ammonium-aided buoyancy

The presence of elevated ammonium concentrations within the hemolymph of only copepods known to undergo diapause in winter was confirmed in this study (Table 1; see 12 for review of previously measured values). Simultaneously, Na^+ and to a lesser extent Mg^{2+} and Ca^{2+} concentrations were reduced in relation to both seawater and the hemolymph of non-diapausing copepods. Variable amounts of K^+ are not further discussed in this study, since elevated concentrations are most likely the result of injured body tissue in the course of the hemolymph extraction, allowing the leakage of K^+ from the intracellular into the extracellular space.

Our findings of cation replacement in the hemolymph of diapausing copepods are in good accordance with changes in the ion composition in body fluids from a range of marine organisms known to use low-density fluids for buoyancy regulation [13,15,16,21]. For comparison, concentration of NH_4^+ in the deep-sea shrimp *N. gibbosus* was 296 ± 51 mmol L^{-1} in the carapace fluid and 217 ± 54 mmol L^{-1} in the hemolymph [15].

Compared to the extra cost of swimming or the accumulation of low-density organic compounds such as lipids, the energetic costs involved in the production of ammonium are low, since ammonium is a waste product from the catabolism of proteins and amino acids. Furthermore, in contrast to other buoyancy mechanisms, ammonium-aided buoyancy is independent from high ambient pressures and rapid changes in depth [13] and is therefore well suited for the extensive vertical migrations of copepods.

pH of hemolymph

Ammonia is highly toxic for most organisms and the concentration in the hemolymph of aquatic crustaceans is generally below 0.8 mmol L^{-1} [17]. In decapod shrimps for instance, 96-h LC_{50} values for adult *Penaeus paulensis* and juvenile *Litopenaeus vannamei* were, respectively, 2.4 mmol L^{-1} [36] and 2.2 mmol L^{-1} total ammonia [37]. The protonation of ammonia to form the comparatively less toxic ionized form NH_4^+ is strongly dependent on the H^+ concentration, and to a lesser extent upon temperature and salinity of the respective solution. At a pH of 7.8, 0°C and 32-40 psu salinity (pK ≈ 10.16), 99.6% of total ammonium exists in the ionic form NH_4^+, whereas 0.4% is present as NH_3 [38]. At ammonium concentrations of ≈ 500 mmol L^{-1} as measured in the hemolymph of diapausing copepods in this study, 0.4% would result in a NH_3 concentration of 2 mmol L^{-1}, which far exceeds the general hemolymph concentration of ≤ 0.8 mmol L^{-1} in aquatic crustaceans [17]. Under acidic conditions of pH ≈ 6.0, as measured in the hemolymph of diapausing copepods with elevated ammonium concentrations, only 0.007% of total ammonia is present as toxic NH_3 [38], resulting in a concentration of 0.035 mmol L^{-1}. Since NH_3 is lipid-soluble, uncharged and therefore easily diffusible across phospholipid membranes, it is regarded as the most toxic form in fish [18] and aquatic crustaceans [19].

To avoid ammonia toxicity, ammoniacal deep-sea crustaceans and squids sequester ammonium fluids in specialized vacuoles, chambers or gelatinous layers [14,15,39]. So far, there is no evidence for the existence of such a cellular unit in copepods.

The present study revealed very low pH values only in the hemolymph of diapausing copepods (Table 1, Figure 2). Moreover, the amount of ammonium was correlated to the respective hemolymph pH in both overwintering species (Figure 3). *C. acutus* and *R. gigas* had pH values of 6.1 to 6.2 in both seasons. These results are comparable to the pH in the ammonium-rich carapace fluid of *N. gibbosus* (pH 6.6 ± 0.08) [15] and in the vacuolar fluid of ammoniacal squid (≥ 5.1) [21]. At such low pH levels, virtually all ammonium is present in the non-toxic ionized form, which additionally reduces the loss of ammonia by diffusion [39,40].

Marine planktonic crustaceans are generally sensitive to low pH conditions and exposure to elevated H^+ concentrations can cause high mortality rates in zooplankton communities [41]. In addition, acidic pH conditions in the intracellular milieu have been shown to be relevant factors depressing metabolic rate in a range of other invertebrates during dormancy or environmental hypercapnia [42–44]. Since metabolic depression in diapausing copepods is essential to save energy during the food-limited winter period, the low pH values found in overwintering copepods might be beneficial for a successful implementation of diapause. Indeed, potential benefits of a low extracellular pH leading to a reduced aerobic energy turnover and thus metabolic depression have already been established for the marine worm *Sipunculus nudus* during anaerobiosis [45–47].

Inter-individual/intra-specific variability

Differences in both hemolymph pH and cation composition were discernible between diapausing and non-diapausing species, whereas no statistical differences could be determined between seasons, sampling depths or ontogenetic stages. According to the assumption that ammonium-aided buoyancy changes with season and that hemolymph pH controls metabolic depression, ammonium accumulation should be a seasonal phenomenon with maximum levels in overwintering stages at depth and minimum levels in active stages at the surface. Deviations from this prediction in the present data may be explained by the fact that our samples were collected in the transitional periods of autumn and spring where considerable variation in the individual life histories could not be excluded. Future studies should focus on ammonium concentration and hemolymph pH of Antarctic copepods during the active phase in summer and during diapause in winter.

In *C. acutus*, copepodite stage CV represents the main overwintering cohort. The fact that maximum ammonium concentration in *C. acutus* was observed in copepodite stage CV from the deepest water layer supports the idea that ammonium-aided buoyancy is most important in diapausing stages at overwintering depth. Nevertheless, adults have to ascend to the surface in spring with reduced lipid deposits [4,6]. Thus, elevated ammonium levels and high buoyancy is beneficial for the return to the surface. Ammonium levels and/or excretion rates may vary throughout the water column from individual to individual in relation to other factors such as maturity level or lipid content.

In *R. gigas*, intraspecific variation of hemolymph pH was high and most pronounced in stages CIV and CV in spring. Published data show that most likely not all individuals of *R. gigas* overwinter inactively. Resting stages were found at depth in the marginal ice-zone of the southern Scotia Sea [48], whereas actively feeding and apparently reproducing individuals occurred in the area of the Antarctic Peninsula during winter [49]. Unlike *C. acutus*, *R. gigas* has a more flexible, one- or two-year life cycle, meaning that parts of the population can maintain in the surface waters during winter, while the rest descends and enters diapause [3]. In consequence, we possibly caught and measured a “mixture” of individuals with different physiological backgrounds. Nevertheless, our hypothesis was supported by the observation that the highest ammonium content was measured in copepodite stage CIII in autumn (512 mmol L^{-1}, Table 1), which represents one of the main overwintering stages in *R. gigas* [50], whereas minimum concentrations were found in fully developed females (50 - 52 mmol L^{-1}, Table 1), which definitely had passed through diapause, molting and maturation.

In contrast, the correlation between pH and ammonium was stronger in *C. acutus* CV (Figure 3) and is indicative for a clearly defined one-year life cycle in which successful spawning is closely restricted to the beginning of the productive season [3].

Accumulation of ammonium and the replacement of ions with a higher solute mass (e.g. Na^+, Mg^{2+} and Ca^{2+}) in the hemolymph of Antarctic copepod species known to undergo diapause in winter was confirmed in this study. These findings support the idea of a relation between vertical ontogenetic migration and ammonia aided buoyancy in Antarctic copepods. Moreover, low pH values were measured only in the hemolymph of diapausing copepods with elevated ammonium levels. The ammonium content was statistically correlated to the respective pH of the hemolymph sample and increased with lower pH values. Further research should focus on the effect of low pH for metabolic depression and its possible role as the sought-after trigger for controlling diapause in Antarctic copepods.

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Author Contributions

Conceived and designed the experiments: SS SBSS HA FJS. Performed the experiments: SS SBSS HA FJS. Analyzed the data: SS SBSS HA FJS. Contributed reagents/materials/ analysis tools: SS SBSS HA FJS. Wrote the manuscript: SS SBSS HA FJS. participated in research trips to the Antarctic: SS SBSS HA FJS Carried out sampling and experimental work: SS SBSS HA FJS.

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CHAPTER 3

SEASONAL TRENDS OF HAEMOLYMPH PH AND CATION COMPOSITION OF THE ANTARCTIC COPEPODS CALANOIDES ACUTUS, RHINCALANUS GIGAS AND CALANUS PROPINQUUS AND THEIR IMPLICATIONS FOR LIFE-CYCLE ADAPTATIONS

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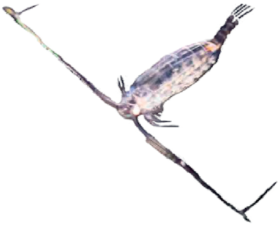

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Chapter 3

Seasonal trends of haemolymph pH and cation composition of the Antarctic copepods Calanoides acutus, Rhincalanus gigas and Calanus propinquus and their implications for life-cycle adaptations

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Abstract

Herbivorous copepod species have developed specific life-cycle strategies to cope with the pronounced seasonality of primary production in the Southern Ocean. Ontogenetic vertical migration (OVM) associated with a resting stage (diapause) at greater depth (>500 m) is a known adaptation to escape food scarcity during winter, although the controlling factors inducing and terminating OVM and diapause are still unknown. This study provides a comprehensive data set of seasonal and depth related dynamics within the life-cycle adaptations of resting and non-resting Antarctic copepod species *Calanoides acutus*, *Rhincalanus gigas* and *Calanus propinquus* from four expeditions, covering austral spring, summer, autumn and winter. High concentrations of ammonium (NH_4^+) with maximum values of up to 530 mmol L^{-1} in winter were found in diapausing species *C. acutus* and *R. gigas* and were associated to acidic pH conditions of pH ≤ 6. These findings support previous investigations on novel hypotheses about the controlling factors of copepod diapause. It is assumed that the replacement of ions with a higher molecular weight and the accumulation of low density ammonium contribute to the achievement of an energy-saving state of neutral buoyancy. Furthermore, low pH conditions might not only be a precondition for ammonium accumulation, but in addition trigger dormancy in copepods.

Keywords: Zooplankton; copepod; Antarctic; diapause; buoyancy; ammonium

1 Introduction

In the Southern Ocean, strong seasonal changes in solar radiation, sea-ice extent and, hence, primary production present a challenge for herbivorous zooplankton and have shaped different species' life cycles and overwintering strategies. The three dominant large Antarctic copepod species *Calanoides acutus*, *Rhincalanus gigas* and *Calanus propinquus* are representatives for different life-cycle strategies and present species- and stage-specific as well as seasonal variations in their vertical and latitudinal distribution (e. g. Voronina 1970, Marin 1988, Atkinson 1991, Marin and Schnack-Schiel 1993, Atkinson 1998), the performance of seasonal/ontogenetic vertical migrations (OVM) (e.g. Bathmann et al. 1993, Atkinson 1998), lipid-biosynthesis pathways and storage patterns (e.g. Hagen et al. 1993, Kattner and Hagen 1995, Lee at al. 2006, Kattner et al. 2012), dietary preferences (e.g. Dalsgaard et al. 2003) and metabolic activities (Drits et al. 1994, Pasternak and Schnack-Schiel 2001). *C. acutus* is a seasonal migrant overwintering at greater depth as late copepodids in a resting stage called diapause. While in diapause, metabolic depression is evident from reduced metabolic rates and swimming activities in order to avoid a depletion of energy reserves. *C. propinquus* overwinters actively feeding in the upper water layers, switching to a more omnivorous diet during winter, whereas *R. gigas* has a more flexible, one- or two year life cycle with parts of its population entering diapause, while the rest remains in the surface layer (Atkinson 1998). Even though our knowledge on life-cycle adaptations of Antarctic copepods has vastly improved over the last decades, the signals and physiological mechanisms triggering ontogenetic vertical migration and diapause are still far from understood.

Only recently, copepod species were found to differ in the ion composition of their haemolymph. High amounts of ammonium (NH_4^+) associated with low pH values of ≤ 6 were measured in the diapausing copepods *C. acutus* and *R. gigas* (Sartoris et al. 2010, Schründer et al. 2013). Additionally, *C. acutus* maintains neutral buoyancy during austral winter only by its biochemical composition without the additional need of swimming movements (Schründer et al. 2014). These findings provide a basis for novel hypotheses, which postulate that the replacement of heavy ions (e.g. Na^+, Mg^{2+}, Ca^{2+}) by ammonium with a lower density reduces the overall density of the copepod and helps maintaining an energy saving state of neutral buoyancy during diapause (Sartoris et al. 2010). A low haemolymph pH is a precondition to maintain and accumulate ammonium in sufficient amounts in the less toxic ionised form NH_4^+. Since low pH values are also known as a relevant factor reducing metabolic rate in other marine invertebrates, they may, in addition, ensure metabolic depression during diapause (Sartoris et al. 2010, Schründer et al. 2013).

Previous studies on life-cycle adaptations were often restricted to the summer months and observations in mid-winter are rare due to logistical constraints. This study provides a comprehensive data set from four expeditions, covering austral spring, summer, autumn and winter (Fig. 1). Copepods from different seasons, developmental stages, sampling depths, and hence, different life-cycle phases were collected and analysed with regard to their extracellular ammonium content and pH. In addition, physiological and biochemical parameter such as metabolic rates, lipid storage patterns and stable isotope ratios were measured. Respiration rates were determined to provide information on the metabolic activity of the copepods, whereas the analysis of lipids and fatty acid compositions was used to identify species-specific lipid storage patterns and selective retention and mobilisation of lipid components during starvation periods. In addition, some fatty acids can be used as trophic biomarkers, as they are characteristic of specific microalgae groups and are incorporated largely unmodified into the storage lipids (Dalsgaard et al. 2003). The isotopic ratio of nitrogen ($\delta^{15}N$: $^{15}N/^{14}N$) is usually indicative for the trophic position of a consumer. Due to the selective retention of the heavier ^{15}N isotope in the body tissues and the excretion of the lighter ^{14}N isotope, the isotopic composition is typically enriched in ^{15}N by 3.4 ± 1.1‰ from one level to the other (Minagawa and Wada 1984).

There are two main objectives of this study. The first objective is a seasonal comparison of the extracellular cation composition and pH to test novel hypotheses on physiological processes, in which diapausing copepods regulate their buoyancy via changes in their ion composition, while an associated pH reduction serves as a potential trigger for metabolic depression. If so, ammonium accumulation and pH reduction should be a seasonal phenomenon with highest ammonium concentrations and lowest pH levels in diapausing individuals at overwintering depth during winter, and normal pH conditions and lowest amounts of ammonium in active individuals from the surface waters in spring and summer. The second objective is to confirm and supplement existing knowledge on life-cycle strategies of polar copepods and to clarify the life-history backgrounds of the investigated copepods, based on supplementary data on respiration rates, lipid content, lipid class and fatty acid composition and stable isotope ratios from different seasons, stations and depth layers.

2 Material and Methods

2.1 Sampling

Copepods were sampled during four expeditions onboard RV *Polarstern* in late summer/early autumn (ANT XXVII/3, February to April 2011), late spring (ANT XXVIII/2, December 2011), winter (ANT XXIX/6, June to July 2013) and early summer (ANT XXIX/9, January to February 2014, Fig. 1).

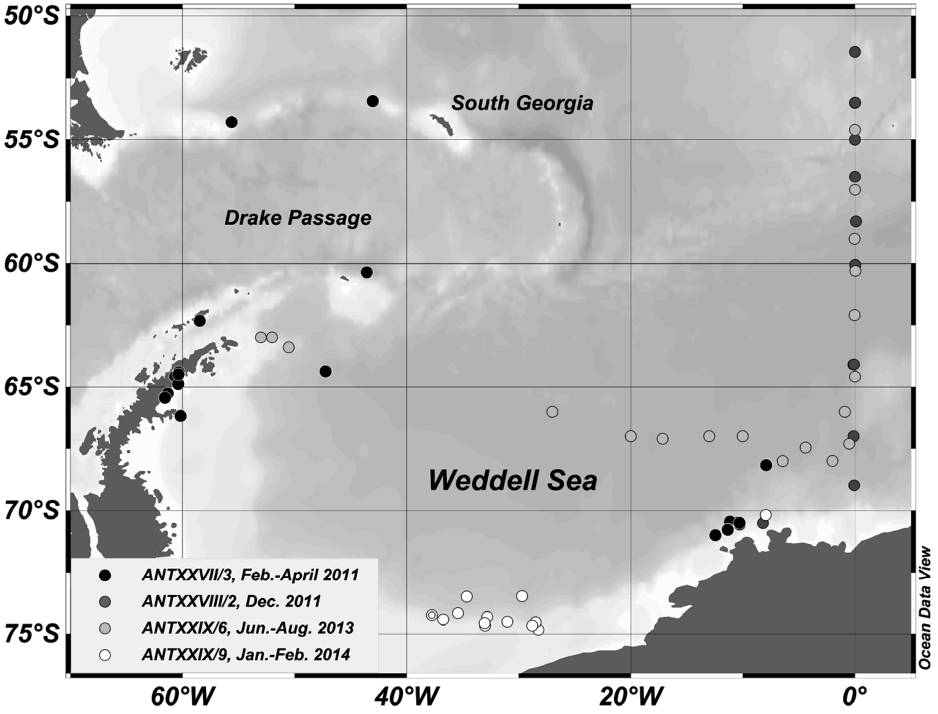

Figure 1. Study area with sampling sites.

Zooplankton samples were collected by stratified vertical hauls from a maximum depth of 2000 m to the surface using multiple/opening closing nets (Multinet Maxi, mouth opening 0.5 m²; Multinet Midi, mouth opening 0.25 m²; 100 µm mesh size for both). Additional hauls from a maximum depth of 300 m to the surface were made with a Bongo net (mouth opening 0.28 m², mesh size 100 µm) and during ANTXXVIII/2 also with a Tucker Trawl (mouth opening 2.25 m², mesh size 1500 µm). Specimens of *Calanoides acutus*, *Calanus propinquus* and *Rhincalanus gigas* were sorted from the samples and identified to sex and copepodite stage (copepodite stage 3-5: CIII-CV; adult females: F). For respiration measurements, haemolymph extraction and pH measurements, live individuals were kept in filtered seawater in a temperature-controlled refrigerator or cooling container at 0°C prior to the measurements. For analyses of dry mass, lipid content and composition and stable isotope signature, specimens were deep-frozen at -80°C.

2.2 Haemolymph extraction and analysis

Haemolymph was extracted manually from individual live copepods using small glass capillaries (for further details see Schründer et al. 2013). Each haemolymph sample was diluted in 40 µl of de-ionised water and the cation composition was analysed by ion chromatography using a DIONEX ICS 2000 with an IonPac CS 16 column and methane sulfonic acid (MSA, 30 mmol L^{-1}) as an eluent at 0.36 ml min^{-1} flow rate. Inorganic ions such as NH_4^+, Na^+, Mg^{2+}, K^+, and Ca^{2+} were identified according to retention times in comparison to a cation standard of known composition (Dionex, Six Cation Standard). Cation concentrations are expressed as mmol L^{-1}.

2.3 Extracellular pH-measurements

Extracellular pH (pH_e) was measured in individual haemolymph samples of at least 500 nl directly onboard using a NanoDrop 3300 fluorometer (Thermo Fischer) and a fluorescent dye (HPTS, 8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt, 1nM) as a pH indicator. pH was determined by calculating the ratios of fluorescence excitation at wavelengths 365 and 470 nm in comparison to a calibration curve with 50 mM Imidazole (Sigma-Aldrich, Steinheim, Germany) buffered seawater. Measurements resulting in pH units below 5.0 or above 8.5 should be interpreted with caution due to limitations of the fluorescent dye HPTS. For a detailed description of the method see Schründer et al. (2013).

2.4 Respiration measurements

Oxygen consumption was measured non-invasively by optode respirometry using several 1-channel Fiber-Optic Oxygen Meters Fibox 3 (PreSens, Precision Sensing GmbH) during expedition ANTXXVII/3 and a 10-Channel Fiber-Optic Oxygen Meter (OXY-10, PreSens, Precision Sensing GmbH) during expeditions ANTXXVIII/2 and ANTXXIX/6. Measurements were conducted in gas-tight glass bottles of 13 ml volume. The bottles were filled with oxygenated and filtered seawater. Depending on individual size, 1 to 22 individual copepods were transferred into each bottle. One or two animal-free controls were measured in each experiment to correct for unspecific microbial respiration. During the measurements, all bottles were kept in a water bath in a dark and temperature-controlled refrigerator at 0.4 °C. The decrease in dissolved oxygen concentration was logged every 1 to 15 sec for up to 12 hours. Data from the first hour of each experiment were excluded from further analysis in order to minimise the effects of handling stress at the beginning of the experiments. After each measurement, individuals were checked for condition and deep-frozen at -80°C for subsequent determination of dry mass.

2.5 Dry mass determination and lipid analysis

For dry mass determination, copepods were lyophilised for 48 h and weighed individually using a Sartorius Microbalance NCII1S with a precision of ± 10 µg.

Total lipid content was measured gravimetrically according to Folch et al. (1957), modified by Hagen (2000). Samples were homogenised with a Potter homogenizer (Braun, Potter S) and an ultrasonic cell disrupter (Bandelin electronic, UW 2070). Lipids were extracted with an organic solvent (dichloromethane/methanol, 2:1 by volume) and washed with aqueous KCl solution (0.88%). The extracts were dried under a stream of nitrogen and weighed.

For the analysis of the fatty acid (FA) and fatty alcohol (FAlc) composition, subsamples of the lipid extracts were transesterified with 3% concentrated sulphuric acid in methanol at 80°C for 4 h according to Kattner and Fricke (1986). Measurements were carried out with a gas chromatograph (Agilent Technologies 7890A) equipped with a DB-FFAP column (30 m length, 0.25 mm diameter) and helium as a carrier gas. Fatty acids and fatty alcohols were identified by comparing their retention times to those of known standards (Menhaden fish oil; natural copepod standard *Calanus hyperboreus*). Total wax ester (WE) content was estimated based on the FAlc content assuming equal carbon chain lengths of the FAlc and FA components in the WE molecules (Kattner et al. 2003). Total wax ester content is expressed as percentage of total lipid (%TL).

A principal component analysis (PCA, Primer 6.1.6) was conducted to identify patterns in the species-specific and/or seasonal fatty acid composition. Prior to analysis, data were arcsin-square-root transformed to compensate for non-homogenous variance of percentage values (Osborne 2002). Fatty acid compositions were analysed following the trophic biomarker concept according to Dalsgaard et al. (2003), with 16:1(n-7), 16:4(n-1), 18:1(n-7) and 20:5(n-3) being tracers for a principal diatom-based diet, 18:4(n-3) and 22:6(n-3) as flagellate markers, 20:1(n-9), 22:1(n-11) and 22:1(n-9) being calanid markers and 16:0, 20:5(n-3) and 22:6(n-3) as principal components of biomembranes (Dalsgaard et al. 2003, Kelly and Scheibling 2012).

2.6 Stable isotope analysis

Deep-frozen copepods were lyophilised for 48 h, weighed and transferred into tin capsules. Stable isotope analyses of nitrogen ($\delta^{15}N$) were carried out at TÜV Rheinland Agroisolab GmbH (Jülich, Germany) by mass spectrometry (Carlo Erba Instruments, EA NA1500 Series 2) with standard AIR (atmospheric air; IAEA-N1, Vienna) as reference and helium as a carrier gas. Isotopic ratios are calculated after Hodum and Hobson (2000).

3 Results

3.1 Haemolymph pH and cation composition

In the haemolymph of diapausing *C. acutus* and *R. gigas*, mean pH_e was significantly lower than in actively overwintering *C. propinquus* (ANOVA, $p<0.0001$) in all developmental stages and months (Table 1, Fig. 2). In the true diapausing species *C. acutus*, the lowest pH_e was measured in winter (pH_e 4.2, Table 1).

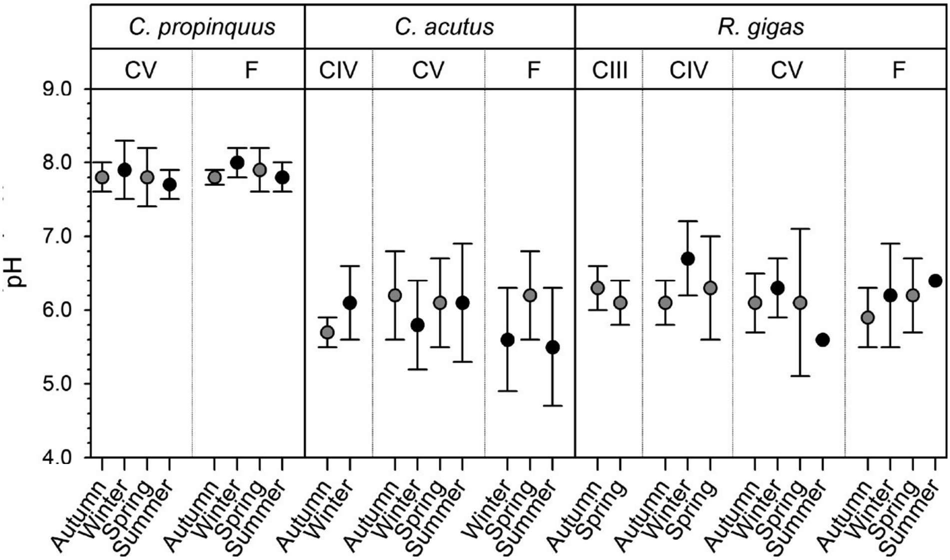

Figure 2. Haemolymph pH of *Calanus propinquus*, *Calanoides acutus* and *Rhincalanus gigas* in different seasons, the present data (black dots) complement published data (grey dots) by Schründer et al. (2013). Mean values ± standard deviation, number of replicates as in Table 1.

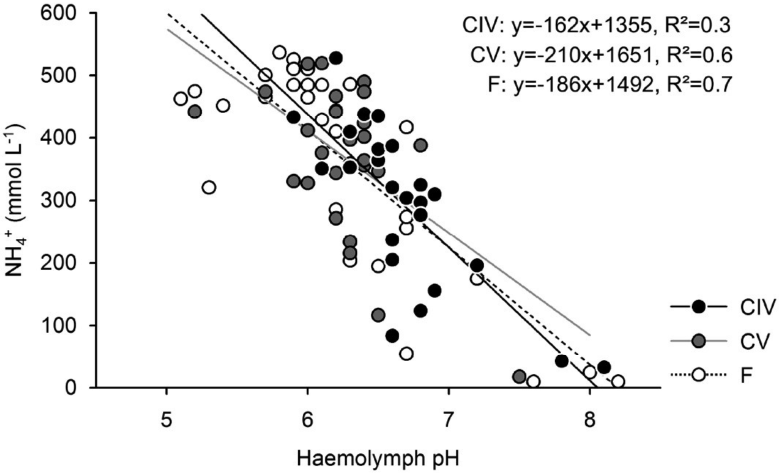

Figure 3. Relationship between pH and concentration of ammonium (NH_4^+, mmol L^{-1}) in the haemolymph of *Rhincalanus gigas* in austral winter (stage CIV y=-162x+1355, R²=0.3, p=0.005; CV y=-210x+1651, R²=0.6, p=0.442; females y=-186x+1492, R²=0.7, p<0.001).

However, in all three species, mean haemolymph pH values remained fairly constant throughout the year, varying between 7.7 to 8.0 in *C. propinquus*, 5.6 to 6.1 in *C. acutus* and 6.2 to 6.7 in *R. gigas*. Seasonal, as well as depth-related differences were not significant. In winter-active *C. propinquus*, ammonium concentrations were low in all developmental stages and during all sampling months, with a maximum of 62 mmol L^{-1} in adult females in winter (Table 1). In *C. acutus* and *R. gigas* by contrast, ammonium concentrations were always significantly higher throughout the year and in all developmental stages (ANOVA, $p<0.0001$). Accordingly, concentrations of the other cations, in particular of sodium, were reduced (Table 1). In the diapausing species *C. acutus*, ammonium concentrations were generally highest in overwintering stages CIV during austral winter with a mean concentration of 415 ± 99 mmol L^{-1} and an individual maximum of 530 mmol L^{-1}, although seasonal and depth-related trends were not significant (Table 1). In *Rhincalanus gigas*, the ammonium content was significantly affected by acidity and increased with lower pH values. The correlation was most pronounced in adult females ($y=-186x+1492$, $R^2=0.7$), followed by CV ($y=-210x+1651$, $R^2=0.6$) and CIV ($y=-162x+1355$, $R^2=0.3$) (Fig. 3).

Table 1. pH and cation composition (mmol L^{-1}) in the haemolymph of *Calanus propinquus*, *Calanoides acutus* and *Rhincalanus gigas* (mean values ± s.d., range in parentheses). CIII - CV: copepodite stages 3 - 5, F: females, n number of analysed individuals, n.d. not determined.

Species/Stage	**Sampling Period**	**pH_e**		**Cation composition (mmol L^{-1})**					
			n	**Na^+**	**NH_4^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**n**
C. propinquus CV	Winter	7.9 ± 0.4 (7.2 - 8.4)	12	500 ± 13 (473 - 526)	17 ± 12 (4 - 54)	14 ± 5 (5 - 27)	12 ± 12 (1 - 46)	8 ± 1 (5 - 10)	26
	Summer	7.7 ± 0.2 (7.4 - 7.8)	4						n.d.
C. propinquus F	Winter	8.0 ± 0.2 (7.9 - 8.1)	3	492 ± 20 (464 - 514)	34 ± 17 (17 - 62)	12 ± 4 (7 - 19)	6 ± 6 (2 - 14)	7 ± 1 (5 - 8)	6
	Summer	7.8 ± 0.1 (7.7 - 7.8)	3						n.d.
C. acutus CIII	Winter		n.d.	142 ± 121 (51 - 350)	397 ± 123 (188 - 495)	5 ± 3 (2 - 8)	5 ± 5 (1 - 10)	2 ± 1 (1 - 3)	5
C. acutus CIV	Winter	6.1 ± 0.5 (4.9 - 7.6)	86	117 ± 90 (16 - 453)	415 ± 99 (41 - 530)	9 ± 5 (2 - 23)	7 ± 8 (1 - 41)	2 ± 2 (1 - 9)	123
C. acutus CV	Winter	5.8 ± 0.6 (4.4 - 7.5)	103	216 ± 136 (18 - 520)	303 ± 147 (8 - 525)	15 ± 6 (3 - 32)	12 ± 10 (1 - 49)	4 ± 3 (1 - 13)	130
	Summer	6.1 ± 0.8 (4.8 - 7.6)	14						n.d.
C. acutus F	Winter	5.6 ± 0.7 (4.2 - 7.6)	79	207 ± 118 (37 - 520)	316 ± 126 (17 - 505)	12 ± 7 (2 - 31)	11 ± 9 (1 - 41)	3 ± 2 (1 - 9)	79
	Summer	5.6 ± 0.8 (4.5 - 7.4)	28						n.d.
R. gigas CIII	Winter		n.d.	273 ± 135 (152 - 480)	246 ± 156 (7 - 386)	6 ± 2 (4 - 9)	20 ± 16 (5 - 44)	5 ± 3 (3 - 11)	7
R. gigas CIV	Winter	6.7 ± 0.5 (5.9 - 8.1)	24	237 ± 110 (99 - 459)	288 ± 120 (33 - 438)	9 ± 7 (2 - 32)	11 ± 9 (2 - 41)	5 ± 3 (2 - 11)	26
R. gigas CV	Winter	6.3 ± 0.4 (5.2 - 7.5)	26	171 ± 104 (51 - 477)	356 ± 116 (18 - 490)	11 ± 6 (4 - 32)	9 ± 9 (2 - 35)	3 ± 2 (1 - 9)	24
	Summer	5.6	1						n.d.
R. gigas F	Winter	6.2 ± 0.7 (4.8 - 8.2)	35	177 ± 150 (27 - 489)	351 ± 167 (10 - 513)	7 ± 3 (2 - 15)	12 ± 14 (1 - 47)	3 ± 3 (1 - 10)	29
	Summer	6.4	1						n.d.

3.2 Respiration rates

Mass-specific oxygen consumption rates were generally higher in smaller copepodite stages, with a maximum of 81±25 µmol O_2 h^{-1} g DM^{-1} in active overwintering *C. propinquus* CIV from the surface layer (200-0m). Overall, highest variation of respiration rates occurred during winter at the surface, ranging from 23 to 112 µmol O_2 h^{-1} g DM^{-1} in actively overwintering *C. propinquus* CIV and from 7 to 64 µmol O_2 h^{-1} g DM^{-1} in *R. gigas* CIV (Fig. 4).

In the diapausing species *C. acutus*, oxygen consumption considerably decreased with depth in autumn, ranging from 32.4±2.8 µmol O_2 h^{-1} g DM^{-1} in the upper 50 m to 7.5±1.0 µmol O_2 h^{-1} g DM^{-1} at 1500-1000 m in CV and from 29.7±9.3 (300-0 m) to 11.0 µmol O_2 h^{-1} g DM^{-1} (1400-1000 m) in CIV. In winter, respiration was generally low throughout the water column and did not exceed 30 µmol O_2 h^{-1} g DM^{-1}, whereas during spring, rates of up to 70 µmol O_2 h^{-1} g DM^{-1} were measured in females from the surface layer (Fig. 4).

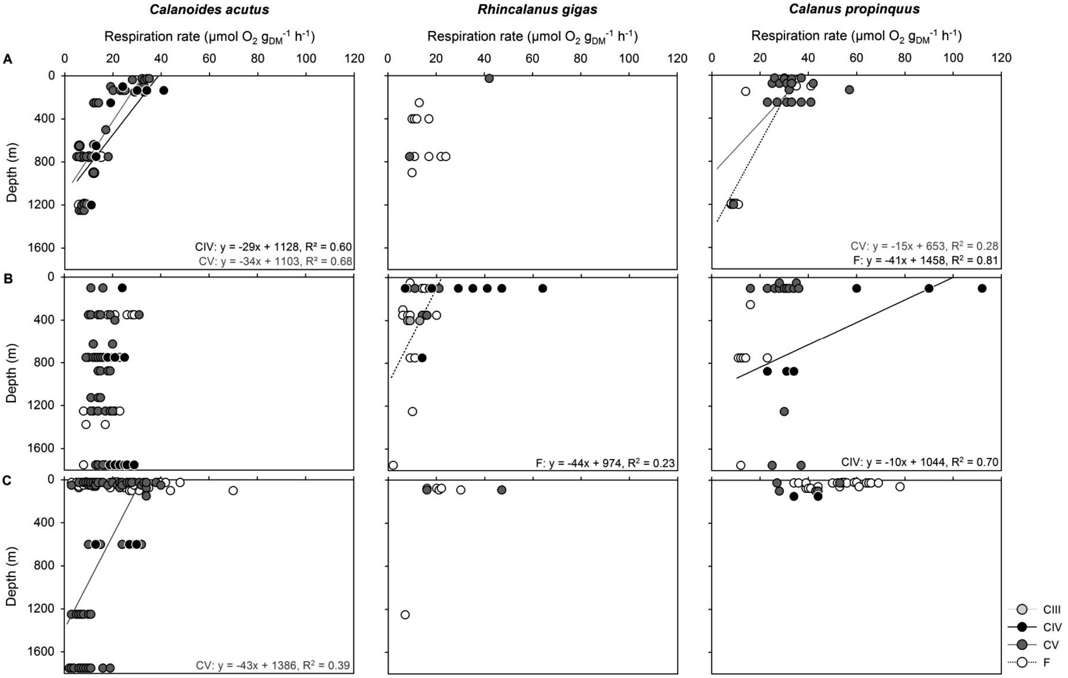

Figure 4. Respiration rates (µmol O_2 gDM^{-1} h^{-1}) of *Calanoides acutus*, *Rhincalanus gigas* and *Calanus propinquus* in autumn (A), winter (B) and spring (C) (CIII-CV: copepodite stages 3-5, F: female).

3.3 Dry mass and total lipid content

In all three species, lipid mass (LM, mg ind.$^{-1}$) was positively correlated to body dry mass (DM, mg ind.$^{-1}$), except for *C. propinquus* in spring (spring: *C. acutus* y=221x+1.52, R^2=0.56, *R. gigas* y=669x+1.97, R^3=0.76; summer: *C. acutus* y=302x+1.43, R^2=0.87, *C. propinquus* y=332x+2.87, R^2=0.60; autumn: *C. acutus* y=185x+1.74, R^2=0.93, *R. gigas* y=656x+2.02, R^2=0.89, *C. propinquus* y=411x+1.57, R^2=0.96; winter: *C. acutus* y=111x+2.18, R^2=0.66, *R. gigas* y=391x+3.35, R^2=0.56, *C. propinquus* y=317x+2.10, R^2= 0.79). Dry masses generally increased from spring towards summer, reached highest values in individuals from deeper water layers (>300 m) from February to April and decreased again towards winter (Fig. 5).

Total lipid content (TL, %DM) accounted for a minimum of 5%DM in *Rhincalanus gigas* during winter to a maximum of 61%DM in *Calanoides acutus* during early summer (January, Fig. 5). In diapausing species *C. acutus*, lipid levels were significantly correlated to depth of occurrence and increased with increasing depth in females in early summer (January) (y = 17x - 150, R^2 = 0.30, $p<0.01$) and CIV and CV during autumn (CIV: y = 20x - 185, R^2=0.77, $p<0.01$; CV: y = 35x - 648, R^2=0.67, $p<0.01$). In *R. gigas*, total lipid content was generally lower than in the other two species, ranging from 5% in winter to 46% in spring (Fig. 5). In *C. propinquus* CV from greater depths, total lipid content clearly increased from December to March/April and decreased towards winter. In females from the surface layers, lipid content gradually increased from spring towards the end of summer (March/April) and at greater depths, TL dropped towards winter by 72%DM (Fig. 5).

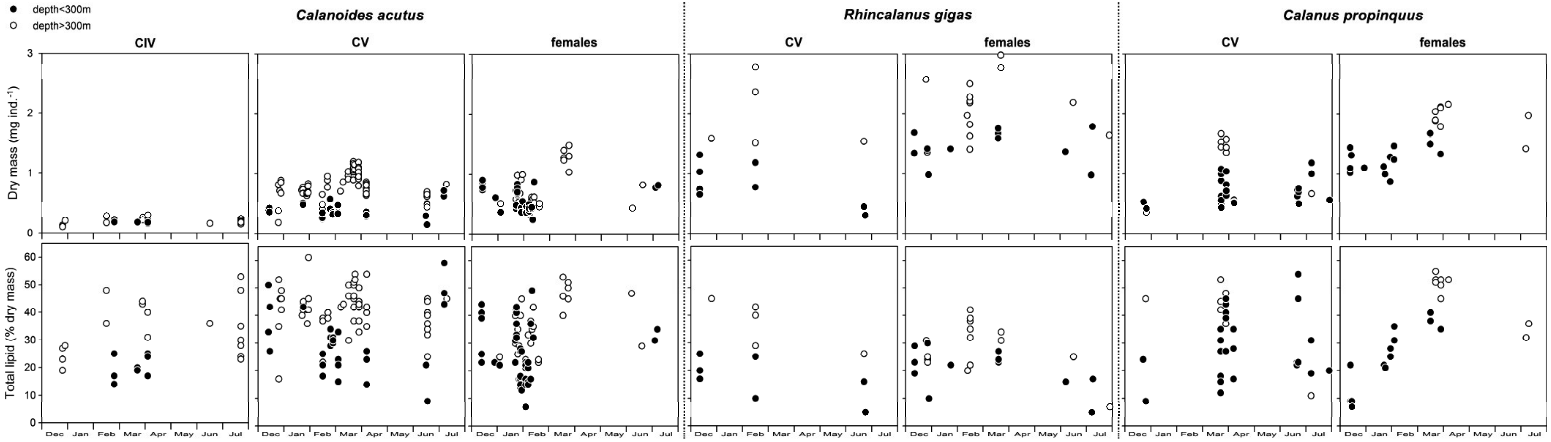

Figure 5. Seasonal trends in dry mass (mg ind.$^{-1}$) and total lipid content (in percent dry mass) of *Calanoides acutus*, *Rhincalanus gigas* and *Calanus propinquus* (CIV and CV: copepodite stages 4 and 5; division into different depth layers: black <300m depth, white >300 m depth).

3.4 Lipid class composition, wax ester content, fatty acid and fatty alcohol composition

The largest differences between the three species were the accumulation of different lipid classes, followed by species-specific as well as stage-dependent and seasonal variations in total lipid content and in the fatty acid/fatty alcohol composition (Table 2).

Both inactively overwintering species *C. acutus* and *R. gigas* accumulated very high amounts of wax esters with up to 85% of total lipid in *C. acutus* and 84%TL in *R. gigas*. In contrast, no wax esters were found in actively overwintering *C. propinquus*.

The fatty acid composition of the diapausing species *C. acutus* and *R. gigas* were generally dominated by the polyunsaturated fatty acid 20:5(n-3). In *C. acutus*, elevated amounts of very long-chain calanid markers 20:1(n-9), 22:1(n-11) and 22:1(n-9) were found. In contrast such long-chain moieties were nearly absent in *R. gigas* (Table 2). In *C. propinquus* overall mayor fatty acids were in decreasing order 16:0, 22:1(n-9), 20:5(n-3), 22:1(n-11), and 22:6(n-3) and overall most pronounced seasonal differences in fatty acid composition were found in adult females. Here, samples from the surface in spring clearly differed from the other samples, having severely reduced levels of calanid markers 22:1(n-11) and 22:1(n-9), and in turn extra ordinary high percentages of phospholipids 22:6(n-3), 20:5(n-3) and 16:0 (Table 2).

A PCA was conducted to compare the samples of all species and cruises with regard to fatty acid composition and the first two principal components are shown in Fig. 6. PC1 explains 47.7% and PC2 26.9% of the total variance of FA compositional data. PC1 was characterized by negative loadings of calanid markers 20:1(n-9), 22:1(n-11), 22:1(n-9) and 16:0, and positive loadings for all remaining fatty acids contributing less than 2% to total fatty acids. All samples were grouped in three well defined species-specific clusters according to the fatty acid profiles. Especially *Rhincalanus gigas* was tightly grouped together, whereas in *C. propinquus* three female samples from the surface layers in spring and a deep-water sample of *C. acutus* CV in winter deviated from the other samples (Fig. 6).

Table 2a. Seasonal trends of dry mass (DM), total lipid content (TL), wax ester content (calculated from fatty alcohols), fatty acid and fatty alcohol composition (<2% not presented) of *Calanoides acutus* (a), *Rhincalanus gigas* and *Calanus propinquus* (b). TFA: total fatty acid; TFAlc: total fatty alcohols; mean±standard deviation for n≥3; for n=2, sample 1 data/sample 2 data; n (ind): number of samples (total number of individuals); n.a.: not available; "-": traces or not detectable.

Species	***Calanoides acutus***										
Stage	CIV			CV				F			
Season	Autumn	Winter	Spring	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer
n (ind)	8 (131)	5 (80)	4 (89)	32 (194)	9 (41)	8 (45)	4 (60)	3 (8)	1 (3)	4 (17)	17 (327)
Dry mass (mg)	0.21±0.04	0.22±0.02	0.15±0.05	0.66±0.29	0.50±0.18	0.66±0.25	0.68±0.12	0.95±0.40	2.20	0.82±0.08	0.54±0.17
Total lipid (%DM)	28±2	28±5	16±7	37±10	33±12	43±10	45±1	42±16	29	38±8	27±8
Wax ester (%TL)	74.3±5.1	79.8±3.5	67.8±8.2	84.5±5.2	74.1±18.1	82.2±4.9	81.6±1.2	78.1±4.6	82.2	82.1±4.2	70.9±6.4
Fatty acids (%TFA)											
14:0	2.8±0.6	3.5±0.3	4.8±0.5	3.4±0.9	4.7±1.1	2.7±0.6	4.8±0.4	4.8±0.5	6.8	3.3±0.7	5.5±1.4
16:0	7.0±1.0	5.1±0.4	7.5±2.7	4.5±1.5	8.0±4.0	4.3±1.8	3.1±0.1	4.8±1.0	5.0	5.1±0.9	6.5±1.5
16:1(n-7)	9.5±2.4	13.5±0.7	12.1±3.3	7.9±3.6	13.6±3.4	6.5±1.3	5.6±0.8	9.1±3.4	10.2	10.1±1.1	8.2±0.6
16:4(n-1)	5.2±0.8	3.5±1.0	0.4±0.1	3.7±2.1	3.1±1.9	2.1±1.0	0.8±0.2	1.5±0.3	1.7	2.6±1.0	1.1±0.7
18:1(n-9)	3.7±0.3	5.6±0.2	7.9±2.0	4.8±0.8	5.1±1.1	5.1±0.7	6.8±0.8	5.7±1.2	7.2	5.0±0.9	6.3±1.0
18:1(n-7)	1.7±0.3	1.3±0.0	1.6±0.3	1.4±0.3	1.4±0.2	1.2±0.1	1.1±0.1	1.4±0.3	1.5	1.2±0.1	1.6±0.3
18:2(n-6)	1.5±0.7	2.5±0.1	4.1±0.2	1.6±0.6	1.9±0.4	1.8±0.4	2.1±0.2	1.6±0.4	2.4	1.7±0.4	1.8±0.3
18:4(n-3)	3.5±1.2	1.4±0.3	4.0±3.7	6.9±7.3	1.9±0.5	8.9±2.5	10.9±1.4	8.0±7.1	11.0	3.9±0.9	2.6±2.0
20:1(n-9)	8.1±2.7	15.9±1.6	10.6±4.9	19.4±5.7	15.9±6.0	22.3±6.1	22.4±0.8	20.6±1.6	16.3	21.3±4.5	20.5±4.7
20:5(n-3)	29.6±3.5	23.8±2.1	19.0±4.4	21.7±6.9	19.5±4.2	21.0±5.7	12.3±0.7	17.2±4.5	15.4	21.6±5.2	17.3±5.9
22:1(n-11)	4.5±1.7	6.5±0.8	5.6±2.5	8.0±2.0	8.5±3.0	8.3±2.9	9.3±0.6	8.7±1.8	6.5	9.5±1.0	8.5±1.7
22:1(n-9)	1.5±0.5	1.9±0.3	1.5±0.4	2.6±0.9	3.8±3.2	2.7±0.8	2.7±0.2	3.1±1.1	2.1	3.4±0.4	2.7±0.6
22:6(n-3)	13.0±20	8.0±0.4	15.2±3.1	8.9±1.5	6.4±1.3	9.0±2.7	8.6±0.5	8.4±1.5	8.9	7.4±1.5	10.3±4.0
Fatty alcohols (%TFAlc)											
14:0	14.1±2.0	10.8±0.5	17.3±1.4	10.9±2.0	11.6±3.1	10.6±1.2	12.0±0.9	10.7±2.7	15.0	8.8±1.1	7.7±1.6
16:0	16.2±2.2	15.0±0.4	18.9±3.9	11.1±2.3	12.3±3.4	11.4±1.6	10.0±0.2	10.3±1.4	13.2	10.5±0.9	7.7±2.2
16:1	7.4±3.3	5.5±0.5	7.0±2.7	3.2±1.7	4.8±2.4	2.8±0.8	1.9±0.2	2.9±0.7	3.6	3.7±1.2	2.3±1.1
18:1(n-9)	3.2±0.9	2.8±0.1	3.0±0.1	3.3±3.0	2.9±2.8	1.8±0.4	1.7±0.0	1.8±0.2	2.3	1.8±0.2	1.3±0.4
20:1(n-9)	41.0±5.7	47.3±1.4	34.5±5.9	52.8±5.7	50.4±8.2	56.1±1.5	48.7±0.9	53.8±1.8	46.9	52.8±3.4	50.5±2.6
22:1(n-11)	16.4±3.0	17.8±1.5	18.9±1.3	17.4±2.0	16.7±1.8	16.6±2.3	17.2±0.7	19.8±5.7	17.8	20.5±1.6	21.7±3.5

Table 2b

Species	***Rhincalanus gigas***							***Calanus propinquus***						
Stage	CV			F				CV			F			
Season	Autumn	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Autumn	Winter	Spring	Summer
n (ind)	4 (5)	5 (14)	1 (8)	6 (12)	1 (3)	8 (16)	2 (11)	11 (47)	6 (19)	1 (5)	8 (30)	1 (3)	4 (15)	5 (29)
Dry mass (mg)	1.97±0.74	1.07±0.39	0.92	2.09±0.59	2.2	1.61 ± 0.41	1.42/1.98	1.0±0.4	0.8±0.2	0.5	1.8±0.3	2.0	1.2±0.2	1.4±0.8
Total lipid (%DM)	34±9	17±14	n.a.	33±9	25	21 ± 5	22/21	33±13	21±7	24	45±8	37	9±1	25±4
Wax ester (%TL)	82.7±2.5	67.9±10.1	72.4	76.8±10.4	83.9	72.2 ± 4.8	61.9/74.7	-	-	-	-	-	-	-
Fatty acids (%TFA)														
14:0	0.3±0.1	0.8±0.4	1.1	0.7±0.4	0.7	0.9±0.3	1.3/1.1	3.0±1.2	4.3±1.1	2.5	3.1±1.2	3.2	3.4±0.7	2.1±1.4
16:0	2.6±0.6	6.0±2.6	5.6	4.9±3.0	4.0	7.0±3.7	6.1/5.5	18.4±5.0	17.7±0.8	15.0	15.4±1.4	13.9	24.6±5.0	13.5±1.1
16:1(n-7)	15.8±3.0	9.9±2.1	16.4	16.4±1.3	20.9	14.1±4.2	20.0/17.8	4.9±2.8	6.5±0.3	4.8	4.5±0.8	3.5	3.4±1.7	3.9±2.1
16:4(n-1)	6.2±1.0	3.7±1.3	0.7	4.1±1.8	4.4	2.0±0.9	1.0/1.2	0.3±0.3	0.3±0.2	0.6	0.2±0.1	-	0.1±0.1	0.1±0.2
18:1(n-9)	25.3±4.3	17.9±6.9	23.8	22.3±7.2	25.7	22.9±2.2	29.4/25.2	2.6±0.6	3.5±1.0	2.3	3.4±1.1	4.2	2.8±0.9	2.1±1.2
18:1(n-7)	2.5±0.1	3.3±0.4	3.2	2.9±0.7	2.5	3.3±1.0	3.4/2.9	1.5±0.5	1.6±0.2	1.1	1.4±0.3	1.1	2.2±0.1	0.8±0.5
18:2(n-6)	1.7±0.5	2.3±0.4	2.5	2.4±0.4	2.6	3.0±0.7	2.0/2.4	0.9±0.6	2.0±0.1	1.1	1.4±0.4	1.9	1.2±0.4	1.1±0.2
18:4(n-3)	3.0±0.2	4.7±0.5	9.6	5.5±5.8	4.5	9.2±6.9	6.3/7.4	2.3±0.4	4.1±0.8	4.6	2.5±0.9	2.7	0.7±0.3	2.9±1.4
20:1(n-9)	0.4±0.1	0.3±0.2	-	0.6±0.2	0.3	0.7±0.6	0.0/0.3	3.0±0.8	1.8±0.4	2.8	2.9±0.4	2.1	0.6±0.2	2.4±0.9
20:5(n-3)	31.0±4.5	36.8±2.3	23.4	29.0±7.3	24.6	24.0±3.7	16.4/22.9	12.0±1.9	17.5±3.9	19.0	10.7±1.6	7.8	24.0±4.3	9.9±6.1
22:1(n-11)	-	-	-	-	0.2	-	-	17.4±4.7	10.8±1.8	12.8	19.8±3.4	23.8	1.9±2.5	13.0±6.2
22:1(n-9)	-	-	-	-	0.4	-	-	20.3±4.2	10.0±1.8	17.9	21.6±2.2	21.1	2.2±4.1	12.6±7.9
22:6(n-3)	4.7±1.8	10.6±3.8	7.5	6.9±1.9	4.1	8.9±1.7	9.2/7.1	7.9±1.5	13.9±1.4	11.3	7.3±1.5	10.3	30.0±5.8	9.1±5.4
Fatty alcohols (%TFAlc)														
14:0	40.3±5.7	39.3±3.1	54.5	42.2±5.4	53.7	46.7±4.8	61.9/52.6	-	-	-	-	-	-	-
16:0	49.4±2.8	49.1±2.5	40.2	46.5±2.8	38.1	43.6±4.0	33.1/39.1	-	-	-	-	-	-	-
16:1	2.9±1.0	5.1±1.9	2.2	3.9±2.2	4.1	3.7±1.6	1.7/3.6	-	-	-	-	-	-	-
18:1(n-9)	5.0±2.0	3.5±1.1	1.6	4.8±2.6	1.5	2.2±0.5	1.4/1.3	-	-	-	-	-	-	-
20:1(n-9)	2.7±2.1	-	-	-	-	-	-	-	-	-	-	-	-	-
22:1(n-11)	0.4±0.4	-	-	-	-	-	-	-	-	-	-	-	-	-

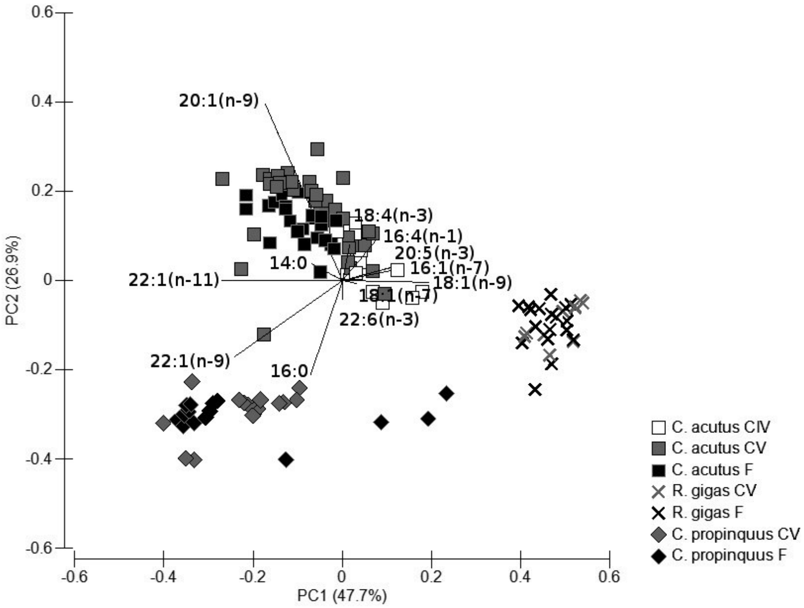

Figure 6. Principal component analysis (PCA) of the fatty acid composition of *Calanoides acutus*, *Rhincalanus gigas* and *Calanus propinquus* (data were arcsine square-root transformed, fatty acids contributing <2% to total fatty acids, and fatty alcohols are excluded).

3.5 Stable Isotopes

Isotopic ratios of $\delta^{15}N$ were negatively correlated with sampling depth in all stages of *C. acutus* in autumn, in copepodids CIV in winter and spring and CV in summer (Fig. 7). Highest $\delta^{15}N$ were measured in copepodids CIV from the surface layer during autumn (9.7 ± 0.5‰, Fig. 7). At 650 to 1250 m depth, overwintering copepodids CIV had a remarkably stable $\delta^{15}N$ ratio of 5.2‰ throughout the year. In *R. gigas*, $\delta^{15}N$ ratios ranged from 2.0‰ in CV during autumn to 7.0‰ in females in spring (Fig. 7). Depth-related trends were less pronounced and variation was especially high in the mesopelagic layer (≈ 400 m) in autumn (1.6 - 7.9‰). Only in spring, $\delta^{15}N$ ratios were positively correlated to sampling depth in adult females (Fig. 7).

Isotopic ratios of $\delta^{15}N$ in actively overwintering *C. propinquus* ranged from 0.3 ± 0.1‰ in copepodids CIV in winter to a maximum of 6.7‰ in an adult female in spring (Fig. 7). $\delta^{15}N$ ratios were positively correlated with sampling depth in copepodids CIV and CV in autumn.

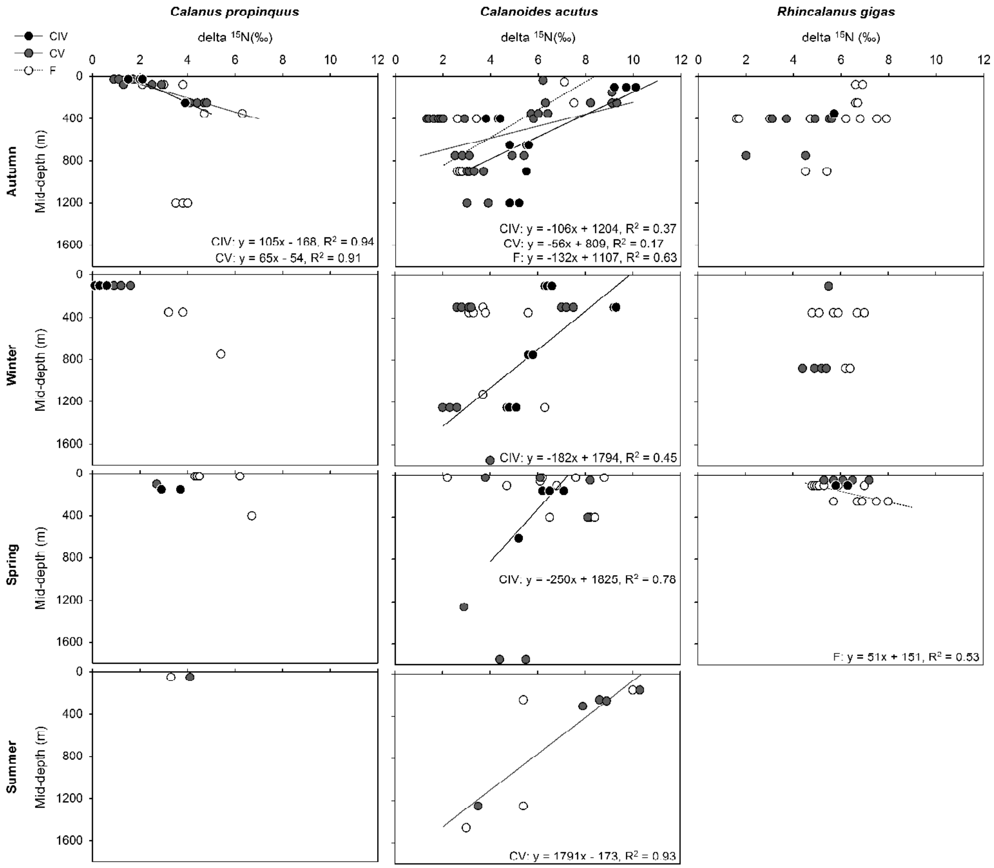

Figure 7. $\delta^{15}N$ (‰) ratios of *Calanoides acutus*, *Calanus propinquus* and *Rhincalanus gigas* at mid-depth of sampling interval (m) in austral autumn (A), winter (B), spring (C) and summer (D) (CIV-CV: copepodite stages 4-5, F: female).

4 Discussion

Herbivorous copepods in polar oceans have to cope with seasonally limited food-supply during the long polar winter. To overcome extended periods of starvation, several species including the Antarctic *Calanoides acutus* and three Arctic *Calanus* species overwinter at depth in a dormant state called diapause, exclusively fuelled by internal energy reserves in the form of lipids (e.g. Conover 1988, Atkinson 1998, Hirche 1998). The mechanisms triggering descent at the end of summer and inducing metabolic depression for diapause are still far from understood.

In a previous study, Sartoris et al. (2010) discovered that diapausing copepod species accumulated large amounts of ammonium ions (NH_4^+) in the haemolymph and postulated that the replacement of cations with a higher molecular weight such as Na^+, Mg^{2+} and Ca^{2+} by low density NH_4^+ may contribute to an energy-efficient buoyancy regulation during diapause.

We (Schründer et al. 2013) showed that ammonium accumulation in diapausing copepods is associated with reduced extracellular pH levels. The distribution and formation of ammonium is strongly linked to the pH. In aqueous solution, ammonia (total ammonia: NH_3/NH_4^+) exists as either ionised NH_4^+ or un-ionised NH_3. Low pH favours the formation of NH_4^+, the less toxic and less diffusive form of ammonia, and, as such, is a prerequisite for the storage of ammonium in large quantities.

In addition, we recorded empirically that diapausing *C. acutus* with high ammonium content had neutral buoyancy without swimming activity during winter, whereas *Calanus propinquus*, which does not store ammonium, was negatively buoyant (Schründer et al. 2014).

In the present study, we focused on seasonal and depth-related variations in cation composition, ammonium content and other ecophysiological and biochemical parameters in order to elucidate how processes of vertical migration, diapause and ammonium accumulation interact during the life cycle of the Antarctic *C. acutus* in contrast to co-occurring copepods *C. propinquus* and *R. gigas* with different adaptive strategies.

Diapausing and non-diapausing copepods differed in their extracellular cation composition. Within the haemolymph of *C. acutus* and *R. gigas*, strongly increased levels of ammonium (NH_4^+) were found, whereas levels of the remaining cations were reduced. Elevated levels of low-density ammonium and minor contributions of heavier ions contribute to buoyancy regulation. Neutral buoyancy provides an advantage during overwintering at greater depth because it reduces energy requirements for constant swimming. In other marine taxa such as squids (Clarke et al. 1979), deep sea shrimps (Sanders and Childress 1988) and protozoan (Denton et al. 1969), the storage of low-density ammonium is a well-known buoyancy mechanism. It provides a cost efficient solution, since ammonium is a waste product of the protein catabolism. Moreover, the buoyancy effect of ammonium is independent of changes in ambient pressure, experienced by copepods during pronounced seasonal vertical migration.

Elevated levels of NH_4^+ were associated with low pH in the haemolymph in both diapausing species. In *R. gigas*, ammonium concentrations were significantly correlated to haemolymph pH in all stages, while in *C. acutus* linear relationships were found in most seasons with the exception of winter (Schründer et al. 2013). The strong causal relationship between pH and ammonium concentration does not necessarily lead to a significant correlation between these properties throughout the year. It is also possible that only a certain threshold in pH must be surpassed for the formation of NH_4^+.

The great variance of ammonium concentrations in our data set may be explained by a combination of inter-annual, spatial and individual variability. We combined data from four different Antarctic expeditions in subsequent years to create a virtual seasonal cycle. Although all expeditions targeted the Weddell Sea, it was not possible to collect samples at exactly the same positions during each cruise. In addition, individual variability in life history between specimens could not be ruled out. The timing and extent of ammonium accumulation as a buoyancy aid are highly individual processes and depend inter alia on the physiological status and the biochemical composition of each individual. Depending on the developmental status and the physiological background, each body component contributes to the overall density of the individual in varying proportions. Moulting, starving, gonad and egg maturation, actually all metabolic processes, change the relative biochemical composition and hence density.

Besides buoyancy regulation, low pH values may also be involved in inducing metabolic depression during diapause. In several invertebrates, for instance brine shrimp *Artemia salina* and snail *Oreohelix* spp., low intracellular pH values are a relevant factor reducing energy turnover and metabolic rates in different body cells and on the organismic level (Busa and

Nuccitelli 1984, Hand and Gnaiger 1988, Hand and Carpenter 1986, Rees and Hand 1990) and potential effects on metabolic processes via changes in extracellular pH have been shown for marine worm *Sipunculus nudus* (Reipschläger and Pörtner 1996). Therefore, it is possible that low extracellular pH conditions in the haemolymph could also trigger dormancy in copepods.

To supplement seasonal trends in haemolymph pH and ammonium concentration, we measured an extensive data set on other physiological and biochemical parameters including respiration rates, lipid content and composition as well as nitrogen stable isotope ratios.

In accordance with previous studies (e.g. Hagen et al. 1993, Kattner and Hagen 1995, Kattner et al. 2012), the three Antarctic copepod species differed in their lipid class composition and lipid storage strategy. While *C. acutus* accumulated wax esters as major lipid class, lipids of *C. propinquus* were dominated by triacylglycerols. *R. gigas* also synthesizes wax esters, but with fatty acids of shorter chain length (Kattner et al. 1994). The different lipid storage strategies have been attributed to differences in life cycle and dietary composition. Wax esters usually serve as long-term energy stores in diapausing species, whereas triacylglycerols cover shorter term energy requirements of *C. propinquus*, which switches to a more carnivorous diet during winter (Metz and Schnack-Schiel 1995, Pasternak and Schnack-Schiel 2001).

Unexpectedly, *C. acutus* from deeper layers (in all stages in autumn, in CIV in winter and spring and CV in summer) had lower $\delta^{15}N$ ratios than conspecifics from the surface. This result was in contrast to other copepods, where $\delta^{15}N$ ratios often increase with increasing depth of occurrence (Laakmann and Auel 2010). Increasing $\delta^{15}N$ ratios with increasing depth of occurrence in zooplankton are usually explained by a decreasing availability of phytoplankton and/or phytodetritus as potential food source at depth and the notion that sinking particulate organic material (POM) is being consumed and reworked several times on its way down, leading to higher $\delta^{15}N$ ratios in deep-sea POM due to repetitive isotopic fractionation. We assume that specific adaptations in the protein and nitrogen catabolism of *C. acutus* related to ammonia accumulation might result in comparatively low $\delta^{15}N$ ratios in overwintering specimens at depth. Further studies are needed to elucidate the metabolic pathways involved in ammonia accumulation and how they affect nitrogen isotopic fractionation in *C. acutus.*

Calanid copepods play important roles in energy transfer in marine ecosystems. They link primary production to higher trophic levels and, via the biological carbon pump, substantially affect biogeochemical cycles and carbon fluxes. In high-latitude ecosystems, global climate change leads to changes in phenology and seasonality. Spring blooms occur earlier due to sooner breakup of sea ice and/or warmer water temperatures earlier in the season (Søreide et al. 2010, Leu et al. 2011). If the termination of diapause and overwintering in dominant herbivorous copepods was determined by internal physiological factors independent from climate change - as indicated by the results of our study - , a shift in the timing of the spring bloom might result in a mismatch between primary production and zooplankton consumption with potential tremendous effects on the pelagic food web. Therefore, it is essential to understand the regulating mechanisms of dominant copepods` life cycles and our results contribute to a better understanding of the ecophysiological factors involved in the regulation of diapause and overwintering.

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4 Synoptic discussion

Herbivorous copepods constitute a key role in the pelagic food web of both polar oceans. Therefore, it is essential to understand the regulating mechanisms of their life cycles to predict ecological consequences of e.g. climate change on the whole polar marine ecosystem. Towards higher latitudes, primary production cycles are limited by the seasonality of light and sea-ice conditions, and herbivorous copepods have adapted to the fluctuating food-supply by different life-cycle strategies. *Calanoides acutus* conducts extensive seasonal vertical migration associated with a diapause at greater depth in the Southern Ocean. Diapause is characterised by reduced metabolic rates and swimming activities and hence neutral buoyancy at overwintering depth. The physiological mechanisms behind buoyancy regulation, as well as the triggering factors terminating migration and the on- and offset of diapause were the subject of this thesis.

The main objectives were to test a set of inter-linked hypotheses on the role of ammonium-regulated buoyancy and an associated decrease of extracellular pH for triggering metabolic depression during diapause. The comprehensive data set results from four Antarctic expeditions. It comprises a species-specific, stage-dependent, as well as seasonal- and depth-related comparison of the extracellular cation composition and haemolymph pH. Supplementary data on physiological and biochemical parameters from the three dominant herbivorous Antarctic copepods *C. acutus*, *Rhincalanus gigas* and *Calanus propinquus* are provided in the context of different life-cycle strategies.

Chapter 1 describes empirical observations on neutral buoyancy in diapausing *C. acutus* during austral winter. Chapter 2 supports novel hypotheses on (i) buoyancy regulation via modification of the extracellular cation composition and (ii) metabolic depression and diapause induction by a reduction of extracellular pH. Chapter 3 integrates a comprehensive seasonal and depth-related comparison of ammonium concentrations and pH levels with other physiological and biochemical parameters to confirm and extend existing knowledge on life-cycle strategies of polar copepods.

4.1 The importance of neutral buoyancy for a successful implementation of diapause

The control of buoyancy and the maintenance of an optimal overwintering depth over a long period of time play a crucial role, driving anatomical, biochemical and behavioral adaptations. Diapausing copepods need to restrain any activities of high energetic expenditure to survive the food-limited winter period without a depletion of their restricted resources. Active swimming has been shown to be responsible for a four- to sevenfold increase of metabolic rate in copepods *Calanus helgolandicus* and *Dioithona oculata* (e.g. Pavlova and Minkina 1987, Svetlichny and Umanskaya 1991, Buskey 1998), and respirations rates of anaesthetised, and hence immobile planktonic crustaceans were about five to six times lower than those of actively swimming specimens (Davenport and Trueman 1985). Hence, mechanisms to obviate or reduce swimming activities to keep energetic demands low are of fundamental importance during diapause. Diapausing individuals slow down their metabolism to a minimum of essential vital functions (Svetlichny and Hubareva 2005) and it is assumed that they remain motionless and neutrally buoyant throughout diapause duration. However, experimental observations of swimming behaviour and buoyancy or density determinations for Antarctic copepods did not exist so far.

The present study contains the first record that diapausing *C. acutus* achieve a state of neutral buoyancy during austral winter and that buoyancy is maintained only by their biochemical composition. In contrast, active-overwintering *C. propinquus* were negatively buoyant (Chapter 1, Schründer et al. 2014). In order to float, diapausing copepods must have the same density as the surrounding seawater. Main contributors to negative buoyancy are proteins and carbohydrates with densities of 1.3 and 1.5 g cm^{-3}, respectively, exceeding the density of seawater (≈1.025 g cm^{-3}). The incorporation of large amounts of low-density lipids is a major contribution to neutral buoyancy and an increasing number of studies focus on the role of low-density lipids for buoyancy regulation during diapause (e.g. Visser & Jónasdóttir 1999, Irigoien 2004). However, a purely lipid-based buoyancy regulation leads to an inherently unstable depth of neutral buoyancy due to the thermal expansion and compressibility of lipids (Visser and Jónasdóttir 1999, Campbell and Dower 2003). It follows, that the maintenance of neutral buoyancy requires at least additional regulation mechanisms.

4.2 Buoyancy regulation by ammonium accumulation and ion replacement

Widely distributed adaptations found in pelagic invertebrates to reduce the overall density are an increase in lipid or water content, a reduction in exoskeleton calcification or the use of gas-filled chambers (Denton and Gilpin-Brown 1961, Denton and Gilpin-Brown 1966, Sanders and Childress 1988). Another approach is the reduction of the density of body fluids by ion replacement, for instance in pelagic deep-water shrimps, cephalopods and phytoplanktonic cells (Denton et al. 1969, Clarke et al. 1979, Sanders and Childress 1988, Boyd and Gradmann 2002). In cranchiid squids and the deep-sea crustacean *Notostomus gibbosus*, nearly all sodium ions (Na^+) are replaced by NH_4^+ in body fluids. The accumulation of up to 500 mmol L^{-1} NH_4^+ and an associated exchange of ions with a higher density results in an uplift of more than 17 mg mL^{-1} and provides neutral buoyancy (Denton et al. 1969, Sanders and Childress 1988).

Within this study, an intense accumulation of ammonium with similar concentrations of up to 530 mmol L^{-1} was found in the haemolymph of both diapausing species *Calanoides acutus* and *Rhincalanus gigas* (Chapter 2 & 3, Schründer et al. 2013, Schründer et al. submitted). The elevated concentrations of NH_4^+ were associated with minor contributions of the remaining cations, in particular Na^+ and divalent Mg^{2+}, Ca^{2+}, hence highlighting a substantial ion replacement. These findings support the idea of a previous study (Sartoris et al. 2010) and establish ion replacement as a buoyancy-regulating mechanism in copepods.

4.3 The inter-linked role of ammonium accumulation and pH reduction

In most marine organisms, including mammals, fish and crustaceans, the concentration of ammonium in body fluids is generally low (50-400 µmol L^{-1}, Weihrauch et al. 2004), due to the potentially toxic effects of its deionised form NH_3. This study extends the hypothesis of ion replacement and ammonium accumulation as a buoyancy aid by the finding that ammonium concentrations were significantly affected by acidity and increased with lower pH values in the haemolymph of diapausing *C. acutus* and *R. gigas* (Chapter 2 & 3, Schründer et al. 2013, Schründer et al. submitted). It proves that pH conditions are kept low to maintain ammonium in the less toxic NH_4^+ ionised form and to avoid a diffusive loss of NH_3.

pH plays a key role in many physiological processes such as enzyme activity, protein function and ion transport (Gattuso and Hansson 2011). Low intracellular pH conditions reduce energy turnover and metabolic rates in several invertebrates, for instance the snail *Oreohelix* spp., and induce dormancy in the brine shrimp *Artemia salina* (Busa and Nuccitelli 1984, Hand and Gnaiger 1988, Hand and Carpenter 1986, Rees and Hand 1990). The modulation of the intracellular pH is a regulated cellular process, which requires a specific energy demand for the ion exchange between intra- and extracellular compartments against an electrochemical gradient (Reipschläger and Pörtner 1996). In contrast, extracellular pH is poorly regulated and underlies proton transfer from the intracellular space depending on the metabolic rate (Pörtner 1993). However, in vitro experiments on isolated body wall musculature of the marine worm *Sipunculus nudus* have shown that low pH_e values support a reduction of ATP (adenosine-5'-triphosphate) turnover by an inhibition of net proton transport across the cell membrane, thus lowering the energy demand of pH_i regulation by a decrease in the overall rate of acid-base regulation and contributing to an energy-saving strategy during metabolic depression (Langenbuch and Pörtner 2002). A decrease of pH_e from a control level of 7.9 to 6.7 resulted in a reduction of the aerobic metabolic rate by 40-45% (Langenbuch and Pörtner 2002). Therefore, it is possible that low pH conditions also act as a trigger for metabolic depression and hence diapause in copepods. The combination of experimental and molecular approaches in future studies will help to investigate whether the importance of pH_e changes for metabolic regulation is as high in diapausing copepods as it was demonstrated for *S. nudus*.

4.4 Seasonal comparison of extracellular cation compositions and pH levels

According to the hypothesis that ammonium accumulation supports neutral buoyancy during diapause and that an associated drop of extracellular pH controls metabolic depression, NH_4^+ accumulation and pH_e reduction should be seasonal phenomena and vary with copepodid development and depth. The expectation would be that ammonium concentrations are highest in overwintering stages at greater depth and that pH conditions are on physiological levels of ≥ 7.8 in active individuals at the surface in spring and summer to prevent metabolic depression. However, ammonium concentrations varied greatly even within individuals of the same species, developmental stage and season, and seasonal as well as depth-related differences were not significant (Chapter 2 & 3, Schründer et al. 2013, Schründer et al. submitted). As already mentioned in Chapter 3, most of the variance can be explained by a combination of individual, inter-annual, and spatial variability. Great variances in ammonium concentration are a common phenomenon in other ammoniacal organisms such as squid (Lipiński and Turoboyski 1983). The extent of ammonium accumulation as a buoyancy-aid is a highly individual process and depends inter alia on the physiological status and the biochemical composition of each individual. During diapause, cessation of feeding and dormancy prevent/minimise compositional changes with regard to water-, ash-, protein-, lipid, carbohydrate-, chitin-, carbon- and nitrogen-content (Donnelly et al. 1994). But at the end of the overwintering period, moulting and reproductive processes have tremendous effects on the relative proportions and volume fractions of all body components. No later than that, the density decreasing effects of ammonium accumulation and ion replacement come into effect. Depending on habitat, latitude and seasonal progress, considerable variation in individual life histories cannot be excluded. Especially during transition periods such as spring, parts of the population might still reside in diapause while others already started awakening. Further bias may occur because samples came from four separate expeditions, and it was not possible to repeat sampling at the same positions.

4.5 The origin and timing of ammonium accumulation

Elevated levels of ammonium as found in diapausing copepod species within this study can have two possible origins. The first potential way is that NH_4^+ is derived from dietary input during the productive season and is accumulated at the surface before copepods start to descend to overwintering depth. The second way is that it originates from the amino-acid catabolism of body proteins during diapause. The degradation of proteins is the major source of nitrogenous compounds in aquatic crustaceans (Weihrauch et al. 2004). However, starvation experiments of Arctic-boreal copepods *Calanus helgolandicus*, *Calanus finmarchicus* and *Calanus glacialis* (Mayor et al. 2011, Tamelander et al. 2006), as well as krill and mysids (Frazer et al. 1997, Gorokhova and Hansson 1999) revealed that non-nitrogenous substrates such as fatty acids are preferably used to meet basal metabolic requirements during starvation, thus saving proteins for growth and reproduction (Roman 1983, Mayor et al. 2011). With regard to the timing of ammonium accumulation, this would mean that most of the NH_4^+ is produced in the active period before descending to overwintering depth. It would at least explain why high levels of ammonium were found throughout the whole year at various depths and not only in overwintering stages at greater depth. However, this sequence would be counterproductive in the process of downward migration, since copepods would descend at times when their ammonium (and lipid) stores and, hence, buoyancy are at maximum.

The timing of OVM and the sequence of physiological adaptations in polar copepods are subject of an ongoing discussion, also with regard to the accumulation of lipids (Visser & Jonasdottir 1999, Irigoien 2004). The lipid stores and, hence, lipid-regulated buoyancy reach a maximum prior to the onset of the downward migration. As a result, instead of sinking passively, copepods need to swim actively until they reach overwintering depth. Similarly, copepods need to ascend in spring with severely reduced lipid levels. Therefore, lipids have often been considered as a barrier for downward migration rather than an aid (Yayanos et al. 1978). An increasing number of studies, however, support the assumption that the physical properties of the accumulated wax esters within the lipid stores play a vital role in the achievement of neutral buoyancy at overwintering depth (Visser and Jónasdóttir 1999, Pond and Tarling 2011, Pond et al. 2012, Pond 2012). Visser and Jónasdóttir (1999) developed a simple three component model to calculate a depth of neutral buoyancy of diapausing *Calanus finmarchicus* in the Faroe-Shetland Channel, based on pressure- and temperature-induced density differences between the accumulated wax esters and the surrounding seawater. They proposed a net effect that wax esters become denser with increasing depth and decreasing water temperature, thus increasing the body density of the individual in order to prevent a hydrostatic lift by the otherwise low-density lipids. Pond and Tarling (2011) and Pond et al. (2012) expand this idea and postulate that wax esters with a certain threshold of >50% unsaturation (with respect to the amount of double bonds between carbon atoms in the WE molecule) are assumed to undergo pressure- and temperature-induced phase transitions from liquid to solid phase at depths ≥500 m. According to Visser and Jónasdóttir (1999), the density difference between seawater at a salinity of 35 and 7°C at the surface and 0°C at 1000 m is 0.0054 g cm^{-3}, whereas the respective density difference for wax esters between the same depths is 0.0193 g cm^{-3}. This would mean that copepods that are positively buoyant at the surface would become neutrally buoyant at greater depths. The effect of on ammonium-containing fluid is probably in a similar range, assuming a density difference of 0.017 g cm^{-3} (ammonium-containing fluid with 1.011 g cm^{-3} at 300 mmol L^{-1} vs. seawater with 1.028 g cm^{-3} at salinity 35 and 0 °C). A combination of both factors, i.e. the increase in wax ester density and low-density ammonium accumulation, could actually represent a possible explanation for the regulatory mechanisms of copepod buoyancy. Both, lipids and ammonium are accumulated during the productive season at the surface and copepods need to swim to a certain depth until a density increase or even a pressure-induced phase-transition of wax esters is achieved. The extent of lipid accumulation and the degree of unsaturation of the wax esters determine the overwintering depth of neutral buoyancy. Lipids are to a certain part utilized at the end of diapause for moulting, growth and reproductive processes so that ammonium accumulation could act as a fine-tuning mechanism and assist in the subsequent upward migration in spring, when lipid levels are severely reduced.

If so, pH reduction needs to precede ammonium accumulation during the active feeding period. Copepods must first reduce their pH to avoid the toxic effects and the diffusive loss of ammonia. A low pH would then represent the trigger for the cessation of feeding and the onset of diapause. This sequence would explain why pH levels did not show a distinct seasonal trend and low pH levels were also found in spring and summer. But it would remain unexplained how copepods with a reduced metabolism and hence swimming activity are able to reach depths of pressure-induced neutral buoyancy, as it is assumed in the above discussed hypothesis. Future studies should focus on a combination of experimental and molecular approaches to actually understand the whole physiological mechanism and sequence of buoyancy regulation.

4.6 Extracellular cation composition and pH in Arctic copepods

In both polar oceans, copepods have developed similar adaptations to the strong seasonality of light and food regime at higher latitudes. In the Arctic, three of the largest copepod species, i.e. *Calanus finmarchicus*, *C. glacialis* and *C. hyperboreus*, are known to perform ontogenetic vertical migration and overwinter in diapause (Conover 1988, Hirche 1998, Kaartvedt 2000, Falk-Petersen et al. 2009). The obvious question is whether ion replacement and pH reduction are potential controlling factors of copepod dormancy and buoyancy regulation also within these boreal and Arctic species.

Indeed, in a recent study (Freese et al. 2015), a clear seasonal pattern of pH_e with values of below pH 6 in boreal winter and higher than pH 7.8 in the surface layers in summer were found in the haemolymph of *C. glacialis*. However, NH_4^+ levels did not follow a seasonal trend and concentrations were generally lower (up to 68.6±20.6 mmol L^{-1}) than in the Antarctic species within this study. Instead, considerable amounts of up to 197 ± 102 mmol L^{-1} of Lithium (Li^+) were found with concentrations being distinctly highest during the overwintering period and lowest when individuals were actively feeding at the surface. The physiological effects of Li^+ could not yet been clarified, but the energy demanding accumulation and a negative correlation to Na^+ concentrations are indicative for a biological function. The authors suggest that Arctic diapausing copepods benefit from the combination of both NH_4^+ and Li^+ accumulation and the replacement of ions with a higher density at the end of the overwintering period by supporting uplift during upward migration (Freese et al. 2015).

The question remains, why a distinct seasonal trend in pH_e was found for Arctic diapausing copepods, but not for Antarctic copepod species in the present study. Within the study of Freese et al. (2015), copepods were sampled in regular time intervals from a seasonally ice-covered fjord in close vicinity to a land-based station. In contrast, samples from the Antarctic study originate from four different expeditions and from transition zones of varying seasonal progress. Hence, profound variations in life-historic backgrounds within the copepod populations cannot be excluded.

5 Conclusions and future perspectives

The present study addresses novel hypotheses about the role of ammonium accumulation and pH reduction as controlling factors for diapause in Antarctic calanoid copepods. It includes the first report that diapausing *Calanoides acutus* achieve a state of neutral buoyancy during austral winter by its biochemical composition alone without the additional need of energy-requiring swimming movements. In contrast, actively overwintering *Calanus propinquus* are characterised by negative buoyancy.

It could be confirmed that diapausing and non-diapausing copepod species differ in their extracellular cation composition. Within the haemolymph of diapausing *C. acutus* and *Rhincalanus gigas*, strongly increased levels of ammonium (NH_4^+) were found, whereas levels of the remaining cations were reduced. The replacement of cations with a higher molecular weight such as Na^+, Mg^{2+} and Ca^{2+} by low density NH_4^+ decreases the overall density of the diapausing copepod and supports the achievement of neutral buoyancy during diapause and upward vertical migration in spring.

This is the first study to show that the accumulation of ammonium ions was related to severely decreased extracellular pH levels of pH ≤6 to favour the formation of less toxic ammonium ions and to prevent diffusive loss. Other studies have shown that low intra- or extracellular pH conditions are responsible for metabolic reduction and the induction of dormancy in a range of invertebrates. Furthermore, in Arctic diapausing *Calanus glacialis*, a seasonal trend of extracellular pH was found, even though cation concentrations were independent from pH changes. Thus, it is possible, that low pH_e levels are not only a precondition for ammonium accumulation and buoyancy regulation, but in addition induce metabolic depression and diapause. To assess the role of low pH_e conditions as a potential trigger of copepod dormancy, future studies should be combined with molecular and experimental approaches to gain more precise information on the physiological processes during diapause.

Future scenarios about CO_2 concentrations in the Earth's atmosphere predict a fourfold increase due to anthropogenic emissions from the beginning of the industrial revolution to the year 2100 (Plattner et al. 2001). Once dissolved in seawater, CO_2 acts as a weak acid, influencing the bicarbonate/carbonate ratio and leading to a decline in seawater pH. Mean pH in surface waters has decreased from approximately 8.2 to 8.1 between pre-industrial time and the 1990s and is expected to reach 7.8 in year 2100 (Gattuso and Lavigne 2009), with severe effects on the acid-base status, calcification and photosynthetic processes of affected organisms (reviewed by Gattuso and Hansson 2011). Even though potential physiological effects of a decrease in extracellular pH levels still need to be further confirmed, it remains astonishing how copepods cope with a reduction of more than 1 pH unit as found in this study. Recent studies investigated the influence of environmental conditions such as increased temperatures (5 and 10°C) and/or elevated pCO_2 of 2300 ppm on the extracellular pH of *C. glacialis* during and at the end of diapause (Tonkes et al. 2015). They did not find any response of pH_e, leading to the assumption that *C. glacialis* is capable of regulating pH_e even at environmental conditions predicted for the end of the century. Only at higher food concentrations, an increase of pH_e was found, indicating that feeding is crucial in the transition from diapause towards activity (Tonkes et al. 2015).

Copepods may remain relatively unaffected from the threat of ocean acidification on a physiological level, but this does not count for changes in food quality, seasonal shifts in food availability etc. Increasing atmospheric CO_2 concentrations, accompanied by increasing temperatures bear the risk of a drastic reduction in sea-ice extent and thickness in polar oceans, altering the onset, duration and magnitude of primary production due to earlier ice break-ups. A temporal mismatch between the timing of phytoplankton blooms and the reproductive cycles of copepods may have dramatic consequences for the entire marine food web (Søreide et al. 2010) and make studies on copepod physiology, adaptive potential and potential implications of climate change on the life cycles of dominant herbivorous copepods an urgent task in polar marine sciences.

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Eidesstattliche Erklärung

(Gem. § 6(5) Nr. 1-3 PromO)

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel:

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