Life in cold oceans: activity dependent on extracellular ion regulation?

Die Rolle der extrazellulären Ionenregulation in der Kältetoleranz mariner Crustaceen

Dissertation
zur Erlangung des akademischen Grades
– Dr. rer. nat –

dem Fachbereich 2 Biologie/Chemie
der Universität Bremen
vorgelegt von

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Diplom-Biologin

Bremen 2010

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Tag des Promotionskolloquiums: 5. November 2010

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ASW	artificial sea water
ASW –Mg^{2+}	artificial sea water with reduced magnesium concentration
ßO_2	oxygen solubility (µmol L^{-1} $Torr^{-1}$)
bpm	beats per minute
CI	crab I, first juvenile instar
CII	crab II, second juvenile instar
C:N ratio	Carbon to nitrogen ratio, is a proxy for the lipid:protein ratio
DW	dry weight
frq	frequency
FW	fresh weight
C_{O_2}	oxygen concentration (µmol L^{-1})
HLP_{O_2}	haemolymph oxygen partial pressure (kPa)
ind	individual
M	megalopa stage
M_{O_2}	oxygen consumption, respiration, metabolic rate (µmolO_2 h^{-1} ind^{-1})
Mya	million years ago
n	number of individuals
n.d.	not determined
NSW	natural sea water
NSW +Mg^{2+}	natural sea water of increased [Mg^{2+}]
P_B	barometric pressure (Torr)
P_{H_2O}	water vapour pressure (Torr)
Q_{10}	temperature-velocity relationship, is a measure of thermal sensitivity
sc	scaphognathite
s.d.	standard deviation
s.e.	standard error
t	time
T	temperature
T0	time point directly after hatching of larvae
V	volume of the respiration chamber (L)
ZI	zoea I, zoeal stage I
ZII	zoea II, zoeal stage II

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Summary

It has been hypothesized that the capacity for extracellular ion regulation of marine crustaceans is a factor that is key in determining their cold tolerance and biogeography in the Southern Ocean. Groups exhibiting low extracellular magnesium concentrations (e.g. caridean shrimps, amphipods and isopods) are thought to be more cold tolerant and able to thrive in extremely cold waters of the Antarctic shelf (-1.8°C). Groups displaying high extracellular magnesium concentrations (brachyuran and anomuran lithodid crabs) are constrained to the warmer regions of the sub-Antarctic and Antarctic (> 0°C). This thesis investigates whether there is a relationship between extracellular ion regulation, activity and thermal tolerance in a temperate brachyuran crab (*Carcinus maenas*, Decapoda, Brachyura, Carcinidae) and in crustaceans from the Southern Ocean. In the sub-Antarctic stone crab *Paralomis granulosa* (Decapoda, Anomura, Lithodidae) lecithotrophic larvae as well as juvenile stages were considered, as thermal tolerance of larval development may strongly influence the distribution of decapod crustaceans. To test the hypothesis that cold tolerance is dependent on haemolymph magnesium concentration, haemolymph magnesium concentration was experimentally altered. Adult specimens were incubated in artificial sea water with a reduced magnesium concentration similar to a level of that of caridean shrimps (6 mmol L^{-1}). Larval *P. granulosa* did not develop in artificial sea water irrespective of the magnesium concentration, thus it was only possible to study effects of increased magnesium concentration (97 mmol L^{-1}).

Haemolymph ion composition was determined in an array of Antarctic and sub-Antarctic crustaceans. Antarctic amphipods hyporegulated extracellular magnesium to the same extent as their temperate counterparts. Antarctic isopods exhibited similarly high haemolymph magnesium concentrations as the sub-Antarctic lithodids. Therefore, high haemolymph magnesium concentration does not represent a constraint for isopods to occur at water temperatures below 0°C.

In early stages of *P. granulosa* effects of low temperature and increased ambient magnesium concentration on survival, developmental time, organic (C, H and N) composition, oxygen consumption, spontaneous and forced activity, heart and ventilation rates as well as haemolymph ion composition were determined. The results suggest that low temperature and high magnesium concentration synergistically impede larval survival and development. The temperature-dependent effect of magnesium was most prominent during forced swimming activity of the zoea I, but was hardly detectable in the resting juvenile. This may indicate that highly active larval stages, despite their higher haemolymph magnesium concentrations, are more susceptible to magnesium than the more advanced life stages.

Thermal tolerance at two ambient magnesium concentrations was determined in adult male specimens of *P. granulosa* using an acute stepwise temperature protocol. Arterial and venous haemolymph oxygen partial pressure, heart rate, ventilation rate and haemolymph cation composition were measured at rest and after a forced activity (righting) trial. The data suggest that within the experimental time frame neither magnesium concentration nor oxygen delivery set limits to cold tolerance in the adult stage of *P. granulosa*. Significantly increased extracellular potassium concentrations after activity at the lowest temperatures, may denote difficulties to maintain cellular potassium homeostasis.

Effects of temperature and reduced magnesium concentration on haemolymph ion composition, spontaneous walking speed and food consumption of the shore crab *C. maenas* were studied using a step-wise temperature protocol. Whereas walking speed was not significantly affected by magnesium concentration, food consumption remained significantly higher at reduced magnesium concentration than under control conditions at low temperature. Acute cold exposure and online recording of arterial oxygen partial pressure, ventilation and heart beat frequency at rest revealed a significant effect of reduced magnesium concentration on ventilation and heart rates, but this translated only into an insignificant increase of arterial oxygen partial pressure at low temperatures. There was no evidence for seasonal effects on haemolymph ion composition in *C. maenas*. In this study, besides the change in food consumption, there was no clear indication for an effect of magnesium concentration on cold tolerance of *C. maenas*.

As seen in isopods, life in the extremely cold continental shelf areas of the Antarctic is generally possible despite high haemolymph magnesium concentrations. The most active first larval stage of *P. granulosa* seems to be most susceptible to magnesium, but it is doubtful that the high haemolymph magnesium concentration of this species plays a role in its geographic distribution. Other decapod crustaceans might however be constrained by low temperature, if their lifecycle includes planktotrophic larvae with a low capacity for magnesium regulation.

Zusammenfassung

Es wurde postuliert, dass die Fähigkeit zur Magnesiumexkretion ein wichtiger Faktor ist, der die Biogeographie mariner Crustaceen im Südlichen Ozean beeinflusst. Crustaceen, die eine hohe Magnesiumkonzentration in der Hämolymphe aufweisen (decapode anomure und brachyure Krebse), kommen nur in wärmeren Gewässern der Subantarktis und Antarktis (> 0°C) vor, während Gruppen, die zur starken Hyporegulation befähigt sind (Amphipoda, Isopoda, decapode Garnelen), auch in den Schelfbereichen der Weddell und Ross Meere abundant sind (-1.8°C). Ziel der Arbeit war es, zu prüfen, ob ein Zusammenhang zwischen der Fähigkeit zur Regulation der extrazellulären Ionenkonzentrationen, dem Aktivitätsniveau und der Kältetoleranz der Tiere besteht. Dies wurde sowohl an der Strandkrabbe *Carcinus maenas* (Decapoda, Brachyura, Carcinidae) aus den gemäßigten Breiten als auch an der subantarktischen Steinkrabbe *Paralomis granulosa* (Decapoda, Anomura, Lithodidae) untersucht. Da die Verbreitung einer Art von der Temperaturtoleranz verschiedener Entwicklungsstadien abhängen kann, wurden auch die lecithotrophen Larvalstadien sowie Juvenilstadien von *P. granulosa* betrachtet. Um zu untersuchen, ob die Kältetoleranz mit der Magnesiumkonzentration der Hämolymphe in Zusammenhang steht, wurde die Magnesiumkonzentration des Seewassers verändert. Die Magnesiumkonzentration der Hämolymphe von adulten Tieren wurde durch Inkubation der Tiere in künstlichem Seewasser mit einer reduzierten Magnesiumkonzentration (6 mmol L^{-1}) auf Werte gebracht, die vergleichbar sind mit denen von Garnelen. Larvale *P. granulosa* entwickelten sich im künstlichen Seewasser nicht. Deshalb wurden an ihnen nur Effekte einer erhöhten Magnesiumkonzentration (97 mmol L^{-1}) untersucht, wofür natürliches Seewasser verwendet werden konnte. Außerdem wurde die Ionenzusammensetzung der Hämolymphe einiger Amphipoden, Isopoden und Lithodiden aus subantarktischen und Antarktischen Gewässern bestimmt.

Die untersuchten antarktischen Amphipodenarten wiesen ähnlich niedrige Hämolymph-Magnesiumkonzentrationen auf wie Arten aus den gemäßigten und subtropischen Breiten. Im Gegensatz dazu lag die Magensiumkonzentration der Hämolymphe bei den betrachteten antarktischen Isopoden im gleichen Bereich wie die der subantarktischen Lithodidenarten. Eine hohe Magnesiumkonzentration schränkt Isopoden also nicht in ihrer Verbreitung auf wärmere Gewässer ein.

Effekte von niedriger Temperatur und erhöhter Magnesiumkonzentration auf Larval- und Juvenilstadien von *P. granulosa* wurden anhand von Überlebens- und Entwicklungsraten, des Gehalts organischer Bestandteile (C, H und N), des Sauerstoff-Verbrauchs, der Spontan- und erzwungenen Schwimmaktivität, der Ventilations- und

Herzschlagfrequenzen und anhand der Ionenzusammensetzung der Hämolymphe bestimmt. Die erhöhte Magnesiumkonzentration und die niedrige Temperatur wirken synergistisch und beeinträchtigen das Überleben und die Entwicklung der Larven. Der temperaturabhängige Effekt von Magnesium wurde in den hochfrequenten Schwimmbewegungen der Zoea I am stärksten ersichtlich, jedoch kaum im inaktiveren Juvenilstadium. Dies könnte bedeuten, dass das erste und aktivste Larvenstadium trotz seiner höheren Hämolymph-Magnesiumkonzentration empfindlicher auf Magnesium reagiert als spätere Stadien.

Der Effekt von Magnesium auf die Temperaturtoleranz von adulten *P. granulosa* wurde anhand des arteriellen und venösen Sauerstoffpartialdrucks, der Herzschlag- und Ventilationsfrequenzen und der Kationenkonzentrationen der Hämolymphe unter Ruhebedingungen und nach erzwungener Aktivität bestimmt. Die Kältetoleranz der Tiere stand weder mit der Sauerstoff-Versorgung noch mit der Magnesiumkonzentration in Zusammenhang. Signifikant erhöhte Kaliumkonzentrationen in der Hämolymphe nach Aktivität in der Kälte könnten ein Hinweis darauf sein, dass die zelluläre Kaliumhomöostase schwerer zu halten war, und könnte die Verlangsamung der Tiere in der Kälte erklären.

Ionenzusammensetzung der Hämolymphe, Spontanaktivität und Nahrungsaufnahme wurden bei *C. maenas* bei verschiedenen Temperaturen und Magnesiumkonzentrationen des Wassers bestimmt. Die Reduktion der Magnesiumkonzentration führte bei niedrigen Temperaturen zwar zu keinem signifikanten Anstieg der Spontanaktivität, die Nahrungsaufnahme war jedoch signifikant höher als bei der Kontrollgruppe. Ventilations- und Herzschlagfrequenz sowie der arterielle Sauerstoffpartialdruck wurden unter Ruhebedingungen abhängig von der Magnesiumkonzentration gemessen, während die Temperatur erniedrigt wurde. Die niedrige Magnesiumkonzentration im Wasser führte zu einem signifikanten Anstieg der Ventilationsfrequenz in der Kälte, jedoch führte dies nur zu einem wenig erhöhten Sauerstoffpartialdruck in der Hämolymphe. Außerdem gab es keine Hinweise auf eine saisonale Änderung der Ionenkonzentrationen der Hämolymphe. Auch bei der adulten Strandkrabbe konnte daher, abgesehen von der veränderten Nahrungsaufnahme, kein deutlicher Zusammenhang zwischen der Magnesiumkonzentration und der Kältetoleranz festgestellt werden.

Da Isopoden in hochantarktischen Gewässern vorkommen, scheint es generell möglich zu sein, trotz hoher Hämolymph-Magnesiumkonzentration die erforderliche Kältetoleranz zu entwickeln. Vor allem die Untersuchungen an adulten *P. granulosa* sprechen dafür, dass die Magnesiumregulation für die Verbreitung dieser Art keine Rolle spielt. Die Beeinträchtigung der Schwimmbewegungen des ersten Larvalstadiums durch Magnesium

könnte jedoch einen Hinweis darauf geben, dass eine fehlende Fähigkeit zur Magnesiumregulation die Verbreitung von Arten mit planktotrophen Larven einschränkt.

1. INTRODUCTION

1.1. Biogeography of crustaceans and the sub-Antarctic and Antarctic thermal regimes

In the Antarctic marine environment the diversity of decapod crustaceans is low compared to the sub-Antarctic (Gorny 1999). More than 130 benthic and pelagic decapod crustacean species are found in the Southern Ocean, but only about 30 species occur south of the Polar Frontal Zone (Figure 1.1). Whereas brachyuran and anomuran crabs are diverse and abundant in tropical, temperate and subpolar continental shelf regions, only 9 species of the anomuran family Lithodidae have been identified to date and brachyuran crabs are missing altogether south of the Polar Frontal Zone (Gorny 1999; Astorga et al. 2003). Therefore, the lithodid crabs represent the southernmost group of "reptant"[1] crabs. Specimens have been found near islands of the Scotia Arc and on the continental shelf of the Western Antarctic Peninsula in the Bellingshausen Sea (Klages et al. 1995; García Raso et al. 2005; Thatje et al. 2008). The most diverse and abundant decapods in the Antarctic are benthic and pelagic shrimps of the infraorders Penaeoidea and Caridea (Gutt et al. 1991; Klages et al. 1995; Gorny 1999; García Raso et al. 2005; Thatje et al. 2005b). In contrast, the peracarid amphipods and isopods are highly abundant and speciose in the Antarctic with over 500 and 400 species, respectively (Brandt 1999; Held 2000; Gutt et al. 2004). The present invertebrate fauna of the Antarctic continental shelf consists mainly of groups that are typical for the deep-sea, like e.g. echinoderms and peracarids (Brandt 1991; Aronson et al. 2007) and includes a large number of eurybathic species (Brey et al. 1996).

The most conspicuous characteristic of the Antarctic benthic environment is its extremely low water temperature, which changes only little with season (Knox 2007; Barnes and Peck 2008). The isolation of the continent by the deep sea, by the Antarctic Circumpolar Current and the Antarctic Convergence (or Polar Front) contribute to the constancy of conditions (Lawver and Gahagan 2003). The Polar Front divides the sub-Antarctic from the Antarctic region at about 50°S and may form an oceanographic barrier (Orsi et al. 1995). At the Polar Front warm water masses from the north (surface temperature ca. 8°C) meet cold

[1] Refers to the former taxon Reptantia, which included the infraorders Brachyura, Anomura, Astacidea and Palinura as opposed to the Natantia, which included the Caridea, Penaeidea and Stenopodidea. Currently the infraorders are divided into the Dendrobranchiata (infraorder Penaeidea) and the Pleocyemata (infraorders Brachyura, Anomura, Caridea, Stenopodidea, Astacidea, Palinura and Thalassinidea) (Kästner 1993). In the present text "reptant" denotes brachyuran and anomuran crabs only, because astacid and palinurid lobsters exhibit low haemolymph magnesium concentrations (Robertson 1949, 1953).

water masses from the south (surface temperature ca. 2°C). Near the bottom this difference in temperature however is less pronounced. At the Polar Front potential seabed temperatures are ca. 2°C on continental shelf (0 – 1000 m), 0 – 2°C on continental slope (1000 - 3000 m) and -1 – 0°C in deep-sea regions (> 3000 m). Potential bottom temperatures in high Antarctic continental shelf areas are highest at the Western Antarctic Peninsula (ca. 1°C) and lowest in the Weddell and Ross Seas (ca. -1.5°C, Clarke et al. 2009). In contrast, in the sub-Antarctic Magellanic Province at the tip of South America and the Falkland Islands water temperature in shelf areas changes seasonally from 2 - 4°C in winter to 9 - 11°C in summer (Boschi 1979; Hoggarth 1993; Lovrich and Vinuesa 1993; Arntz et al. 1999; Boschi 2000; Arkhipkin et al. 2004).

Figure 1.1: Map of Antarctica and the Southern Ocean.
From http://mappery.com/Antarctica, modified by Volker Hein.

1.2. Temperature effects and thermal tolerance

Temperature is thought to be one of the major determinants of the geographical distribution of ectothermic species (Somero 1997; Astorga et al. 2003; Pörtner and Knust 2007; Hall and Thatje 2009; Calosi et al. 2010, Tittensor et al. 2010). Ectothermic animals are not capable to control their body temperature independently of the ambient temperature. Therefore, temperature directly affects physiological rates and the velocity of biochemical reactions in these animals. This usually results in the acceleration of metabolic rate by a variable factor (Q_{10}) of usually between two and four in response to a temperature increase by 10°C (Eckert

et al. 2000). Furthermore, temperature directly influences the structure of biomolecules, which may have implications for membrane fluidity and permeability, which in turn may affect ion and osmoregulatory as well as nervous functions (Hochachka and Somero 2002).

Marine ectotherms display thermal tolerance windows, which match the range and variability of their habitat temperatures (Frederich and Pörtner 2000; Anger 2001; Pörtner 2002; Melzner et al. 2006a,b; Pörtner and Farrell 2008; Schröer et al. 2009; Pörtner 2010). Various life stages of a species may differ in their thermal tolerance range and show adaptations to e.g. seasonal changes in temperature (Anger 2001; Pörtner and Farrell 2008). Thermal tolerance is thought to be set at the highest level of organismal complexity, the whole organism, where the capacity of the cardiorespiratoy system plays a major role (Frederich and Pörtner 2000; Pörtner 2002; Pörtner 2010). Beyond the upper pejus temperature (pejus (*lat.*) = getting worse) the onset of a mismatch between oxygen supply and demand of the tissues during warming leads to progressive loss of performance and finally to the transition to anaerobic metabolism at the critical temperature (oxygen- and capacity-limited thermal tolerance, Figure 1.2). The same has been observed upon cold exposure in various temperate marine species (Sommer et al. 1997; Frederich and Pörtner 2000; Melzner et al. 2006a,b). The capacities of ventilatory and circulatory systems, which facilitate oxygen delivery, are based on properties of the tissues, cells and molecules involved, but in turn are dependent on oxygen supply themselves. Accordingly, acclimatization and adaptation to different temperature regimes is achieved through changes on the tissue, cellular and molecular levels (Tschischka et al. 2000; Hochachka and Somero 2002; Pörtner 2002). Adjustments of both aerobic and anaerobic energy metabolism are involved (Sommer et al. 1997; Tschischka et al. 2000; Sokolova and Pörtner 2001; Sommer and Pörtner 2002; Sokolova and Pörtner 2003). Recent examinations of temperature-dependent changes in arterial haemolymph P_{O_2} in the cold-eurythermal spider crab *Hyas araneus* indicate that cold acclimatization or adaptation of the oxygen supply system may support oxygen delivery even at the lowest experimental temperature (0°C, Walther et al. 2009). However, oxygen limitation may still occur in peripheral organs, presumably due to a low oxygen diffusion velocity at low temperatures, and could not be definitely excluded, because data on venous haemolymph P_{O_2} and tissue oxygenation were missing (Walther et al. 2009; Pörtner 2010).

Exposure of ectotherms to low temperature slows metabolism, growth, ontogenetic development, locomotory activity as well as ventilation and circulation (Frederich et al. 2000a; Frederich and Pörtner 2000; Frederich et al. 2000b; Anger 2001; Bock et al. 2001; Anger et al. 2003; Barnes and Peck 2008). Furthermore, locomotion and the cardiovascular system are controlled by the nervous system, which itself is subjected to thermal effects. Thermal

sensitivity of neuromuscular systems is determined mainly by the function of the neuromuscular synapse, as nerve conduction and direct stimulation of the muscle are less sensitive to temperature (Prosser and Nelson 1981). This may be due to the fact that signal transduction at the synapse is a complex process, which involves enzyme synthesis and function, protein-protein interaction, exocytosis, phosphorylation states of channels and transmitter-receptor binding at pre- and postsynaptic sites (Macdonald 1981; White 1983; Dunn and Mercier 2003). The speed of neuromuscular transmission is primarily influenced by the speed of transmitter release (Millar and Atwood 2004). The reduction of signal transduction velocity at low temperature is based on declined quantal content of the synapse, which is thought to result from the reduction of calcium conductivity at the presynaptic membrane (Dunn and Mercier 2003).

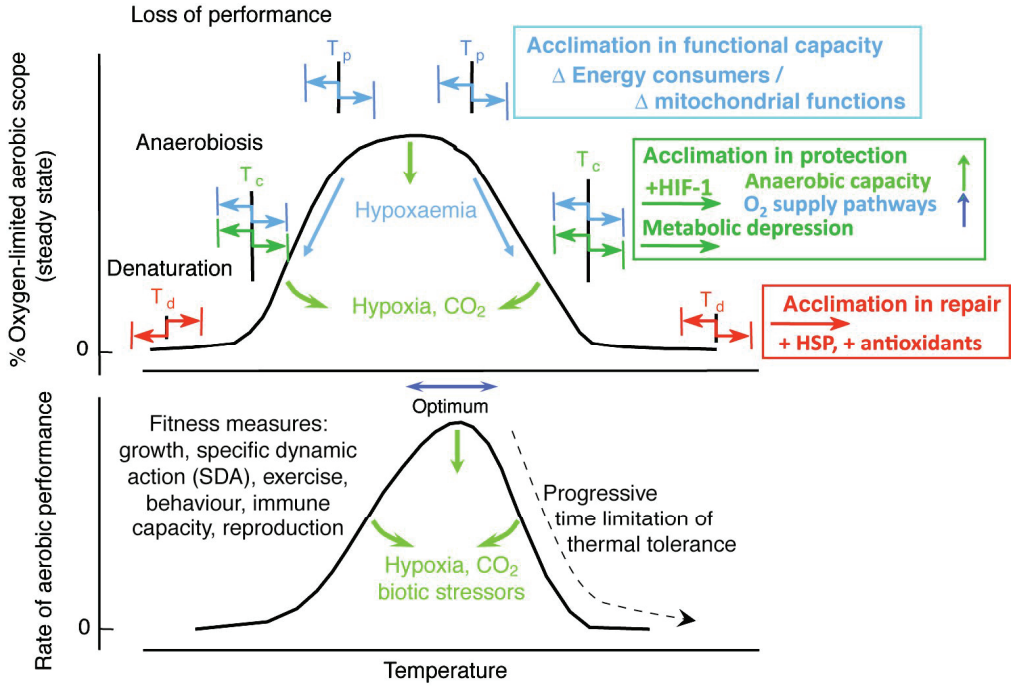

Figure 1.2: Oxygen- and capacity-limited thermal tolerance in ectothermic animals. Optimal oxygen supply of the tissues (determined by measuring oxygenation of body fluids) between pejus thresholds allows for maximal aerobic performance (activity, growth and reproduction etc.) close to the upper pejus temperature. At temperatures beyond pejus temperatures a mismatch between oxygen supply and demand develops (hypoxaemia) and tolerance is time limited, especially once critical temperatures are surpassed and the organism relies on anaerobic energy production. T_p: pejus temperature, pejus (*lat.*): getting worse, T_c: critical temperature, T_d: temperature of denaturation, HIF: hypoxia inducible factor, HSP: heat shock protein, horizontal arrows: shift of optimum and temperature thresholds due to acclimation, green vertical arrows: depression of performance due to biotic and abiotic stressors. From Pörtner (2010).

1.3. The magnesium hypothesis

Magnesium has been known for its anaesthetic effect for more than 50 years (Katz 1936; Waterman 1941; Boardman and Collier 1946; Pantin 1948). Similar to low temperature, it may

act at both pre- and postsynaptic sites by impeding calcium influx during excitation (Hagiwara and Takahashi 1967; Dudel et al. 1982; Iseri and French 1984; Ushio et al. 1993; Parnas et al. 1994). Robertson (1949, 1953) determined the inorganic ion composition of the body fluids in a variety of marine invertebrates and noted that sluggish marine invertebrates, like echinoderms and molluscs exhibit high haemolymph magnesium concentrations, while many decapod crustacean species display low magnesium concentrations. He was the first to suggest a correlation between the haemolymph magnesium concentration and the activity level of decapod crustaceans. Lithodid and majid crabs are generally characterized by a cryptic sluggish lifestyle and high haemolymph magnesium concentrations (20 - 50 mmol L^{-1}). More active shrimps, amphipods and isopods are thought to hyporegulate haemolymph concentration to 5 - 20 mmol L^{-1} (Mantel and Farmer 1983; Tentori and Lockwood 1990; Burton 1995; Frederich 1999). Evidence for a negative relationship between activity and magnesium concentration was later provided by measuring walking and righting speeds, oxygen consumption and the scope for heart rate in decapod and amphipod crustaceans (Walters and Uglow 1981; Morritt and Spicer 1993; Spicer et al. 1994; Sartoris et al. 1997; Watt et al. 1999).

The paralysing effect of magnesium may be even more distinct at low temperature (Lagerspetz and Tiiska 1996). Both high magnesium concentration and low temperature may reduce the calcium conductivity at the synapse and may therefore act synergistically to reduce activity and overall physiological performance. All muscular systems, including those for ventilation and circulation, may be affected by combined effects of these two factors (Frederich 1999; Frederich et al. 2000a,b). According to the concept of oxygen- and capacity-limited thermal tolerance, aerobic performance in species displaying high haemolymph magnesium concentrations may be limited already at higher temperatures than in species with low haemolymph magnesium levels (Figure 1.3). In the cold-eurythermal majid crab *Hyas araneus* righting speed was three times slower at -2°C than at temperatures above 0°C (Frederich et al. 2000b). The experimental reduction of haemolymph magnesium concentration from the natural 50 mmol L^{-1} to 6 mmol L^{-1} resulted in an increase of righting speed at sub-zero temperatures, so that it remained constant in the entire investigated range from -2 to 6.5°C. Furthermore, the lower pejus threshold of the warm-eurythermal spider crab *Maja squinado* was shifted from 8 to 6°C, because reduced haemolymph magnesium concentration induced a threefold increase in cardiac output (Frederich et al. 2000a). Hence, it was hypothesized that crustacean groups displaying a high capacity for magnesium hyporegulation (amphipods, isopods, caridean shrimps) are more cold tolerant than those with a low capacity (anomuran and brachyuran crabs, Figure 1.3; Sartoris et al. 1997; Frederich 1999; Frederich et al. 2000b; Frederich et al. 2001). The ability to regulate

magnesium was proposed to be a major determinant of the biogeography of crustaceans in the Southern Ocean, which seems to be widely accepted today (Thatje et al. 2005a; Aronson et al. 2007; Lovrich et al. 2007). However, cold tolerance and the capability to regulate ions have been investigated in only few subpolar and polar crustaceans (Mackay and Prosser 1970; Foyle and O'Dor 1989; Sartoris and Pörtner 1997a,b; Frederich et al. 2000a,b; McAllen et al. 2005; Webb et al. 2007). Furthermore, none of the southernmost anomuran lithodid crabs had been examined with respect to this hypothesis.

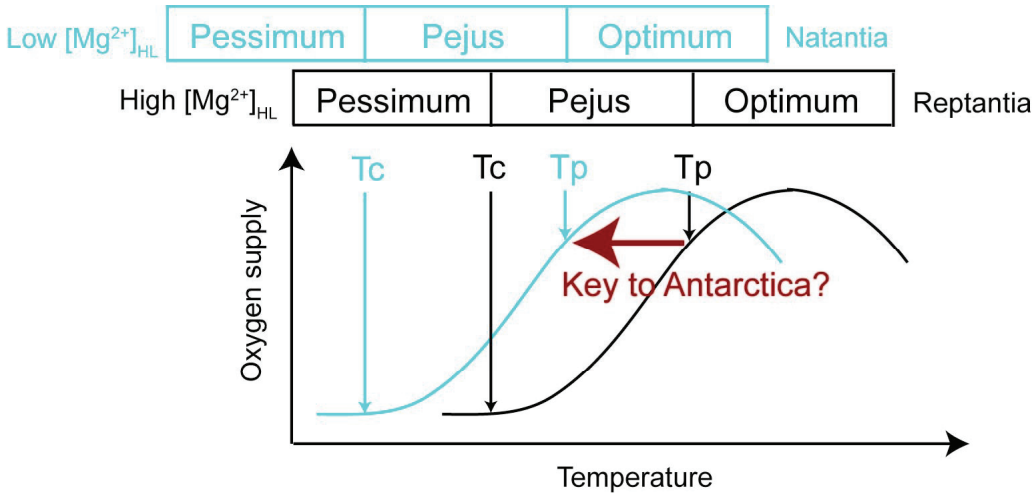

Figure 1.3: Magnesium hypothesis for the biogeography of marine decapod crustaceans in the Southern Ocean.
High haemolymph magnesium concentration leads to anaesthesia at low temperatures and hampers oxygen supply. Hence, anomuran and brachyuran crabs ("Reptantia") are less cold tolerant than caridean shrimp species ("Natantia"). This may explain the absence of "reptant" crabs in extremely cold Antarctic regions. Redrawn after Frederich et al. (2001).

1.4. Ion regulation and thermal tolerance

In marine crustaceans ionic and osmotic regulation and excretion take place across specialized epithelia of the gills and branchial chambers, the antennal glands (maxillary glands in isopods), the gut and the hepatopancreas (Mantel and Farmer 1983; Freire et al. 2008). These epithelia possess large amounts of the enzyme Na^+/K^+-ATPase, which provides a majority of the driving force for transepithelial ion transport (Lucu and Towle 2003; Khodabandeh et al. 2005a). Branchial tissues may facilitate sodium, chloride and calcium regulation. Magnesium and sulphate are excreted along with the urine by the antennal glands (Holliday 1980; Zanders and Martelo 1984). In addition, potassium and calcium are reabsorbed in these organs (Freire et al. 2008). The hepatopancreas is involved in sulphate excretion and is a major site for calcium storage during the moulting cycle (Ahearn 1996; Gerencser et al. 2001).

The mechanism for magnesium excretion in crustaceans is still unknown. Genes for a number of magnesium channels, exchangers and transporters have been identified in bacteria, unicellular eukaryotes, plants and mammals (including humans, Haynes et al. 2002; Gardner 2003; Maguire 2006), but only little information seems to be available for marine vertebrates and invertebrates. In marine fish, divalent ions are excreted via the kidney. Current understanding suggests magnesium entry into renal cells following an electrochemical gradient and subsequent excretion via energy-consuming active transport on the apical side against an electrochemical gradient. There is also evidence for the sequestration of magnesium into vesicles in renal cells of marine fish, which may contribute to magnesium excretion through an endocytosis-exocytosis mechanism. This would allow a high rate of magnesium excretion without endangering housekeeping functions of the cell by high intracellular free magnesium concentrations (Hentschel and Zierold 1994; Chandra et al. 1997; Beyenbach 2000). Magnesium extrusion in excitable invertebrate cells (squid axon and barnacle muscle cells) was postulated to take place via an electroneutral magnesium exchanger and may directly or indirectly involve sodium, potassium and chloride entry. Magnesium transport in these cells is ATP-dependent, but relies on kinase activity (phosphorylation of proteins) rather than ATPase activity (Rasgado-Flores and Gonzalez-Serratos 2000). It remains to be examined whether this mechanism is also employed in crustacean renal cells.

Environmental temperature may influence both passive (permeability of epithelia) and active (ion transport) components of ionic and osmotic regulation, but mechanisms are not well understood (Mantel and Farmer 1983; Burton 1986; Pequeux 1995; Charmantier et al. 2009). As the temperature-velocity relationship (Q_{10}) for passive diffusion of ions is smaller than the Q_{10} for active, ATP-dependent ion transport, at low temperatures ectothermic animals may face the problem that diffusion exceeds ion transport processes. Therefore, the energy consumed for ionic regulation may take up a larger proportion of the metabolic rate in the cold than in the warmth. Alternatively, if this is not completely compensated, animals may lead a sluggish lifestyle in cold regions (Burton 1986; Hochachka 1988). This may not only be crucial for intracellular ion homeostasis, but also for that of the extracellular compartment, because extracellular ion regulation is at least in part driven by transcellular processes (Freire et al. 2008). Effects of temperature may differ dependent on season, size, exposure time and the considered ions. Winter-acclimated individuals of the temperate marine shrimp *Palaemon elegans* are for example more tolerant to low salinities at low temperature than animals collected in spring (Janas and Spicer 2008). With respect to haemolymph magnesium regulation, acute warm or cold exposure can lead to an increase in haemolymph magnesium concentration in caridean shrimp species (Sartoris and Pörtner

1997a,b). Long-term (>24 h) cold exposure resulted in an increase in magnesium concentration if the temperature difference was large in brachyuran crabs, caridean shrimps and amphipods (Zanders and Martelo 1984; Campbell and Jones 1989; Tentori and Lockwood 1990; Spicer et al. 1994), but temperate and arctic caridean shrimps recovered to control levels within 6 days (Sartoris and Pörtner 1997a,b).

1.5. Ontogeny and thermal tolerance

Reproductive and developmental traits have been discussed to play a major role in the adaptation of crustaceans to the (sub-) polar environment (Thatje et al. 2003b). Low temperature and a pronounced seasonality of primary productivity may have selected for species with fewer but larger eggs, lecithotrophy and the abbreviation of larval development (Thorson 1950; Mileikovsky 1971; Clarke 1988; Thatje et al. 2003b). Decapod crustaceans exhibit these characteristics to various degrees (Anger 2001; Zaklan 2002; Thatje et al. 2003b). For example, the sub-Antarctic lithodid crab *Paralomis granulosa* only reproduces biennially and carries few (1000-10000) and large (diameter ca. 2 mm) eggs (Lovrich and Vinuesa 1993, Zaklan 2002, Figure 1.4). Larval development of *P. granulosa* is fully lecithotrophic, so entirely independent of food supply, and abbreviated to only two zoeal stages and a megalopa stage (Campodonico and Guzman 1981; Calcagno et al. 2003; Kattner et al. 2003; Calcagno et al. 2004). Species of the genus *Paralomis* are usually found in continental slope and deep-sea habitats. *P. granulosa* is the only species of this genus, which occurs in shallow water and even in the intertidal of the Magellanic Province (0 – 100 m; Boschi 1979; Hoggarth 1993; Lovrich and Vinuesa 1993; Boschi 2000; Macpherson 2004).

Successive larval stages may have different temperature-dependent growth optima, which may be adapted to seasonal temperature change (Anger 2001). Larvae are considered to be more temperature sensitive. Thus, larval thermal tolerance may represent a bottleneck for species distribution (Frederich 1999; Anger 2001; Anger et al. 2003; Thatje et al. 2003b; Pörtner and Farrell 2008; Hall and Thatje 2009; Storch et al. 2009a,b). In the kelp crab *Taliepus dentatus*, the megalopa stage is the most temperature sensitive amongst the larval stages (Storch et al. 2009b). Mechanisms, which determine thermal tolerance in early developmental stages are currently under investigation. Since the surface to volume ratio decreases substantially during ontogeny, developmental changes in the oxygen supply system (integument and gills, circulatory system, respiratory pigment) and organs and tissues involved in ion regulation (gills, hepatopancreas and antennal glands) may be involved.

It has been hypothesized that the organism is mainly dependent on diffusion during early development. With proceeding ontogeny a cardiorespiratory system develops, which facilitates oxygen and energy supply of the tissues by convective processes (Spicer 1994;

Reiber and Harper 2001; Spicer and Eriksson 2003). Furthermore, evidence suggests that there is a transition of the heart pacemaker from the heart muscle (myogenic) to the cardiac ganglion (neurogenic) during juvenile development in crustaceans (Yamagishi and Hirose 1997; Reiber and Harper 2001). The respiratory pigment haemocyanin is already present in oocytes and capable to bind oxygen in zoeae of *Cancer magister*. However, oxygen affinity is lower in larval than in juvenile and adult haemocyanins (Terwilliger and Dumler 2001; Spicer and Eriksson 2003). Developmental changes in oxygen binding properties complement changes in haemolymph magnesium regulation (high magnesium concentrations increase oxygen affinity of the pigment). This results in the conservation of oxygen-binding properties of the whole haemolymph throughout life history (Brown and Terwilliger 1992, 1998).

The capacity for extracellular osmotic and ionic regulation may change during ontogeny while regulatory epithelia differentiate (Felder et al. 1986; Lignot and Charmantier 2001; Cieluch et al. 2004; Cieluch et al. 2005; Khodabandeh et al. 2005b; Khodabandeh et al. 2006). Osmoregulatory capacity is mainly due to the capability to regulate sodium and chloride concentrations, and is assessed by determining the gradient between external and internal (haemolymph) osmolarity in a salinity cline. It does not in all cases linearly improve with life history stage, but is adapted to the environmental conditions each larval stage faces. Conditions may differ greatly if life history is spent in a number of different habitats, including marine, estuarine, freshwater or even semi-terrestrial environments (Charmantier 1998; Cieluch et al. 2004; Anger et al. 2008; Charmantier et al. 2009). Much less is known about developmental changes in the regulation of single ion species. The haemolymph ion composition in zoeal stages of brachyuran and anomuran crab species has not been studied previously. A progressive decrease in haemolymph magnesium and sulphate concentrations has been observed throughout larval development of the lobster *Homarus gammarus* (Newton and Potts 1993), from megalopa, juvenile to adult stages of the crab *Cancer magister* (Brown and Terwilliger 1992) and from juvenile to adult snow crabs (*Chionoecetes opilio*, Charmantier and Charmantier-Daures 1995). The heavy ion sulphate may influence the buoyancy of larval stages (Newton and Potts 1993). Similar to adults, magnesium may affect the activity level, the capacity of the cardiorespiratory system and thereby thermal tolerance of larvae and juveniles.

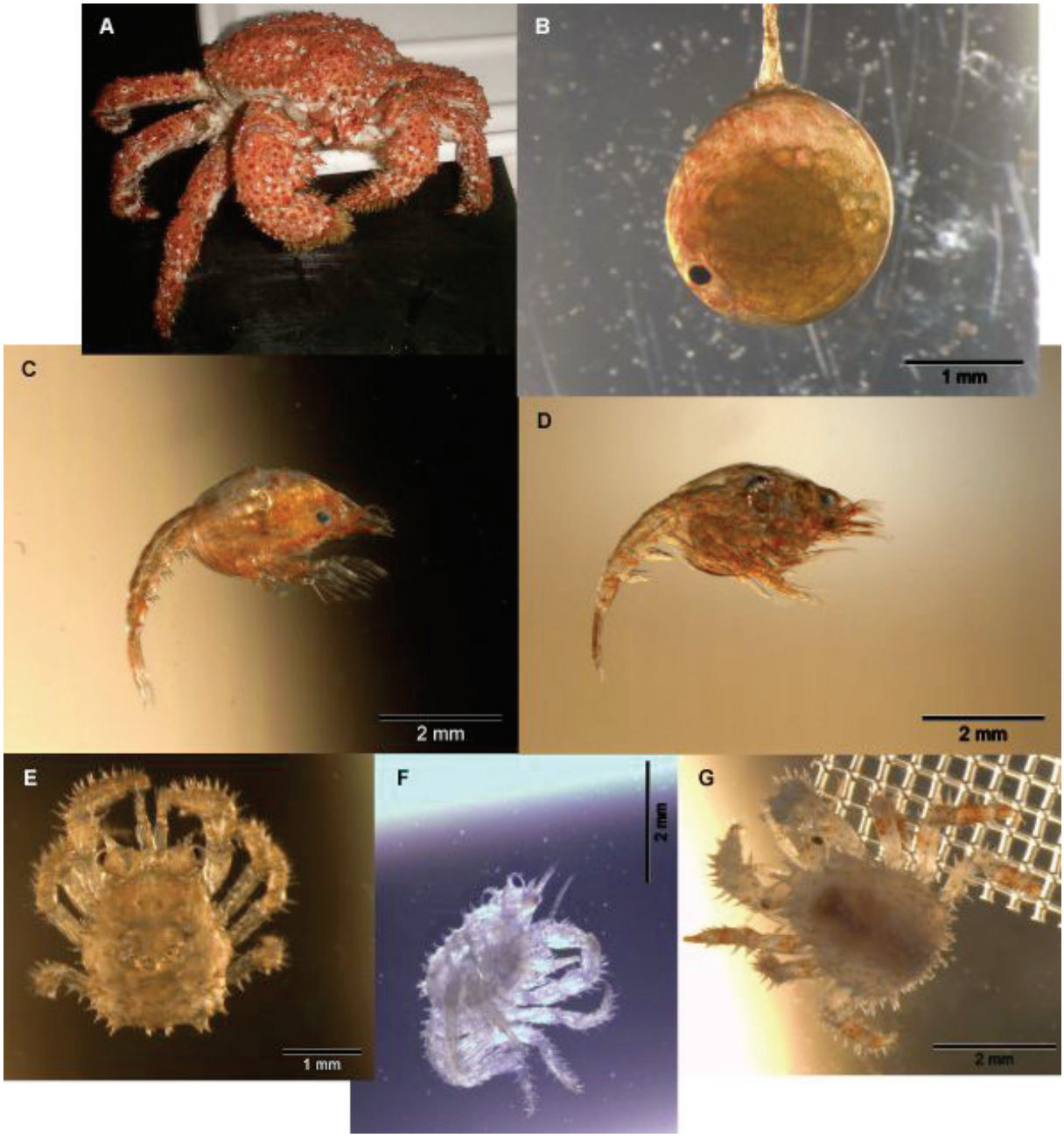

Figure 1.4: Lifecycle of *Paralomis granulosa* (Jaquinot).
A: adult female, carapace length 9 cm, B: advanced embryo, ca. 2 weeks prior to hatching, C: zoea I, third maxilliped without setae, rudimentary pleopods, D: zoea II, third maxilliped bears setae, pleopods developed, E: megalopa, dorsal view, F: megalopa, lateral view, note well-developed pleopods, G: first juvenile instar (crab I). Note the consumption of yolk globules located anterior and lateral to the heart during fully lecithotrophic larval development (C-E).

1.6. Key questions

This study was designed to improve the understanding of the role which extracellular magnesium concentration plays in determining the distribution patterns of crustaceans in the Southern Ocean. As the biogeography of species is greatly impacted by temperature, effects of magnesium on thermal tolerance of multiple life history stages were considered. More specifically the following questions were addressed:

Is there a simple correlation between the extent to which haemolymph magnesium concentration is hyporegulated, cold tolerance and the biogeography of crustaceans from the Southern Ocean?

Inorganic ion composition of the haemolymph of a variety of sub-Antarctic and Antarctic amphipod, isopod and decapod crustaceans was determined and related to the collection sites of the specimens.

Does the capacity for extracellular ion regulation change during ontogeny? Does it relate to the activity levels of the stages? Is inorganic ion homeostasis of the haemolymph affected by temperature?

Ion composition of the haemolymph was studied in early developmental stages of the lithodid crab *Paralomis granulosa*. The results were discussed with respect to changes in activity during development. It was hypothesized that the capacity for magnesium excretion is temperature-dependent. Increased magnesium concentrations may contribute to the slowing of systemic parameters and locomotion in the cold. This was studied on short time scales in adult specimens of the decapods *P. granulosa* and *Carcinus maenas* and on a longer time scale in juvenile *P. granulosa*. Long-term effects are discussed concerning implications for the moulting cycle.

How cold tolerant are lithodid crabs? Is the cold tolerance of early life history stages affected by magnesium concentration?

Larval culture was not successful in artificial sea water, which was used to produce an incubation medium with a low magnesium concentration to affect a reduction of haemolymph magnesium concentration. Thus, effects of elevated magnesium concentration on cold tolerance of early life history stages of *P. granulosa* were studied. Larval survival, duration of development, oxygen consumption, elemental (CHN) composition, dry weight as well as swimming activity, ventilation and heart rates were evaluated during cold exposure at two magnesium concentrations.

Will "reptant" decapods get more cold tolerant if their haemolymph magnesium levels are reduced to those of caridean shrimps?

Potential changes in thermal tolerance due to experimental reduction of haemolymph magnesium concentration were studied in adult *C. maenas* and *P. granulosa* by assessing activity patterns, heart and ventilation rates, oxygen delivery and haemolymph inorganic ion composition.

2. MATERIALS AND METHODS

2.1. Acquisition and maintenance of animals

Lithodes confundens, *Paralomis formosa*, *Paralomis spinosissima* and *Peltarion spinosulum* were collected during the ICEFISH Cruise in June 2004 (http://www.icefish.neu.edu/). Specimens of *Notocrangon antarcticus*, *Eurythenes gryllus*, *Abyssorchomene plebs*, *Eusirus propeperdentatus*, *Glyptonotus antarcticus*, *Natatolana* sp. and *Ceratoserolis trilobitoides* were obtained during Polarstern expedition ANT XXIII/8 in the Antarctic summer 2007. Haemolymph samples were collected either on board (RV Nathaniel B. Palmer or RV Polarstern) or from live animals transported back to the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven, Germany (AWI) after an acclimation period of one week at 0°C and 32.5 PSU.

Male and ovigerous female *Paralomis granulosa* were obtained from local fishermen in Punta Arenas, Chile in April 2008. The animals were transported to the AWI on board RV Polarstern (ANT-XXIV/4) and thereafter kept in a recirculated aquarium system at 4°C, 32.5 PSU and an artificial 12:12 h light:dark cycle. Two females were transferred to the biological laboratory on Helgoland, Germany (BAH) in June and placed in individual flow-through tanks (20 L) at 6°C, 33 PSU and an artificial 12:12 h light:dark cycle. Water temperature was raised to 9°C at 1°C day^{-1} one week after arrival of the animals on Helgoland and subsequently kept constant at this temperature. All laboratory-kept animals were fed *ad libitum* with pieces of mussels, shrimps or isopods.

Freshly hatched larvae of *P. granulosa* were collected every morning in separate sieves (300 µm mesh size), which received overflowing water from the aquaria of each female. Actively swimming larvae were randomly selected and placed in groups of 10 animals in 400 mL glass bowls (diameter 10.5 cm) filled with filtered natural sea water (NSW) or natural sea water to which $MgCl_2*6H_2O$ was added and which was subsequently diluted with deionised water to a salinity of 32.5 PSU (NSW +Mg^{2+}, for sea water ion composition see Table 2.1). Larvae were raised at 1, 4 or 9°C without food and water was changed every other day. The bowls were checked for deaths and moults daily to record survival and developmental time. When the larvae reached the megalopa stage, they were provided with pieces of nylon mesh as a substrate. After metamorphosis to the first juvenile stage, animals were fed with *Artemia* sp. nauplii (Sanders Brine Shrimp Company) and water was changed daily.

Carcinus maenas were collected near Carolinensiel, Germany for a survey of haemolymph ion composition in March 2007 and 2008 at a water temperature of 7°C and in July 2008 at a water temperature of 15°C. Samples were taken right after collection.

Specimens of *C. maenas* used for experiments were obtained from the BAH in February 2007 and 2008 and kept in a recirculated aquarium system at the AWI at 10°C, 32.5 PSU and an artificial 12:12 h light:dark cycle.

Table 2.1: Ion composition (mmol L^{-1}) of incubation media.
NSW: natural sea water, salinity 32.5 PSU, NSW +Mg^{2+}: natural sea water to which $MgCl_2$ * 6 H_2O was added and which was subsequently diluted to 32.5 PSU, ASW: artificial sea water 32.5 PSU (recipe from Langer et al. (2006)), ASW –Mg^{2+}: artificial sea water with reduced magnesium concentration, NaCl was added to keep salinity at 32.5 PSU.

Medium	Na^+	Cl^-	K^+	Mg^{2+}	Ca^{2+}	SO_4^{2-}
NSW	473	538	9.9	51	9.9	27
NSW +Mg^{2+}	383	580	7.7	97	7.8	24
ASW	442	530	9.5	51	10.0	27
ASW –Mg^{2+}	516	530	10.5	5.9	9.6	28

2.2. Haemolymph collection

Adult specimens were blotted dry before haemolymph was withdrawn either with a syringe and hypodermic needle, which was inserted through an arthrodial membrane at the coxa of a walking leg (crabs), in the heart region (*N. antarcticus, E. gryllus, E. propeperdentatus*) or by inserting a pointed glass capillary dorsally into the heart region of the animals (remaining species). A known volume of haemolymph was immediately diluted in 50 µl of deionised water to prevent agglutination.

Larvae and juveniles of *P. granulosa* were superficially dried on filter paper and immersed in mineral oil to prevent evaporation and desiccation. Remaining sea water adhering to the carapace was quickly removed by use of a finely drawn out glass microcapillary. An unknown volume of haemolymph of each individual was drawn either from the heart (zoeae) or from the base of the last fully developed pereiopod (megalopae and juveniles) with a new glass microcapillary, and blown out into a centrifuge tube containing 20 µL of deionised water. All samples were stored at -20°C.

2.3. Ion chromatography

Ion composition of haemolymph and sea water was determined by ion chromatography (ICS-2000, Dionex®, Idstein, Germany) after the dilution of the samples with deionised water. A conductivity cell and a self-regenerating suppressor were used to reduce background conductivity. Cations (Na^+, K^+, Mg^{2+}, Ca^{2+}) were separated on an IonPac® CS16 column with

methane sulphonic acid (30 mmol L^{-1}) as an eluent at a flow rate of 0.36 mL min^{-1} at 40°C. Anions (Cl^-, SO_4^{2-}) were separated on an IonPac® AS11-HC column with potassium hydroxide (30 mmol L^{-1}) as an eluent at a flow rate of 0.30 mL min^{-1} at 30°C. Ion concentrations were calculated in mmol L^{-1} relative to the Dionex® Six Cation-II or Five Anion Standards. As the initial dilution of the haemolymph of larvae and juveniles of *P. granulosa* was unknown, the concentrations of the ions determined in each individual sample were summed and set to 100%. Subsequently, the fractional (%) concentrations of the ions were calculated to allow the comparison of means (publication II).

2.4. Experiments on early developmental stages of *Paralomis granulosa*

Experiments were carried out on several life stages of *P. granulosa* (see Table 2.2). It was not possible to raise larvae in artificial sea water irrespective of the magnesium concentration. Therefore it was decided to carry out all experiments on the early stages only in NSW and NSW +Mg^{2+}.

2.4.1. CHN contents and dry mass

Individual larvae and juveniles of *P. granulosa* were rinsed with deionised water over a 300 µm mesh sieve, blotted dry and stored in weighed out tin cartridges (HEKAtech GmbH, Wegberg, Germany) at -20°C. The samples were freeze-dried for 24 h at 0.05-0.12 mbar using a Christ Alpha 1-4 LDC-1M vacuum drier and subsequently dry weight was determined on a Sartorius 4504MP8 super micro balance. Carbon (C), hydrogen (H) and nitrogen (N) content was measured in a Euro EA 3000 elemental analyser (HEKAtech GmbH, Wegberg, Germany) using acetanilide as a standard. C:N ratios were calculated as a proxy for changes in the lipid:protein ratio during development dependent on temperature and magnesium concentration. The sum of C, H and N was subtracted from the dry weight to evaluate the build-up of other, mainly inorganic, compounds.

2.4.2. Oxygen consumption

Oxygen consumption measurements of individual larvae and juveniles of *P. granulosa* were carried out in 5-mL Hamilton syringes (Hamilton Bonaduz AG, Bonaduz, Switzerland), similar to the method established by Thatje et al. (2003a). Air saturation was recorded using needle-type fibre-optic oxygen micro-optodes connected to a Microx TX3 unit (Presens GmbH, Regensburg, Germany). The temperature-compensated calibration of the optodes was carried out using a saturated ascorbic acid solution (0% air saturation) and water vapour (100% air saturation) prior to measurements. Single individuals were introduced into each syringe and the water volume was adjusted to 300 µl by carefully moving the plunger.

Keeping the syringe submerged in sea water, the side of the cannula was sealed with a rubber septum. The needle of the oxygen sensor was inserted through this septum and the tip of the sensor was positioned in the middle of the chamber. Syringes were placed upside down in Erlenmeyer flasks filled with air-saturated filtered sea water. The flasks were kept in a temperature-controlled bath containing cooling fluid. Oxygen depletion in the chamber was recorded down to only 80% air saturation to keep stress and CO_2 accumulation at a minimum. Blanks were run before and after the experiments to evaluate microbial respiration. Oxygen consumption (MO_2, $\mu molO_2\ h^{-1}\ individual^{-1}$) was calculated from the slopes of the readings using the following equations:

$$MO_2 = \Delta CO_2\ V\ \Delta t^{-1}$$

$$CO_2 = (P_B - PH_2O)\ 0.2095\ ßO_2$$

With

CO_2: oxygen concentration ($\mu mol\ L^{-1}$)

V: volume of the respiration chamber (L)

t: time (h)

P_B: barometric pressure (Torr)

PH_2O: water vapour pressure (Torr), from Boutilier et al. 1984

$ßO_2$: oxygen solubility ($\mu mol\ L^{-1}\ Torr^{-1}$), from Boutilier et al. 1984.

2.4.3. Spontaneous swimming activity of zoeal stages at culture temperatures

The culture bowls were filled with sea water to about 1 cm water depth so that vertical movements of the larvae were greatly excluded, and placed on a piece of laminated graph paper. Groups of up to five larvae of each female were placed in separate bowls and allowed to acclimate overnight. Watching each larva, the number of 5 mm grids passed by the animal within 5 min was counted. Afterwards, the larva was removed to prevent counting the same animal twice.

2.4.4. Activity of zoea I and crab I during acute cold exposure

Zoeae and first instar juveniles reared at 9°C in either NSW or NSW $+Mg^{2+}$ were fixed in a temperature-controlled microchamber underneath a stereomicroscope (Olympus SZX16) equipped with a digital video camera (Olympus DP71) and software Cell D (Soft Imaging System) similar to the method described in Storch et al. (2009b). The animals were superglued to a pointed glass microcapillary attached to a small glass table, which was placed to the centre of the micro chamber filled with the respective culture sea water. Care was taken to fix an individual to the capillary at the dorsal side of the cephalothorax slightly anterior to the heart region, which allowed the animals to freely move their appendages.

Underneath the stereomicroscope the animals were observed laterally with the heart, scaphognathite, first maxillipeds (zoea I) and antennules (crab I) being clearly visible. In the evening, the animals were placed in the chamber, which was covered with a piece of cloth to prevent visual disturbance of the animal and left to acclimate to the experimental control conditions at 9°C overnight (at least 12 h). The next day, the chamber was sequentially cooled to 6, 3, 1 and -1°C, with each temperature change taking 30 min and each acclimation period taking 90 min at the respective temperature steps. At the end of each acclimation period, beginning at the control temperature of 9°C a 2 min video was recorded at 30 frames s^{-1}. All heart, scaphognathite, maxilliped and antennule beats during the 2 min videos were counted manually and are given as beats s^{-1}.

Table 2.2: Overview of experiments and of parameters determined in the respective developmental stages of *P. granulosa*.
All measurements were undertaken during intermoult. NSW: natural sea water, NSW +Mg^{2+}: sea water with increased magnesium concentration, ASW –Mg^{2+}: artificial sea water with reduced magnesium concentration (see Table 2.1 for ion composition). T: temperature, ZI: zoea I, ZII: zoea II, M: megalopa, CI: crab I, CII: crab II, A: adult, frq: frequency, *animals were raised at 9°C and acclimated to 1 or 4°C for 2 wk prior to measurement.

Determined parameters	Conditions		ZI	ZII	M	CI	CII	A
	T (°C)	Culture media						
Survival & developmental time	1	NSW & NSW +Mg^{2+}	x	x				
	4	NSW	x	x				
	9	NSW & NSW +Mg^{2+}	x	x	x			
CHN composition	1	NSW	x	x	x			
	1	NSW +Mg^{2+}	x					
	4	NSW	x	x	x			
	9	NSW & NSW +Mg^{2+}	x	x	x	x		
Oxygen consumption	1	NSW	x	x	x	x*		
	1	NSW +Mg^{2+}	x					
	4	NSW	x	x	x	x*		
	9	NSW & NSW +Mg^{2+}	x	x	x	x		
Haemolymph ion composition	4	NSW						x
	9	NSW	x	x	x	x	x	
Haemolymph [Mg^{2+}]	9	NSW +Mg^{2+}	x			x		
Haemolymph ion composition after 3 wk cold exposure	1	NSW				x		
	4	NSW				x		
	9	NSW				x		
Spontaneous swimming activity at culture temperatures	1	NSW & NSW +Mg^{2+}	x	x				
	4	NSW	x	x				
	4	NSW +Mg^{2+}	x					
	9	NSW & NSW +Mg^{2+}	x	x				
Activity rates during acute cold exposure:								
Heart beat frq	-1, 1, 3, 6, 9	NSW & NSW +Mg^{2+}	x			x		
Scaphognathite beat frq	-1, 1, 3, 6, 9	NSW & NSW +Mg^{2+}	x			x		
Maxilliped beat frq	-1, 1, 3, 6, 9	NSW & NSW +Mg^{2+}	x					
Antennule beat frq	-1, 1, 3, 6, 9	NSW & NSW +Mg^{2+}				x		

2.5. Cardiorespiratory physiology and activity of adult *P. granulosa*

2.5.1. Preparation of animals and experimental protocol

For repeated measurements of arterial haemolymph P_{O_2}, a small hole was drilled through the carapace above the pericardial sinus without injuring the hypodermis (Figure 2.1 D). The hole was covered with a piece of latex dam. After a recovery period of three days one animal at a time was examined in a 100-L-experimental chamber (Figure 2.1 A). The individuals were fixed to a plastic grid by two pereiopods and the chelipeds to immobilize them. A plethysmograph infrared sensor (iSiTec, Bremerhaven, Germany; Depledge and Andersen, 1990) was superglued to the carapace posterior to the latex dam cover to monitor heart frequencies. Additional sensors were placed above each scaphognathite to record ventilation frequencies (Figure 2.1 C, D). After an acclimation period of 24 h at 4°C in the experimental chamber filled with NSW, haemolymph was withdrawn for the determination of arterial and venous haemolymph P_{O_2} and cation composition at rest. The animal was released from the plastic grid and a righting trial was carried out. Subsequently another set of haemolymph samples was taken. The animal was again fixed to the plastic grid and given 60 min to recover from handling stress, before the ramp to the next temperature step was started.

Water temperature was controlled with a thermostat (Lauda T1200) and Wintherm software (version 2.2). The temperature was reduced at a rate of 1°C h^{-1} to 1°C. After an acclimation period of 20 h measurements and sampling were repeated as stated above. The same procedure was used to collect data at -1°C after 20 h of acclimation. Then temperature was returned by 1°C h^{-1} to 4°C. The animal was released from the experimental chamber for two days and water was exchanged. In the second week the same animal was reintroduced into the chamber filled with NSW at 4°C and the protocol was repeated for the successive temperature steps 7, 10 and 13°C.

To bring haemolymph magnesium concentration down to a level similar to that in caridean shrimps, each animal was first transferred to a separate recirculated system at 4°C filled with artificial sea water with reduced magnesium concentration (ASW $-Mg^{2+}$, see Table 2.1 for ionic composition) for 6 days, until haemolymph magnesium concentration had reached a new and constant value (Figure 2.2). Subsequently, each animal was again examined in the experimental chamber filled with ASW $-Mg^{2+}$ at 4, 1 and -1°C with 20 h intervals for acclimation in between using the same protocol as above. All animals survived experimentation.

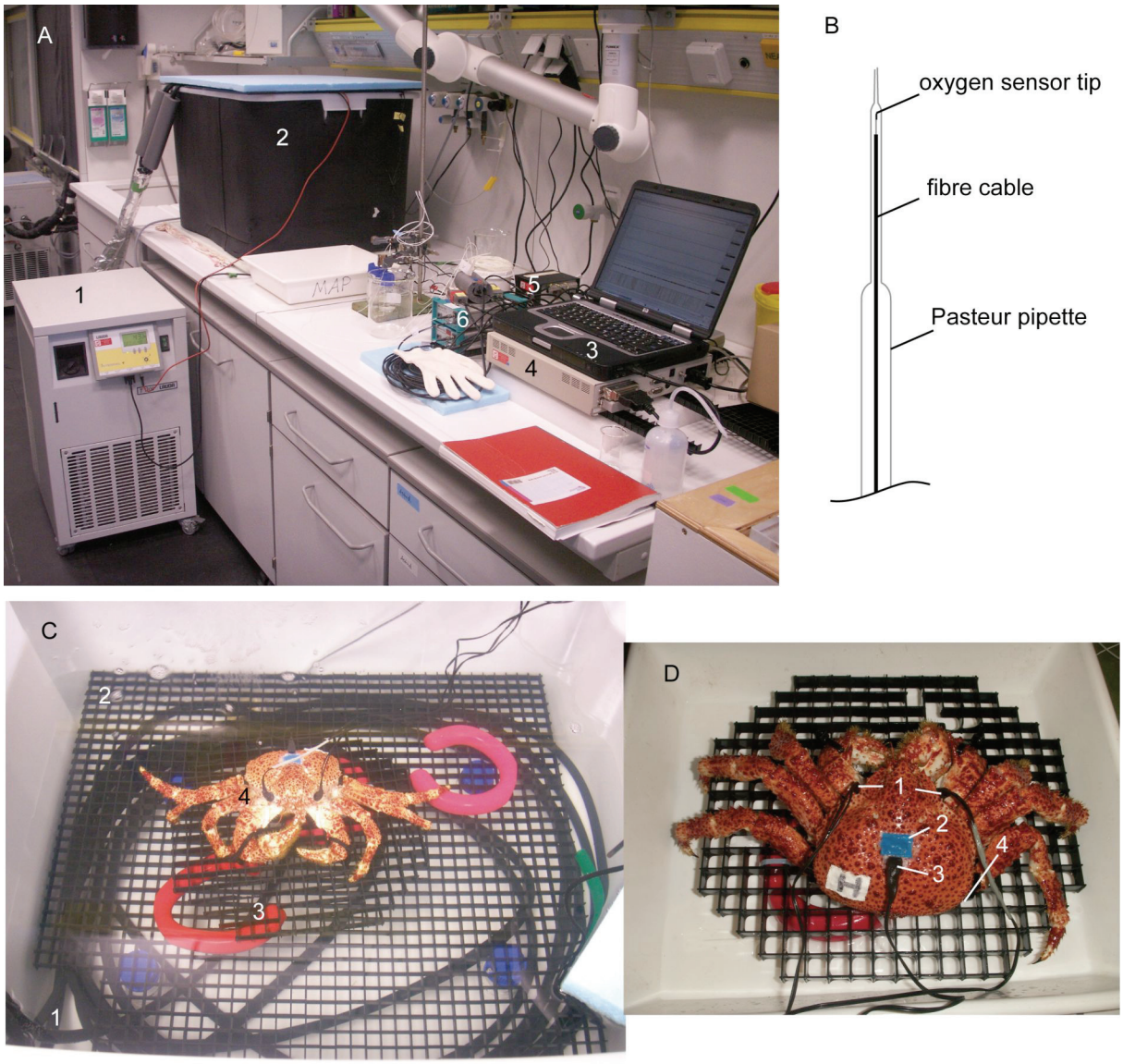

Figure 2.1: Experimental setup for measurement of temperature-dependent cardiorespiratory parameters.
A: Setup in the laboratory. 1 thermostat, 2 incubation chamber (100 L), 3 computer, 4 PowerLab, 5 amplifier for infrared phototransducers (plethysmograph), 6 Microx TX3 oxygen meters. B: Implantable oxygen fibre-optic microsensor housed in a drawn-out glass Pasteur pipette. C: View into the incubation chamber. 1 hose for refrigerant circulation, 2 supportive plastic grid, 3 plastic grid to which the animals is fixed, 4 experimental animal, CL approximately 10 cm. D: Positions of infrared sensors and haemolymph withdrawal. 1 sensors for scaphognathite beat frequency (ventilation) recordings, 2 latex dam covering hole in carapace giving access to pericardial sinus (arterial haemolymph), 3 sensor for heart beat frequency recording, 4 base of last fully developed pereiopod, location for withdrawal of venous haemolymph.

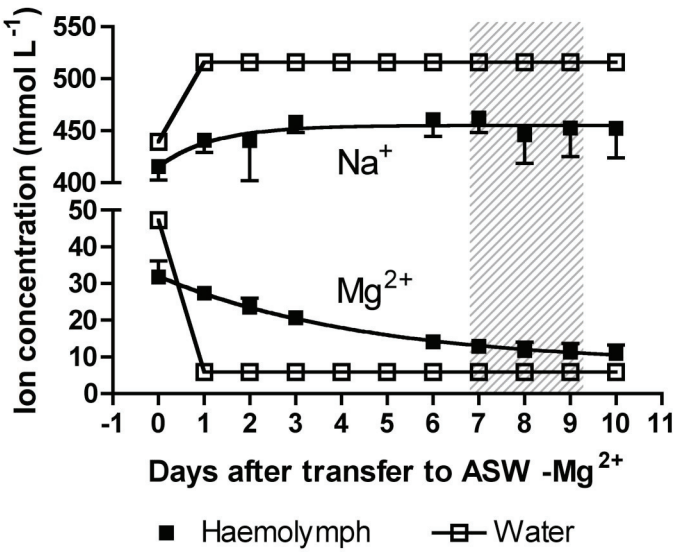

Figure 2.2: Na^+ and Mg^{2+} concentrations (mmol L^{-1}) of 4°C-acclimated adult *P. granulosa* incubated in ASW –Mg^{2+}.
Time course of equilibration with the external medium and reduction of haemolymph magnesium concentration, which was a prerequisite for the experiment. Values are means ± s.d. of 8 individuals. If error bars are not visible, they are smaller than the plot symbols. Day 0: Animals kept in NSW. Shaded area: experimental exposure to 4, 1 and -1°C on days 7, 8 and 9, respectively.

2.5.2. Haemolymph P_{O_2}

Haemolymph P_{O_2} was measured using oxygen optodes (sensor type IMP-PSt1-L5-LIC0-BFG3-TF-YOP), connected to Microx TX3-AOT instruments with Oxyview TX3-v6.02 Software (PreSens, Regensburg, Germany). Temperature-compensated two-point calibration was carried out daily in water vapour (100% air saturation) and saturated sodium sulphite solution (0% air saturation) prior to measurements. Drawn-out Pasteur pipettes equipped with oxygen sensors (Figure 2.1 B) were used to withdraw haemolymph from the pericardial sinus (arterial haemolymph) or from the ventral sinus at the base of a pereiopod (venous haemolymph). After haemolymph collection (up to 10 µl), the tip of the pipette was immediately plugged with dental wax and submerged in the experimental chamber to control the temperature during the measurement. Data were recorded within 5 min after haemolymph collection, before clotting or air diffusion could have obscured the results.

2.5.3. Righting response

The animals were detached from the plastic grid and allowed to recover for 10 min. They were turned to the dorsal side of the carapace and placed on the bottom of the experimental chamber. The time (s) was recorded until the animals had returned to an upright position and all of the pereiopods touched the ground. This was done seven times with 30 s intervals between trials, unless the animals did not right themselves twice consecutively within 180 s.

The mean time-to-right was calculated as the mean of all seven bouts recorded in each animal.

2.5.4. Analyses of heart and scaphognathite activity

A PowerLab system with Chart v5.5.6 Software (AD Instruments, Spechbach, Germany) was used for the continuous recording of heart and ventilation rate. The peak detection routine of the programme was employed to determine heart and ventilation rates (bpm). Average heart and scaphognathite frequencies at rest were determined over 30 min immediately prior to taking the first set of haemolymph samples. Uninterrupted heart and scaphognathite frequencies at rest were determined over 20 - 60 s intervals within the 30 min period prior to the first haemolymph sampling. After the righting trial, heart and scaphognathite frequencies were determined over 10 min immediately after the animals had been reintroduced into the experimental chamber after taking the second set of haemolymph samples. The ventilation rate of each animal was calculated as the mean of the left and right scaphognathite beat frequencies. The frequency of the pauses in heart beat and ventilation at rest (bpm) were calculated by subtracting the rates at rest from the rates at rest excluding pauses. The scopes for heart and ventilation rates (bpm) were calculated by subtracting the minimal rates at rest from the maximal rates recorded after the righting trial.

2.6. Experiments on *Carcinus maenas*

2.6.1. Spontaneous walking activity, food consumption and extracellular ion regulation

Intermolt male crabs of 30 - 90 g body weight were incubated at control (ASW) or reduced sea water magnesium concentration (ASW –Mg^{2+}, see Table 2.1 for ion composition). After an acclimation period of 3 d to the respective medium at 10°C (acclimation temperature, Figure 2.2), temperature was lowered in a stepwise procedure at a rate of 1°C per hour. At each temperature step (10, 7, 5, 3, 1°C) animals were incubated for 20 h, then spontaneous walking activity was recorded by use of a time lapse video recording system at night time when animals are most active (Naylor 1958). Up to 8 animals were observed at a time at a maximum of 3 animals per tank. The next morning, small amounts (up to 10 µl) of haemolymph were taken from each animal by puncturing the arthrodial membrane at the basis of a walking leg with a finely drawn out glass Pasteur pipette. Afterwards animals were fed with a weighed amount of thawed *Cerastoderma edule* and temperature was lowered further. The next day the remains of the food were collected and weighed.

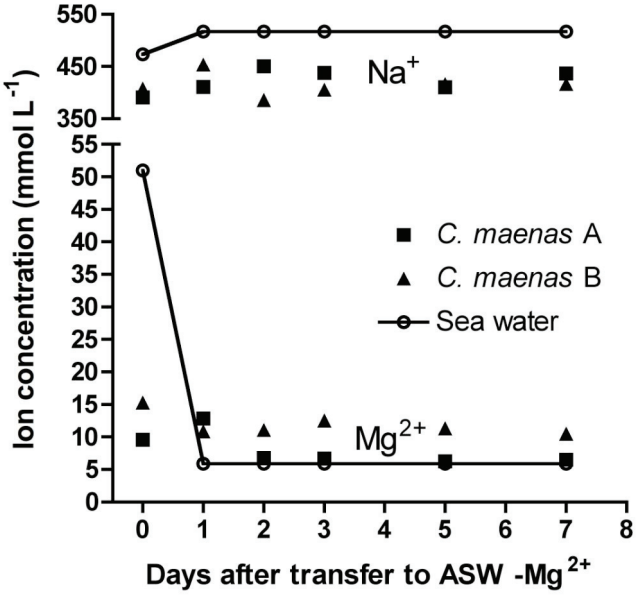

Figure 2.3: Na^+ and Mg^{2+} concentrations (mmol L^{-1}) of adult *C. maenas* incubated in ASW –Mg^{2+} at 10°C.
Time course of equilibration with the external medium and reduction of haemolymph magnesium concentration, which was a prerequisite for the experiment. Values are observations of two individuals (A and B). Day 0: animals kept in NSW.

2.6.2. Cardiorespiratory physiology of *C. maenas*

A small hole was drilled through the carapace above the pericardial sinus, another one about 5 mm posterior to this hole above the posterior aorta. After a 3-d-preincubation period in ASW or ASW –Mg^{2+} the unfed animals were fixed to a plastic grid. For continuous recording of arterial HLP_{O_2} a calibrated (see above) and heparinised (10 MU) needle-type oxygen optode connected to a Microx TX3 unit (Presens GmbH, Regensburg, Germany) was implanted to the pericardial sinus of the animals. A laser-Doppler sensor (Periflux System 5000 PF5010 Laser Doppler Perfusion Monitor, Perimed AB, Stockholm, Sweden) was fixed above the posterior aorta to record heart rate. Two plethysmograph infrared sensors (iSiTec, Bremerhaven, Germany) were attached to the carapace above the scaphognathites to record ventilation rate. Two animals at a time were introduced to the temperature-controlled incubation chamber and were left for 24 h to acclimate. An acute temperature ramp was run from 10°C to 0°C and back to 10°C at 1°C h^{-1}. Continuous data recording and analysis of 6 min (= 0.1°C) intervals were carried out as described above for adult *P. granulosa*.

2.7. Statistics

Before calculating means and standard deviation (s.d.) or standard errors (s.e.) outliers were removed after identifying them at the 95% significance level using Nalimov's test. Statistical

analyses at the $p < 0.05$ level as well as regression and Pearson correlation analyses were carried out using Graph Pad Prism 4.0a.

One-way ANOVA and post hoc Dunett's multiple comparison tests were used to compare means of percentages of ions in haemolymph of sub-Antarctic and Antarctic crustaceans with those of sea water (always 100%, publication I).

One-way ANOVA and post hoc Tukey's multiple comparison tests were run to identify significant changes of haemolymph composition during ontogeny of *P. granulosa* and upon cold exposure of crab I (publication II). Two-way ANOVA and Bonferroni post-tests were performed to analyse spontaneous swimming speed of zoeal stages I and II. Two-way ANOVA for repeated measures and Bonferroni post-tests were used to analyse heart rates, scaphognathite beat rates and maxilliped/antennule beat rate of zoea I and crab I, respectively. An F-test was used to identify significant effects of magnesium concentration on slopes and elevations of linear regressions fitted to the heart rates of zoea I and crab I.

One-way ANOVA and post hoc Tukey's multiple comparison tests were carried out to identify significant effects of temperature on physiological parameters as well as righting speeds of adult *P. granulosa* (publication III). In case the data did not fulfil the prerequisite of equal variances for this test, the nonparametric Kruskal-Wallis and a subsequent Dunn's multiple comparison tests were used. Two-way ANOVA and Bonferroni post-tests were performed to analyse effects of temperature, magnesium concentration and righting activity. An F-test was used to identify significant effects of righting activity on slopes and elevations of linear regressions fitted to the heart and ventilation rates. Discontinuities in heart and ventilation rates were identified according to (Yeager and Ultsch 1989). Resulting regressions were tested for significant differences using an F-test (Nickerson et al. 1989).

Differences in larval survival were identified by use of a Chi-square test on the raw data (publication IV). Survival (number of individuals which moulted to the next stage) is given as the percentage of the number of individuals at the beginning of the experiment. Two-way ANOVA and subsequent Bonferroni tests were carried out on dry weight, biochemical (CHN) composition, C:N ratios, oxygen consumption and duration of development of early stages of *P. granulosa* as well as on walking activity, food consumption and physiological parameters of *C. maenas* dependent on temperature and magnesium concentration (*C. maenas*: additional results). Prior to analysis C:N ratios were transformed using an arc sine function. One-way ANOVA and post hoc Tukey's multiple comparison tests were performed on temperature-dependent haemolymph ion composition of *C. maenas.*

3. PUBLICATIONS

List of publications and my contribution towards them:

Publication I

Wittmann AC, Held C, Pörtner, HO, Sartoris FJ (2010) Ion regulatory capacity and the biogeography of Crustacea at high southern latitudes, Polar Biology 33: 919-928, DOI 10.1007/s00300-010-0768-1

The concept of this study was developed by FJS and myself. I carried out the measurements and analysed the data. I wrote the manuscript, which was revised together with FJS, CH and HOP.

Publication II

Wittmann AC, Storch D, Anger K, Pörtner HO, Sartoris FJ, Temperature-dependent activity in early life stages of the stone crab *Paralomis granulosa* (Decapoda, Anomura, Lithodidae): a role for ionic and magnesium regulation?, submitted to JEMBE

The concept and design of this study were developed by myself and FJS in cooperation with DS and KA. I conducted the experiments and the analysis of the data. The manuscript was written by myself and revised by FJS, DS, KA and HOP.

Publication III

Wittmann AC, Pörtner HO, Sartoris FJ, The role of oxygen delivery and extracellular magnesium in thermal tolerance of the sub-Antarctic stone crab *Paralomis granulosa* (Jaquinot), submitted to JEB

The concept of this study was developed by myself and FJS. I developed the experimental design, carried out the measurements and analysed the data. I wrote the manuscript, which was revised by FJS and HOP.

Publication IV

Wittmann AC, Anger K, Storch D, Pörtner HO, Sartoris FJ, Effects of temperature and magnesium on metabolism and elemental (CHN) composition during early development of the sub-Antarctic stone crab *Paralomis granulosa* (Jaquinot), in preparation

I developed the concept of this study together with FJS, DS and KA. I carried out the experiments and analysed the data. The manuscript was written by myself and was revised by all co-authors.

Publication I

Ion regulatory capacity and the biogoegraphy of Crustacea at high southern latitudes

Astrid C. Wittmann, Christoph Held, Hans O. Pörtner, Franz J. Sartoris

(2010)

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ORIGINAL PAPER

Ion regulatory capacity and the biogeography of Crustacea at high southern latitudes

Astrid C. Wittmann · Christoph Held · Hans O. Pörtner · Franz J. Sartoris

Received: 7 September 2009 / Revised: 4 January 2010 / Accepted: 17 January 2010 / Published online: 6 February 2010

Abstract Brachyuran and anomuran decapod crabs do not occur in the extremely cold waters of the Antarctic continental shelf whereas caridean and other shrimp-like decapods, amphipods and isopods are highly abundant. Differing capacities for extracellular ion regulation, especially concerning magnesium, have been hypothesised to determine cold tolerance and by that the biogeography of Antarctic crustaceans. Magnesium is known to have a paralysing effect, which is even more distinct in the cold. As only few or no data exist on haemolymph ionic composition of Sub-Antarctic and Antarctic crustaceans, haemolymph samples of 12 species from these regions were analysed for the concentrations of major inorganic ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}) by ion chromatography. Cation relationships guaranteed neuromuscular excitability in all species. Sulphate and potassium correlated positively with magnesium concentration. The Antarctic caridean decapod as well as the amphipods maintained low (6–20% of ambient sea water magnesium concentration), Sub-Antarctic brachyuran and anomuran crabs as well as the Antarctic isopods high (54–96% of ambient sea water magnesium concentration) haemolymph magnesium levels. In conclusion, magnesium regulation may explain the biogeography of decapods, but not that of the peracarids.

Keywords Antarctic · Haemolymph ion composition · Decapoda · Isopoda · Amphipoda · Magnesium

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Introduction

Decapod crustacean diversity is low in Antarctic compared to Sub-Antarctic regions (Gorny 1999). Over 130 benthic and pelagic decapod species occur in the Southern Ocean, but only 27 species are present south of the Polar Frontal Zone (PFZ). Brachyuran crabs are completely absent, whereas at least nine species of the anomuran family Lithodidae have been found south of the PFZ (Gorny 1999; García Raso et al. 2005; Thatje et al. 2005). Anomuran and brachyuran crabs still inhabited nearshore habitats of Antarctica in the late Eocene (Feldmann and Zinsmeister 1984a, b). The extinction or migration of brachyuran crabs, which today are restricted to warmer shallow waters of the Sub-Antarctic (Gorny 1999), likely happened during cooling trends in the Miocene, when isopods radiated in the Antarctic and occupied ecological niches vacated by the decapods (Aronson et al. 2007 and references therein; Brandt 1999; Held 2000). Accordingly, amongst the Crustacea the taxon Peracarida is the most abundant and speciose in the Antarctic today, with over 400 isopod and over 500 amphipod species (Brandt 1999; Gutt et al. 2004).

Antarctica is encircled by a strong water current (Antarctic Circumpolar Current or ACC), which developed during the Oligocene and led to climatic cooling of the Southern Ocean (Lawver and Gahagan 2003). At about 50°S cold water masses coming from the South (surface temperature ca. 2°C) meet warmer waters from the North (surface temperature ca. 8°C; Orsi et al. 1995). The sharp change in water temperature is detectable to significant depth and may pose an oceanographic barrier, called the Polar Front. However, near the bottom this difference will be less distinct, depending on the depth of the seabed (Orsi et al. 1995). Potential seabed temperatures at 50°S are ca. 2°C on continental shelf (0–1,000 m), 0–2°C on continental slope

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Abstract

Brachyuran and anomuran decapod crabs do not occur in the extremely cold waters of the Antarctic continental shelf whereas caridean and other shrimp-like decapods, amphipods and isopods are highly abundant. Differing capacities for extracellular ion regulation, especially concerning magnesium, have been hypothesised to determine cold tolerance and by that the biogeography of Antarctic crustaceans. Magnesium is known to have a paralysing effect, which is even more distinct in the cold. As only few or no data exsist on haemolymph ionic composition of Sub-Antarctic and Antarctic crustaceans, haemolymph samples of 12 species from these regions were analysed for the concentrations of major inorganic ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}) by ion chromatography. Cation relationships guaranteed neuromuscular excitability in all species. Sulphate and potassium correlated positively with magnesium concentration. The Antarctic caridean decapod as well as the amphipods maintained low (6 – 20 % of SW), Sub-Antarctic brachyuran and anomuran crabs as well as the Antarctic isopods high (54 – 96 % of SW) haemolymph magnesium levels. In conclusion, magnesium regulation may explain the biogeography of decapods, but not that of the peracarids.

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The repeated extension and retreat of the Antarctic shelf ice and formation and melting of a multiyear sea-ice layer during earth history might have contributed to the current distribution pattern of Antarctic crustaceans. Expansion periodically reduced the space of the shelf habitat and due to a decline of light penetration decreased primary productivity. This may have selected for species, which were able to adapt to or were already adapted to continental slope or deep-sea environments. This may explain why the recent invertebrate shelf fauna is characterised by a large number of eurybathic species (Brey et al. 1996) and of groups, which are important components of the deep-sea fauna, like echinoderms and isopods (Aronson et al. 2007). Furthermore, skeleton-crushing predators

amongst crabs and fish are missing. These predators disappeared at about the same time when climatic cooling occurred. Declining predation pressure caused a fundamental shift in the structure of the Antarctic benthic community and a reestablishment of its archaic character that we observe today (Aronson et al. 2007).

South of the PFZ, species-level endemism is high in the ocean (Arntz et al. 1997). However, endemism may have been overestimated and there are species, which occur both north and south of the PFZ (Barnes and Peck 2008; Thatje et al. 2005). Furthermore, larvae of South American decapod species have been found in Antarctic water masses (Thatje and Fuentes 2003). This indicates that isolation of the Antarctic continent may not be as pronounced as formerly thought and that reinvasion is possible (Clarke et al. 2005). However, the establishment of a species on the Antarctic shelf requires adaptations to constantly low temperature, high pressure and pronounced seasonality of availabe resources (Aronson et al. 2007; Clarke 1988; Clarke et al. 2009).

Reproductive and developmental adaptations have been discussed to determine decapod distribution patterns (Thatje et al. 2003). Many subpolar and polar marine invertebrate groups have evolved a high degree of endotrophy and an abbreviation of larval development to compensate for scarcity and pronounced seasonality of food supply (Thorson's rule, Mileikovsky 1971; Clarke 1988). Whereas lithodid crab species as well as caridean shrimp species have adopted these characteristics to various degrees, there are only few subpolar brachyuran species, which have developed these traits (Thatje et al. 2003). By contrast, peracarids keep their young in a brood pouch until they have completed direct development to juveniles (Luxmoore 1982; Ruppert and Barnes 1994). The female will protect their young from predators and might even provide nutrition to the more advanced offspring (Heilmayer et al. 2008; Janssen and Hoese 1993). It is interesting to note in this context, that similar to the echinoderms of the Antarctic shelf (Poulin et al. 2002), the number of crustacean species with planktonic larvae (decapods) has declined during earth history, whereas brooding crustaceans (peracarids) have radiated.

The biogeography of lithodid crabs is probably constrained by temperature, as this group has only been found in waters warmer than 0 °C (Hall and Thatje 2009) with their southernmost habitat being the continental slope of the western Antarctic Peninsula in the Bellingshausen Sea (García Raso et al. 2005; Thatje et al. 2008). In contrast, caridean decapods as well as amphipods and isopods tolerate temperatures as low as -1.8°C, and are frequently observed in shallower waters of the continental shelf of Antarctica (Brandt 1999; Gutt et al. 1991). Most rates of locomotory activity as well as metabolic and developmental rates are slower in polar than in temperate species with similar ecological function. This indicates that

these processes are not or only poorly temperature-compensated (Barnes and Peck 2008; Young et al. 2006).

Furthermore, the activity level (quantified as righting or walking speed, relative heart rate and oxygen consumption) is negatively correlated with haemolymph magnesium concentration in decapods (Sartoris et al. 1997; Walters and Uglow 1981; Watt et al. 1999) and amphipods (Spicer et al. 1994), as reviewed by Morritt and Spicer (1993). This might be based on the fact that magnesium slows down neuromuscular transmission by blocking calcium channels, which makes it useful as anaesthetic (Iseri and French 1984; Katz 1936; Lee et al. 1996; Pantin 1948; Waterman 1941). Quantal content of crayfish axons, a direct measure of transmitter release, is reduced at high extracellular magnesium concentration (Parnas et al. 1994). Magnesium inhibits the secretion of neurohormones from the X-organ-sinus gland of the land crab *Cardisoma carnifex* at physiological extracellular levels of 10-15 mmol L^{-1} by bocking calcium currents (Richmond et al. 1995). Similarly, low temperature reduces the amount of transmitter release in crayfish axons, which is thought to be the result of reduced calcium influx through calcium channels (Dunn and Mercier 2003). High haemolymph magnesium concentration and low temperature may therefore work in concert to decline neuromuscular transmission and rates of activity. For example Frederich et al. (2000b) observed that the spider crab *Hyas araneus* was threefold slower at righting itself at -2 °C (18.5 s) than at temperatures above 0 °C (6.5 s). When magnesium concentration was experimentally reduced from the natural level of 50 mmol L^{-1} to 6 mmol L^{-1}, the mean time-to-right remained at 6.5 s over the entire investigated thermal range (-2 to 6.5 °C).

Moreover, all muscular systems, including those of ventilation and circulation may be affected (Frederich 1999; Frederich et al. 2000a, b). Low temperature may constrain physiological functions and this may influence the distribution pattern of crustaceans, following the rationale of the concept of oxygen limited thermal tolerance (Pörtner 2002). In the temperate spider crab *Maja squinado*, tolerance to cold was constrained by inefficient ventilation of the gills and reduced circulation of the haemolymph, which led to a decline of haemolymph oxygenation and finally to the onset of anaerobic metabolism during progressive cooling (Bock et al. 2001; Frederich and Pörtner 2000). Judged from a threefold increase in mean cardiac output, the low threshold for optimal performance (pejus temperature) of *Maja squinado* was shifted from 8 to 6 °C in an incubation of low magnesium concentration (6 mmol L^{-1}) compared to natural conditions (50 mmol L^{-1}, Frederich et al. 2000a). Based on these and further results, the hypothesis was brought forward that crustaceans which are thought to have a high capacity for haemolymph magnesium extrusion (caridean shrimps, amphipods and isopods) would be more cold tolerant than those crustaceans which are thought to be poor magnesium regulators (brachyuran and

anomuran crabs, Sartoris et al. 1997; Frederich et al. 2000b). Today, this seems to be accepted as the primary explanation for the biogeography of crustaceans in Antarctica (Aronson et al. 2007; Thatje et al. 2005). Whereas there is experimental evidence for the relationships between temperature, magnesium and physiological functions in temperate and subpolar brachyuran crabs (Frederich et al. 2000a,b), temperate and polar caridean shrimps (Sartoris and Pörtner 1997a,b) and temperate amphipods (Spicer et al. 1994), we do not know whether there is a relation between temperature, the capacity for magnesium extrusion and other physiological functions in anomuran crabs, amphipods, isopods and other crustacean groups from the Southern Ocean.

Primary sites for extracellular ion regulation are the gills and the antennal (decapods, amphipods) or maxillary (isopods) glands (Ruppert and Barnes 1994). These tissues possess high concentrations of the enzyme Na^+/K^+-ATPase, which provides at least part of the driving force for transepithelial ion transport (Khodabandeh et al. 2005; Lucu and Towle 2003). Sodium, chloride and calcium ion uptake and secretion may take place across the gill epithelium. In osmoregulating brachyuran crabs, ionocytes are especially abundant in the posterior gills. These exhibit a higher Na^+/K^+-ATPase activity than the anterior portion of the gills, which are characterized by a thin epithelium faciliating gas exchange (Copeland and Fitzjarrell 1968; Neufeld et al. 1980). Urine formation together with magnesium and sulphate excretion occurs in the antennal glands. Furthermore, calcium and potassium ions may be reabsorbed in exchange for sodium at this site (reviewed by Freire et al. 2008).

In those crustaceans, which have been investigated so far, haemolymph sodium, chloride and potassium concentrations are usually kept at levels similar to those in sea water (Mantel and Farmer 1983). Extracellular calcium concentration varies during the molt cycle (Robertson 1960). Magnesium concentration is strongly hyporegulated in caridean shrimp, amphipod and most isopod species ($[Mg^{2+}]_{HL}$ < 20 mmol L^{-1}) and to a much lesser extent in anomuran and brachyuran crab species ($[Mg^{2+}]_{HL}$ = 20 – 50 mmol L^{-1}, Burton 1995 and references therein; Frederich 1999; Robertson 1953). Sulphate seems to show the same pattern, but only few measurements have been undertaken, particularly few in amphipods and isopods (Mantel and Farmer 1983).

In this study we present the first analyses of ionic composition of the haemolymph of Antarctic amphipods and isopods and of lithodid crab species occurring near Sub-Antarctic islands. Altogether, we provide information on 12 species of decapods, isopods and amphipods and discuss the results with a focus on the hypothesis that extracellular magnesium regulation shapes the biogeography of crustaceans in the Southern Ocean.

Materials and Methods

Sample collection

Haemolymph samples of *Lithodes confundens, Paralomis formosa, Paralomis spinosissima* and *Peltarion spinosulum* were collected during the ICEFISH Cruise in June 2004 (http://www.icefish.neu.edu/). Specimens of *Notocrangon antarcticus, Eurythenes gryllus, Abyssorchomene plebs, Eusirus propeperdentatus, Glyptonotus antarcticus, Natatolana* sp. and *Ceratoserolis trilobitoides* were obtained during Polarstern expedition ANT XXIII/8 in the Antarctic summer 2007. Haemolymph samples were collected either on board directly (RV Nathaniel B. Palmer or RV Polarstern) or from live animals transported back to the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven, Germany (AWI) after an acclimation period of one week at 0 °C and 32.5 ppt. Male specimens of *Paralomis granulosa* were obtained from local fishermen in Punta Arenas, Chile in April 2008, the collection site is therefore not known precisely (Table 1). These animals were transported to the AWI on board RV Polarstern and kept in a recirculating aquarium system at 4 °C and 32.5 ppt for one year until haemolymph samples were taken. Laboratory-kept animals were fed *ad libitum* with pieces of *Mytilus edulis*, *Cerastoderma edule* or *Crangon crangon*.

Animals were blotted dry before haemolymph was withdrawn either with a syringe and hypodermic needle, which was inserted through an arthrodial membrane at the coxa of a walking leg (crabs), in the heart region (*N. antarcticus, E. gryllus, E. propeperdentatus*) or by inserting a pointed glass capillary dorsally into the heart region of the animals (remaining species). Samples were stored at -20 °C or -80 °C until being analysed.

Sea water ion composition at 35 ppt salinity was taken from Atkinson and Bingman (1997) for comparison with field-sampled individuals and calculated for 32.5 ppt for comparison with laboratory-kept animals (Table 2).

Ion chromatography

Ion composition of haemolymph was determined by ion chromatography (ICS-2000, Dionex®, Idstein, Germany) after dilution of the samples with deionised water. A conductivity cell and a self-regenerating suppressor were used to reduce background conductivity. Cations (Na^+, K^+, Mg^{2+}, Ca^{2+}) were separated on an IonPac® CS16 column with methane sulfonic acid (30 mmol L^{-1}) as eluent at a flow rate of 0.36 mL min^{-1} at 40 °C. Anions (Cl^-, SO_4^{2-}) were separated on an IonPac® AS11-HC column with potassium hydroxide (30 mmol L^{-1}) as eluent at a flow rate of 0.30 mL min^{-1} at 30 °C. Ion concentrations were

calculated in mmol L^{-1} relative to the Dionex® Six Cation-II or Five Anion Standards and are also given in percent of the respective sea water ion concentrations to make data of field-sampled and laboratory-kept animals comparable.

Statistical analyses

Before calculating means ± standard deviation (SD), outliers were identified by use of the Nalimov test on the sum of all ions of each individual. One-way ANOVA and post hoc Dunett's multiple comparison tests were run to compare means of percentages of ions in haemolymph with those of sea water (always 100%). Differences were termed "significant" if p-values were below 0.05. ANOVA as well as linear regression and Pearson correlation analyses were performed by use of Prism 4.0a.

Results

For each collection site monthly means of water temperatures at the respective depth were taken from Locarnini et al. (2006, Table 1). Anomuran and brachyuran decapods were found in waters with temperatures above 0 °C while the caridean decapod *Notocrangon antarcticus* as well as most of the peracarids were collected in waters of or below 0 °C.

All groups except *Peltarion spinosulum* displayed significantly lower haemolymph magnesium levels compared to sea water (Fig. 1, Table 2). Despite of this, there are differences in the extent of downregulation of magnesium between groups. Whereas brachyuran and anomuran decapods as well as isopods maintained rather high haemolymph magnesium levels between 54 ± 2% and 82 ± 6% of sea water, those of the caridean decapod *Notocrangon antarcticus* and the amphipods were well below half of the value of sea water, between 6% (*E. propeperdentatus*) and 20 ± 2% (*E. gryllus*).

Likewise, the haemolymph sulphate content of all species except that of *Peltarion spinosulum* was significantly lower than that in sea water (Table 2). The amphipod *E. gryllus* exhibited the lowest value of 7 ± 2%.

Species which maintained low haemolymph magnesium concentrations usually maintained low sulphate levels as well (Fig. 2). This resulted in a significantly ($p = 0.0023$) positive, linear correlation between magnesium and sulphate percentages with Pearson $r = 0.8147$ and $r^2 = 0.6638$ where all investigated species means were considered, except *E. propeperdentatus*, which was excluded because of the small sample size. Only the isopod

Natatolana sp., which showed a significantly reduced sulphate percentage in spite of a high magnesium percentage deviated from this relationship.

Haemolymph sodium content was equal to or slightly lower than in sea water in most species and only significantly reduced in *Natatolana* sp. and *C. trilobitoides* with values of 87 ± 10% and 87 ± 6% (Table 2).

In most investigated species haemolymph calcium was not significantly different from the sea water level. Only *Peltarion spinosulum* exhibited a remarkably low value of 54 ± 34% of sea water calcium concentration (Table 2).

Haemolymph potassium concentrations were close to sea water level in the majority of species. However, *E. propeperdentatus* displayed an extremely low value of 59%, *N. antarcticus, A. plebs* and *E. gryllus* had significantly decreased potassium values and *L. confundens* had a significantly increased level compared to sea water (Table 2). Furthermore, there was a significantly ($p = 0.0044$) positive correlation between magnesium and potassium percentages amongst all species in this study (*E. propeperdentatus* was excluded) with Pearson $r = 0.7824$ (Fig. 3).

Haemolymph chloride levels of most investigated animals were equal to or lower than that in sea water. In *N. antarcticus, A. plebs, Natatolana* sp. and *C. trilobitoides* haemolymph chloride content even was significantly below that of sea water and comprised only 82 ± 9% of the sea water concentration in *N. antarcticus* (Table 2).

Discussion

Sub-Antarctic lithodid crab species exhibited a relatively low but significant capability for magnesium extrusion from the haemolymph. Our data compare well to those obtained in previous studies on the northern species *Lithodes maja* (where $[Mg^{2+}]_{HL} = 50$ mmol L^{-1}, which corresponds to about 92% of sea water concentration, Robertson 1953), *Paralithodes camtschatica* ($[Mg^{2+}]_{HL} = 37$ mmol L^{-1}, ≈ 69%, Mackay and Prosser 1970) and *Neolithodes grimaldii* collected during summer ($[Mg^{2+}]_{HL} = 33$ mmol L^{-1}, ≈ 61%, McAllen et al. 2005). However, our value for *P. granulosa* is twice as high as that recorded by Frederich (1999) for this species (17-24 mmol L^{-1}, ≈ 31- 44%). This difference might be attributable to a different nutritional state of the animals because McAllen et al. (2005) found significantly lower haemolymph magnesium levels ($[Mg^{2+}]_{HL} = 20$ mmol L^{-1}, ≈ 37%) in *N. grimaldii* along with significantly lower haemolymph protein levels during spring when food was presumably scarce. Extracellular protein concentration decreases during starvation (Dall 1974). Whereas the activation of energy demanding magnesium excretion appears paradoxical during food

deprivation, its physiological role could be an activation of the organism to trigger foraging activity (McAllen et al. 2005). An increase in walking activity was observed when brachyuran crabs were experimentally exposed to artificial sea water with reduced magnesium concentration (Frederich et al. 2000b).

The haemolymph magnesium concentration of the brachyuran *Peltarion spinosulum* from the Falkland Islands did not differ significantly from that of sea water. Our data were similar to those previously recorded for *P. spinosulum* and for other subtidal brachyuran species from the Sub-Antarctic (e.g. *Eurypodius latreillei*; Frederich 1999) and from temperate northern latitudes (e.g. *Dromia vulgaris, Hyas araneus*; Robertson 1953; Frederich 1999).

Previous analyses of haemolymph ion composition in isopods were focused on species from intertidal, estuarine or semiterrestrial habitats. These species regulate magnesium down to below 20 mmol L^{-1} (Burton 1995 and references therein; Parry 1953; Ziegler et al. 2000). In contrast, the extracellular magnesium concentration of the deep-sea isopod *Bathynomus doderleini* does not differ from that of sea water (F.-Tsukamoto et al. 2000). Despite this, the relatively well-developed ability to downregulate magnesium in temperate intertidal isopods has been extrapolated to be valid for polar species and has served as a possible explanation for their advantage over decapod crabs to colonise high Antarctic waters (Frederich et al. 2000b; Thatje et al. 2005). Here, we show that the capacity for magnesium regulation of the polar isopods was in the same range as that of the lithodid crabs. A high level of magnesium in the haemolymph therefore does not constrain the isopods to warmer waters. However, a correlation between the general life style/activity and haemolymph magnesium concentration may be postulated. *Glyptonotus antarcticus* exhibited a relatively low magnesium fraction and is described as a "rude carnivorous benthic scavenger and predator" (Janssen and Hoese 1993) and actively forages for food (C. Held, pers. obs.). *Natatolana* sp. possessed a high haemolymph magnesium fraction and are burrow dwellers similar to *Natatolana borealis* from Scottish waters (Taylor and Moore 1995). These animals adopt a sit-and-wait strategy (C. Held, pers. obs.): When dwelling in the burrow, the animals remain inactive except for ventilatory burrow irrigation. However, they exhibit excellent swimming behaviour once carrion or prey is detected by them. After feeding, they return to an inactive mode and digest while staying in their burrows. High extracellular magnesium concentration and reduced activity levels may increase tolerance to hypoxia (Sartoris and Pörtner 1997a), which is frequently encountered by infaunal species. Whereas investigations on the relationships between nutritional state, activity, cold tolerance and haemolymph magnesium levels in isopods are still missing, we may hypothesise that neuromuscular transmission of isopods is less sensitive to magnesium than that of decapods. Furthermore, it is possible that haemolymph magnesium concentration varies according to

the nutritional condition of the animals and that this influences their activity level. These adaptations may allow them to thrive in the extremely cold waters of the Anarctic shelf despite relatively high haemolymph magnesium concentration as observed in our well-fed laboratory animals.

The Antarctic amphipods as well as the caridean shrimp were found to be excellent magnesium regulators similar to their temperate counterparts (Mantel and Farmer 1983; Normant et al. 2005) or tropical oceanic relatives (Tentori and Lockwood 1990). Therefore, ion regulation is not constrained by low temperature but is compensated by these cold adapted species. This was also found in the Arctic amphipod *Apherusa glacialis* (Kiko et al. 2009), the northern caridean prawn *Pandalus borealis* (Sartoris and Pörtner 1997b) and the Antarctic caridean shrimp *Chorismus antarcticus* (Frederich 1999). In contrast, when the temperate amphipod sandhopper *Talitrus saltator* was exposed to winter cold, it ceased to extrude magnesium from the haemolymph and fell into a torpor state (Spicer et al. 1994). Similarly, the capacity for magnesium regulation was hampered in tropical pelagic amphipods, when experimentally exposed to temperatures far below their natural temperature range (Tentori and Lockwood 1990). Amongst the caridean shrimps, the temperate *Crangon crangon* exhibited increased haemolymph magnesium concentrations during short-term exposure to cold (Sartoris and Pörtner 1997a).

The strong positive correlation between haemolymph magnesium and sulphate levels indicates that sulphate is regulated in parallel to magnesium for compensation of osmotic equilibrium (Robertson 1953). The mechanisms for magnesium and sulphate excretion in the antennal gland are not known in detail, but there is evidence that they function independently of each other. When exposed to salinity variations, sulphate extrusion ceased earlier than magnesium extrusion in e.g. an amphipod (Kiko et al. 2009) and a brachyuran decapod (Zanders 1980). Furthermore, it is known from both lobster hepatopancreas and flounder kidney, that sulphate is exported by use of sulphate-anion exchangers (Gerencser et al. 2001). Sulphate excretion in the antennal gland may be based on a similar mechanism and therefore may work independently of magnesium transport.

Extracellular sodium, chloride and potassium were kept close to equilibrium with sea water in most species. This is a general pattern found in marine crustaceans (Mantel and Farmer 1983). Calcium is the major component of the carapace, therefore extracellular concentrations vary during the molt cycle (Mantel and Farmer 1983; Robertson 1960). Cation relationships were similar to those collected by Burton (1995) in over 70 sea water and freshwater crustacean species. He concluded that "haemolymph composition has evolved in such a way as to preserve the transmembrane potential" across the cell membrane. Maintenance of the transmembrane potential is crucial for animals, because it guarantees the

excitability of nerve and muscle and drives ion transport processes (Eckert et al. 2000). If so, we can conclude from this, that our sampled individuals were in good health.

In summary, our results comply with previous assumptions and findings (Frederich et al. 2000b; Thatje et al. 2005), that lithodid crabs from the Southern Ocean are rather poor haemolymph magnesium regulators and do not thrive in waters colder than 0 °C and that caridean shrimps which exhibit a high capacity for magnesium regulation can be found in high Antarctic waters of temperatures below 0 °C. Amongst the peracarids, the Antarctic amphipod species displayed a magnesium regulatory capacity similar to species examined in previous studies from tropic, temperate and polar latitudes (Kiko et al. 2009; Mantel and Farmer 1983; Spicer et al. 1994; Tentori and Lockwood 1990). In contrast, the ability to regulate extracellular magnesium was similarly poor in Antarctic isopods and the lithodids. The isopods must therefore possess different physiological and ecological adaptations, which give them an advantage over the decapod crabs and which enable them to thrive in high Antarctic waters. Concerning the physiology, this could be a reduced sensitivity of neuromuscular systems to magnesium compared to the lithodid crabs. Apart from this, different reproductive traits, like a direct development of the young, which may remain rather inactive as they are carried in a brood pouch or different food preferences could have contributed to the success of the peracarids in the Antarctic (Janssen and Hoese 1993; Brandt 1999).

Acknowledgements

We would like to thank Timo Hirse and Florian Leese for sample collection. We greatly appreciate the help of the crew of RV Polarstern during the transport of live animals. The experiments comply with the current laws of the country in which they were performed. This study was supported by Deutsche Forschungsgemeinschaft grants no. SA 1713 and He 3391/3 and by National Science Foundation grant no. OPP V01-32032. The authors declare that they have no conflict of interest. This is publication 25 of the ICEFISH cruise 2004.

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Table 1 Collection sites, mean fishing depth (m) of the sampled crustaceans and approximate water temperature (°C) at respective depths at the time of collection (Locarnini et al. 2006).

Taxon	Species	Collection site	Depth (m)	Temperature (°C)
Decapoda, Brachyura	*Peltarion spinosulum*	51°41′ S, 57°27′ W	130	6.0
Decapoda, Anomura	*Lithodes confundens*	53°39′ S, 40°44′ W	410	2.0
	Paralomis formosa	56°19′ S, 27°27′ W	340	1.0
	Paralomis granulosa	Magellan region	unknown	5.0 to 8.0?
	Paralomis spinosissima	53°39′ S, 40°44′ W	410	2.0
Decapoda, Caridea	*Notocrangon antarcticus*	65° 32' S, 61° 30' W	490	-1.0 to -0.5
Peracarida, Amphipoda	*Abyssorchomene plebs*	60° 57' S, 55° 55' W	231	0.0
	Eurythenes gryllus	62° 58' S, 57° 58' W	839	1.5
	Eusirus propeperdentatus	62° 58' S, 57° 58' W	839	1.5
Peracarida, Isopoda	*Glyptonotus antarcticus*	61° 20' S, 55° 32' W 62° 19' S, 60° 27' W	137 109	-1.0 -1.0
	Natatolana sp.	60° 57' S, 55° 55' W	231	0.0
	Ceratoserolis trilobitoides	70° 31' S, 8° 48' W 61° 22' S, 56° 1' W	297 353	0.0 -0.5

Table 2 Haemolymph inorganic ion composition of Sub-Antarctic and Antarctic crustaceans determined by ion chromatography (mmol L^{-1}) and expressed as percent of the respective sea water ion concentrations given as means ± SD. [1] field sampling, %-values calculated in relation to sea water with salinity 35 ppt, [2] animals kept in the laboratory prior to sampling, fed *ad libitum*, %-values calculated in relation to sea water with salinity 32.5 ppt, n number of individuals sampled, * significantly different from sea water (100%).

	n	unit	Na^+	Cl^-	K^+	Ca^{2+}	Mg^{2+}	SO_4^{2-}
Sea water 32.5 ppt 35 ppt		% mmol L^{-1} mmol L^{-1}	100 446 481	100 522 563	100 9.7 10.4	100 9.8 10.5	100 50.3 54.2	100 26.6 28.6
Peltarion spinosulum [1]	4	% mmol L^{-1}	99 ± 4 477 ± 19	85 ± 7 479 ± 39	112 ± 12 11.6 ± 1.2	51 ± 34* 5.4 ± 3.5	96 ± 4 51.7 ± 1.9	104 ± 12 30.3 ± 3.4
Lithodes confundens[1]	9	% mmol L^{-1}	100 ± 6 478 ± 28	88 ± 7 505 ± 43	133 ± 20* 13.9 ± 2.0	119 ± 14 12.5 ± 1.4	68 ± 15* 37.0 ± 7.6	77 ± 12* 22.4 ± 3.4
Paralomis formosa[1]	7	% mmol L^{-1}	106 ± 5 508 ± 24	100 ± 3 563 ± 17	101 ± 10 10.6 ± 1.0	104 ± 17 10.9 ± 1.8	73 ± 7* 39.5 ± 3.8	79 ± 14* 22.8 ± 4.1
Paralomis granulosa[2]	8	% mmol L^{-1}	97 ± 2 431 ± 11	94 ± 3 491 ± 15	118 ± 13 11.4 ± 1.3	110 ± 5 10.8 ± 0.5	80 ± 4* 40.0 ± 1.9	63 ± 8* 16.9 ± 2.2
Paralomis spinosissima[1]	10	% mmol L^{-1}	99 ± 3 475 ± 15	95 ± 5 533 ± 29	105 ± 10 10.9 ± 1.0	106 ± 16 11.2 ± 1.7	75 ± 5* 40.7 ± 2.6	71 ± 8* 20.5 ± 2.4
Notocrangon antarcticus[1]	6	% mmol L^{-1}	98 ± 7 476 ± 34	82 ± 9* 477 ± 60	72 ± 16* 7.7 ± 1.7	140 ± 20 14.5 ± 2.0	16 ± 11* 11.0 ± 8.5	33 ± 10* 10.6 ± 4.0
Abyssorchomene plebs[2]	5	% mmol L^{-1}	89 ± 5 397 ± 22	77 ± 4* 404 ± 21	60 ± 6* 5.9 ± 0.6	112 ± 16 11.0 ± 1.6	20 ± 2* 10.0 ± 0.8	33 ± 9* 8.9 ± 2.3
Eurythenes gryllus[1]	10	% mmol L^{-1}	101 ± 5 479 ± 29	95 ± 5 530 ± 36	68 ± 4* 7.1 ± 0.4	95 ± 40 9.9 ± 4.0	20 ± 2* 11.1 ± 0.9	7 ± 2* 2.0 ± 0.6
Eusirus propeperdentatus[1]	2	% mmol L^{-1}	119 574	112 629	59 6.2	99 10.4	6 3.5	33 9.7
Glyptonotus antarcticus[2]	5	% mmol L^{-1}	93 ± 4 415 ± 15	93 ± 4 509 ± 53	126 ± 12 12.2 ± 1.2	101 ± 6 9.8 ± 0.6	54 ± 2* 30.4 ± 8.5	31 ± 2* 12.4 ± 9.6
Natatolana sp.[2]	6	% mmol L^{-1}	87 ± 10* 390 ± 47	83 ± 10* 437 ± 52	106 ± 19 10.3 ± 1.8	111 ± 7 10.9 ± 0.7	82 ± 6* 41.0 ± 3.0	46 ± 10* 12.5 ± 2.6
Ceratoserolis trilobitoides[2]	6	% mmol L^{-1}	87 ± 6* 388 ± 25	83 ± 8* 435 ± 41	99 ± 10 9.6 ± 0.92	97 ± 11 9.5 ± 1.0	60 ± 12* 29.8 ± 5.8	69 ± 10* 18.7 ± 2.7

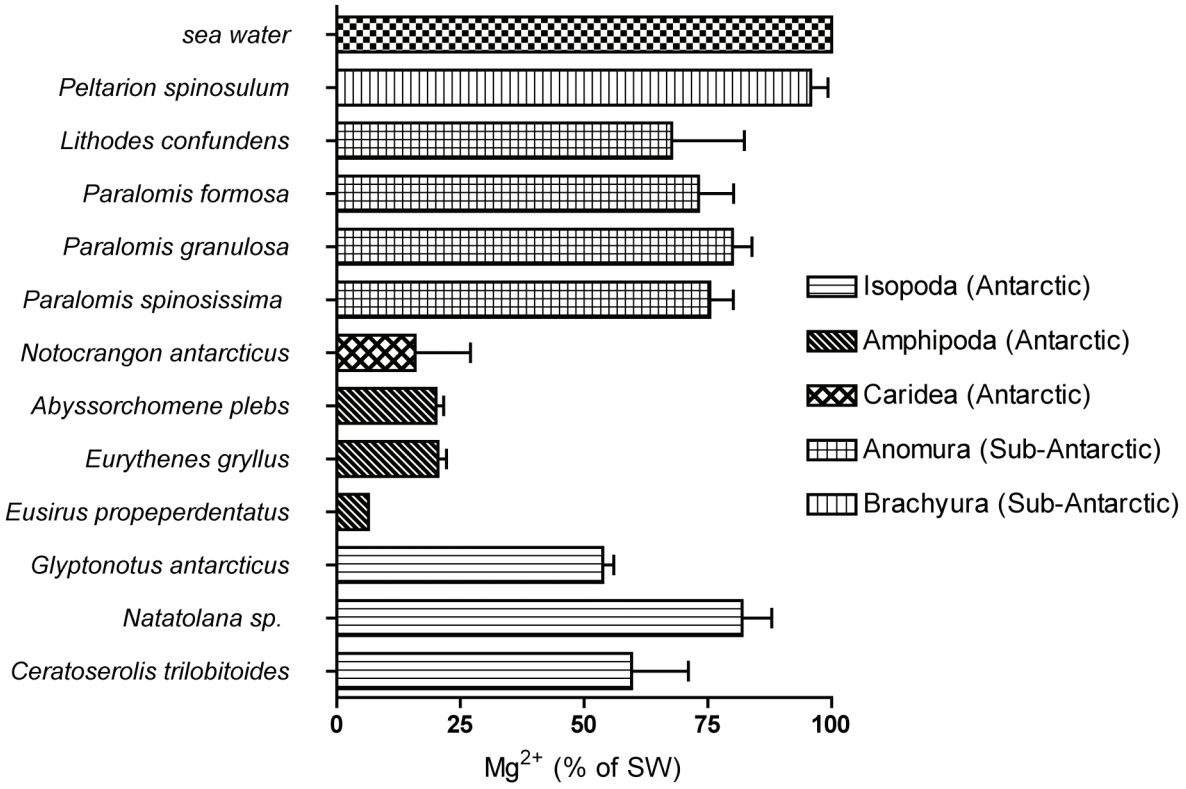

Fig. 1 Magnesium concentration in the haemolymph of Sub-Antarctic and Antarctic crustaceans (% of sea water magnesium concentration). Values are means ± SD. All values except that of *Peltarion spinosulum* are significantly different from sea water (100%).

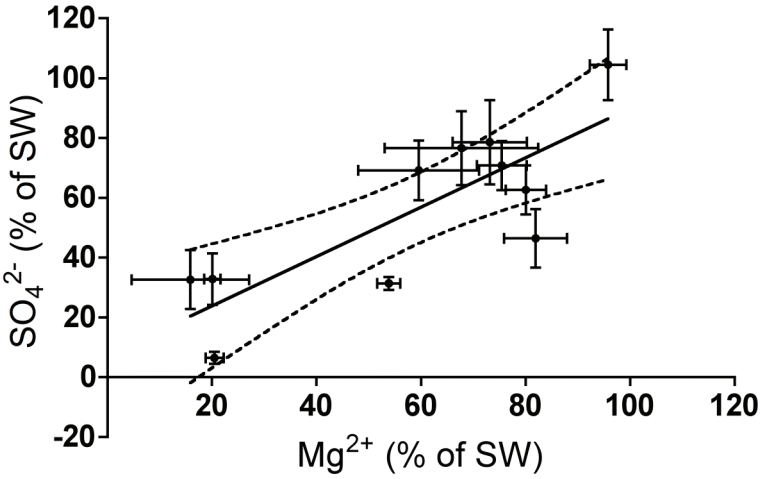

Fig. 2 Relationship between magnesium and sulphate in the haemolymph (% of sea water concentrations) of all species investigated except *E. propeperdentatus*, which was excluded because of the low sample size. Significant positive correlation with Pearson r = 0.8147, p = 0.0023, and linear regression of r^2 = 0.6638 with 95% confidence bands. Values are means ± SD.

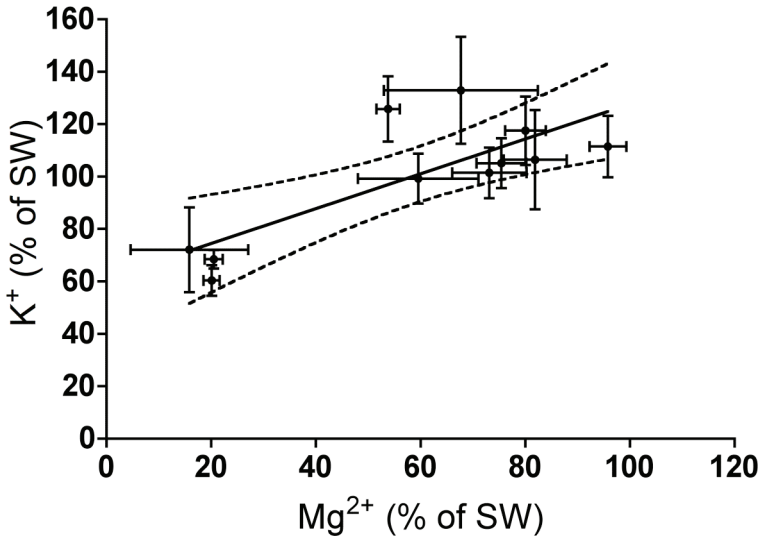

Fig. 3 Relationship between magnesium and potassium in the haemolymph (% of sea water concentrations) of all species investigated except *E. propeperdentatus*, which was excluded because of the low sample size. Significant positive correlation (Pearson r = 0.7824, p = 0.0044) and linear regression of r^2 = 0.6122 with 95% confidence bands. Values are means ± SD.

Publication II

Temperature-dependent activity in early life stages of the stone crab *Paralomis granulosa* (Decapoda, Anomura, Lithodidae): a role for ionic and magnesium regulation?

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(Submitted)

Temperature-dependent activity in early life stages of the stone crab *Paralomis granulosa* (Decapoda, Anomura, Lithodidae): a role for ionic and magnesium regulation?

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Keywords: Antarctic, *Paralomis granulosa*, haemolymph ion composition, heart rate, temperature, magnesium, activity

Abstract

Marine brachyuran and anomuran crustaceans are completely absent from the extremely cold (-1.8 °C) Antarctic continental shelf, but caridean shrimps are abundant. This has at least partly been attributed to low capacities for magnesium excretion in brachyuran and anomuran lithodid crabs ($[Mg^{2+}]_{HL}$ = 20 – 50 mmol L^{-1}) compared to caridean shrimp species ($[Mg^{2+}]_{HL}$ = 5 – 12 mmol L^{-1}). Magnesium has an anaesthetizing effect and reduces cold tolerance and activity of adult brachyuran crabs. We investigated whether the capacity for magnesium regulation is a factor that influences temperature-dependent activity of early ontogenetic stages of the Sub-Antarctic lithodid crab *Paralomis granulosa*. Ion composition (Na^+, Mg^{2+}, Ca^{2+}, Cl^-, SO_4^{2-}) was measured in haemolymph withdrawn from larval stages, the first and second juvenile instars (crab I and II) and adult males and females. Magnesium excretion improved during ontogeny, but haemolymph sulphate concentration was lowest in the zoeal stages. Neither haemolymph magnesium concentrations nor Ca^{2+}:Mg^{2+} ratios paralleled activity levels of the life stages. Long-term (3 wk) cold exposure of crab I to 1 °C caused a significant rise of haemolymph sulphate concentration and a decrease in magnesium and calcium concentrations compared to control temperature (9 °C). Spontaneous swimming activity of the zoeal stages was determined at 1, 4 and 9 °C in natural sea water (NSW, $[Mg^{2+}]$ = 51 mmol L^{-1}) and in sea water enriched with magnesium (NSW + Mg^{2+}, $[Mg^{2+}]$ = 97 mmol L^{-1}). It declined significantly with temperature but only insignificantly with increased magnesium concentration. Spontaneous velocities were low, reflecting the demersal life style of the zoeae. Heart rate, scaphognathite beat rate and forced swimming activity (maxilliped beat rate, zoea I) or antennule beat rate (crab I) were investigated in response to acute temperature change (9, 6, 3, 1, -1 °C) in NSW or NSW + Mg^{2+}. High magnesium concentration reduced heart rates in both stages. The temperature-frequency curve of the maxilliped beat (maximum: 9.6 beats s^{-1} at 6.6 °C in NSW) of zoea I was depressed and shifted towards warmer temperatures by 2 °C in NSW + Mg^{2+}, but antennule beat rate of crab I was not affected. Magnesium may therefore influence cold tolerance of highly active larvae, but it remains questionable whether slow-moving lithodid crabs with demersal larvae would benefit from enhanced magnesium excretion in nature.

Introduction

Marine decapod crustacean diversity is low in Antarctic compared to Sub-Antarctic regions (Gorny, 1999). Over 130 benthic and pelagic decapod species occur in the Southern Ocean, but only 27 species are present south of the Polar Frontal Zone (PFZ). Brachyuran crabs are completely absent, whereas at least 9 species of the anomuran family Lithodidae have been found south of the PFZ (Gorny, 1999; García Raso et al., 2005; Thatje et al., 2005). Anomuran and brachyuran crabs still inhabited nearshore habitats of Antarctica in the late Eocene, but climatic cooling during the Miocene likely caused their extinction in this area (Feldmann and Zinsmeister, 1984a; Feldmann and Zinsmeister, 1984b; Gorny, 1999; Feldmann and Schweitzer, 2006; Aronson et al., 2007 and references therein). Today, brachyuran crabs occur in the warmer waters of the Sub-Antarctic (Gorny, 1999). The biogeography of lithodid crabs is probably constrained by low temperature as well, because this group has only been found in waters warmer than 0 °C (Hall and Thatje, 2009) with their southernmost habitat being the continental slope of the western Antarctic Peninsula in the Bellingshausen Sea (García Raso et al., 2005; Thatje et al., 2008). In contrast, caridean decapods tolerate temperatures as low as -1.8°C, and are frequently observed in shallower waters of the continental shelf of Antarctica (Gutt et al., 1991).

Besides temperature, the capability to regulate extracellular magnesium concentration below the concentration found in sea water (50 mmol L^{-1}) has been proposed to be a factor to influence the biogeography of decapod crustaceans in the Southern Ocean (Sartoris et al., 1997; Frederich, 1999; Frederich et al., 2000a; Frederich et al., 2000b; Frederich et al., 2001). Sub-Antarctic brachyuran and lithodid crab species exhibit high (20 – 50 mmol L^{-1}), Antarctic caridean decapods low (5 – 12 mmol L^{-1}) haemolymph magnesium concentrations (Frederich, 1999; Frederich et al., 2000b; Wittmann et al., 2010). Increased magnesium concentration has a paralysing effect on vertebrates and invertebrates (Katz, 1936; Waterman, 1941; Pantin, 1948; Iseri and French, 1984; Lee et al., 1996). In crustaceans, this is caused by action of the ion at least at two sites, which both are related to the block of calcium channels. Magnesium slows down neuromuscular transmission by reducing transmitter release of synapses (Parnas et al., 1994) and it reduces the contraction of muscle fibres as calcium influx is blocked at the postsynaptic membrane (Hagiwara and Takahashi, 1967; Ushio et al., 1993). Early studies on decapod nerve-muscle preparations showed that a 1.5- to 2-fold increase of external magnesium concentration above natural conditions weakened the response after stimulation, whereas a reduction of magnesium concentration enhanced facilitation of neuromuscular transmission and submaximal tensions of the locomotory musculature (Katz, 1936; Waterman, 1941; Boardman and Collier, 1946).

Robertson (1949, 1953) was the first to note, that there may be an inverse relationship between the haemolymph magnesium concentration and the general activity level of a decapod crustacean species. Furthermore, he suggested that not just extracellular magnesium concentration, but also calcium concentration may play a role in determining the activity of decapods due to the antagonistic effects of these ions (Robertson, 1949; Robertson, 1953; Iseri and French, 1984). Because of this, Robertson (1949, 1953) compared Ca^{2+}:Mg^{2+} ratios interspecifically: subjectively inactive species (e.g. of the genera *Lithodes* and *Maja*) exhibited low (0.19 – 0.31), more active species (e.g. *Cancer*, *Palinurus*) high (0.57 – 2.0) values. Several studies on decapods and amphipods supported this hypothesis quantifying activity by measuring heart rate, oxygen consumption and locomotory activity (Walters and Uglow, 1981; see review by Morritt and Spicer, 1993; Spicer et al., 1994; Sartoris et al., 1997; Watt et al., 1999).

Most rates of locomotory activity as well as metabolic and developmental rates are slower in polar than in temperate species with similar ecological function. These processes thus display little or no compensation for temperature effects (Young et al., 2006; Barnes and Peck, 2008). At the neurophysiological level, low temperature, similar to magnesium, reduces the amount of transmitter released in crayfish axons, which is thought to be the result of declined calcium influx through calcium channels (Dunn and Mercier, 2003). Low temperature and high magnesium concentrations may therefore interact to diminish locomotory activity and affect the entire cardiovascular system of decapods. Referring to the concept of oxygen- and capacity-limited thermal tolerance in crustaceans (Frederich and Pörtner, 2000; Pörtner, 2002), anomuran and brachyuran crab species possessing high haemolymph magnesium concentrations may be constrained in their distribution to the subpolar temperature regime (Frederich et al., 2000a; Frederich et al., 2000b; Frederich et al., 2001). Vice versa, experimental reduction of haemolymph magnesium concentration in adult brachyuran crabs down to the level of caridean shrimp species led to an increase in oxygen consumption, walking and righting activity and cardiac output, extending the thermal tolerance window of these crabs towards lower temperatures and even below 0 °C (Frederich et al., 2000a; Frederich et al., 2000b).

Larval stages are thought to be more sensitive than the adults, therefore the thermal tolerance of larvae should be a crucial factor determining the biogeography of a species (Frederich, 1999; Anger, 2001; Anger et al., 2003; Thatje et al., 2003; Hall and Thatje, 2009; Storch et al., 2009). Hardly any data exist on thermal tolerance of adult and larval lithodid crabs from the Southern Ocean even though this group occurs farther south than the brachyurans. Furthermore, it is not clear whether extracellular ion regulation influences

thermal tolerance and whether and to what extent the capacity for ion regulation changes during ontogeny in species of the family Lithodidae.

Temperature variations affect the regulation of ions, but the magnitude of the effect may depend on the season, exposure time and the ion species considered (Mantel and Farmer, 1983; Burton, 1986; Pequeux, 1995; Charmantier et al., 2009). Both passive (permeability of the epithelio-cuticular complex) and active components (ion transport) could be affected, but so far there is no consistent view on how temperature acts and which mechanisms account for the effects. In adult amphipods and caridean decapods, that haemolymph concentrations of magnesium rises once the animals are exposed to temperatures far below their acclimation temperature for up to one week (Campbell and Jones, 1989; Tentori and Lockwood, 1990, Spicer et al., 1994; Sartoris and Pörtner, 1997). We may therefore hypothesize that low temperature impairs extracellular regulation of this ion, which results in an increase in haemolymph magnesium concentration and subsequent anaesthesia of the animals.

Ontogenetic changes of osmoregulation in decapod crustaceans are well documented (Charmantier, 1998; Charmantier et al., 2009), but only few data exist on the development of the capacity to regulate single ion species (Brown and Terwilliger, 1992; Newton and Potts, 1993). It is known mostly from work on adults, that ion transport and excretion takes place through specialized epithelia in antennal glands, the gut including the hepatopancreas, and the branchial cavity including the gills (Mantel and Farmer, 1983; Gerencser et al., 2001; Freire et al., 2008). These epithelia are rich in the enzyme Na^+/K^+-ATPase, which generates at least part of the driving force for transepithelial ion transport (Lucu and Towle, 2003). Regulation of haemolymph sodium, chloride and calcium levels may take place through uptake and extrusion via the gill epithelium. Antennal glands are involved in magnesium and sulphate excretion via urine production as well as in the reabsorption of calcium and potassium (Freire et al., 2008). Sulphate excretion and calcium storage during the moulting cycle is facilitated by the hepatopancreas (Ahearn, 1996; Gerencser et al., 2001).

In several decapod species, the development of ion regulatory sites during life history, by use of Na^+/K^+-ATPase as a marker, has been shown to correlate with different osmoregulatory capacities of larval, juvenile and adult stages (Felder et al., 1986; Lignot and Charmantier, 2001; Cieluch et al., 2004; Cieluch et al., 2005; Khodabandeh et al., 2005; Khodabandeh et al., 2006). Osmoregulatory capacity does not necessarily improve linearly with stage, but matches environmental conditions, which may be very different for each stage if life history is spent in a variety of habitats (marine, estuarine, limnic or semi-terrestrial, Cieluch et al., 2004; Anger et al., 2008). Similarly, it can be hypothesized that the lifestyle of a given stage is linked to the regulation of single ion species. Planktonic zoeal

stages may benefit from the specific regulation of magnesium and calcium as these ions might influence swimming activity, and from the regulation of sulphate, which influences buoyancy (Newton and Potts, 1993). In agreement with Robertson's rationale, highly active zoeal stages should exhibit low and the settling, predominantly benthic megalopa elevated haemolymph magnesium concentrations. Juveniles should have lower magnesium concentrations than the adults because they can be expected to be more agile than the adults as a result of their higher metabolic rates and associated food requirements in relation to body weight. A prerequisite for this is, however, that the larvae already possess functional tissues for the regulation of these ions. Measurements of haemolymph ion concentrations in the lobster *Homarus gammarus* (Newton and Potts, 1993) and *Cancer magister* (Brown and Terwilliger, 1992) suggest that the capacity for magnesium and sulphate excretion increases during life history as the antennal glands differentiate progressively (Khodabandeh et al., 2006).

Key objectives of this study were to identify ontogenetic and temperature-dependent changes in the capacity for extracellular ion regulation in the Sub-Antarctic lithodid crab *Paralomis granulosa*. Furthermore, we quantified larval activity in relation to haemolymph magnesium concentration and investigated whether it is possible to shift thermal limits of larvae and juveniles by experimentally altering extracellular magnesium concentration. It was not feasible to raise the larvae in artificial sea water, therefore only effects of an increase in magnesium concentration were tested. These investigations intended to elucidate the question whether the capacity for magnesium regulation influences thermal tolerance of larvae and juveniles and thereby the biogeography of lithodid crabs. As a model organism we chose the Sub-Antarctic lithodid crab *Paralomis granulosa*. This species occurs in the Magellanic province, which extends from the north of Chiloé Island in the Eastern Pacific (42°S) to the southern tip of South America, including the Falkland islands, and up to the South-western Atlantic to about 35°S (but here into deeper waters, see Boschi 1979; Boschi 2000). Around the tip of South America and at the Falkland Islands the animals encounter water temperatures in summer of 9 - 11 °C and in winter of 2 - 4 °C (Hoggarth, 1993; Lovrich and Vinuesa, 1993; Arntz et al., 1999; Arkhipkin et al., 2004). Larval development is fully lecithotrophic and highly abbreviated consisting of only two demersal zoeal stages and a megalopa stage (Campodonico and Guzman, 1981). Development of *P. granulosa* is completed in a relatively wide thermal range of 3 - 15 °C with highest survival rates at 6 and 9 °C; the species has therefore been classified as cold-eurythermal (animals from the Beagle Channel, Anger et al., 2003).

We present the first study of haemolymph sodium, magnesium, calcium, chloride and sulphate levels throughout ontogeny in an anomuran crab in relation to activity and

thermal tolerance in its early stages. We discuss the results in the context of the hypothesis that extracellular magnesium regulation co-determines the biogeography of decapod crustaceans in the Southern Ocean.

Materials and Methods

Animals

Male and ovigerous female *Paralomis granulosa* were obtained from local fishermen in Punta Arenas, Chile in April 2008. The animals were transported to the Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, Germany (AWI) on board RV Polarstern (ANT-XXIV/4) and thereafter kept in a recirculated aquarium system at 4 °C, 32.5 PSU and an artificial 12:12 h light:dark cycle. Two females were transferred to the biological laboratory on Helgoland, Germany (BAH) in June and placed in individual flow-through tanks (20 L) at 6 °C, 33 PSU and an artificial 12:12 h light:dark cycle. Water temperature was raised to 9 °C at 1 °C/day one week after arrival of the animals on Helgoland and subsequently kept constant at this temperature. Adult crabs were fed *ad libitum* with frozen mussels, shrimps or isopods.

Freshly hatched larvae were collected every morning in separate sieves (300 µm mesh size), which received overflowing water from the aquaria of each female. Actively swimming larvae were randomly selected and placed in groups of 10 animals in 400 mL glass bowls (diameter 10.5 cm) filled with filtered natural sea water (NSW) or natural sea water to which $MgCl_2*6H_2O$ was added and which was subsequently diluted to 32.5 PSU using deionised water (NSW + Mg^{2+}, for sea water ion composition see Table 1). Larvae were raised at 1, 4 or 9 °C without food. They were checked for deaths and moults daily and water was changed every other day. When the larvae reached the megalopa stage, they were provided with pieces of nylon mesh as a substrate. After metamorphosis to the first juvenile stage, animals were fed with *Artemia* sp. nauplii (Sanders Brine Shrimp Company) and water was changed daily.

Some juveniles of each female which had been raised at 9 °C were transferred to 1 °C and 4 °C respectively about 10 days after they had moulted into the first juvenile instar (for an overview of experiments and determined parameters see Table 2). During a three-weeks-incubation at the respective temperatures until haemolymph sampling, water change and feeding took place every other day.

Haemolymph collection

Haemolymph samples of larvae and juveniles raised in NSW at 9 °C were taken in the middle of each moult-stage (intermoult; Drach and Tchernigovtzeff, 1967) with n = 5 individuals per female. Larval and early juvenile body size is given as carapace length (CL) after McLaughlin et al. (2003): zoea I, two days after hatching (CL 2.0 mm); zoea II, three days after moulting (CL 2.1 mm); megalopa, 15 days after moulting (CL 1.9 mm); juvenile instar I and II, 20 days after moulting (crab I: CL 2.4 mm, crab II: CL 2.8 mm); juvenile instar I after transfer to 4 or 1 °C, 30 days after moulting (n = 3 or 4 of each female at each temperature). Haemolymph samples of zoea I (n = 6 of one female) and crab I (n = 4 of one female) raised in NSW + Mg^{2+} at 9 °C were taken on the same days as those raised in NSW.

Larvae and juveniles were superficially dried on filter paper and immersed in mineral oil to prevent evaporation and desiccation. Remaining sea water adhering to the carapace was quickly removed by use of a finely drawn out glass microcapillary. An unknown volume of haemolymph of each individual was drawn either from the heart (zoeae) or from the base of the last fully developed pereiopod (megalopae and juveniles) with a new glass microcapillary, and blown out into a centrifuge tube containing 20 µL of deionised water.

Haemolymph of adult male and female crabs acclimated to 4 °C was collected from the base of the last walking leg using needle and syringe after animals had been dabbed dry. A known volume of haemolymph was immediately diluted in deionised water to prevent agglutination. Sea water samples (n = 4) were collected for comparison and all samples were stored at -20 °C.

Ion chromatography

Ion composition of haemolymph and sea water was determined by ion chromatography (ICS-2000, Dionex®, Idstein, Germany) after the respective dilution of the samples with deionised water. A conductivity cell and a self-regenerating suppressor were used to reduce background conductivity. Cations (Na^{+}, Mg^{2+}, Ca^{2+}) were separated on an IonPac® CS16 column with methane sulphonic acid (30 mmol L^{-1}) as eluent at a flow rate of 0.36 mL min^{-1} at 40 °C. Anions (Cl^{-}, SO_4^{2-}) were separated on an IonPac® AS11-HC column with potassium hydroxide (30 mmol L^{-1}) as eluent at a flow rate of 0.30 mL min^{-1} at 30 °C. Ion concentrations were calculated in mmol L^{-1} relative to the Dionex® Six Cation-II or Five Anion Standards. As the initial dilution of the haemolymph of larvae and juveniles was unknown, the concentrations of the ions determined in each individual sample were summed and set to

100%. Subsequently, the fractional concentrations (%) of each ion was calculated to allow the comparison of means. The ion composition of sea water and haemolymph in adult specimens is given in mmol L^{-1} and in % fraction of the sum of all ions to illustrate the validity of the calculation (Table 1).

Spontaneous swimming activity of zoeal stages at culture temperatures

The culture bowls were filled with sea water to about 1 cm water depth so that vertical movements of the larvae were greatly excluded, and placed on a piece of laminated graph paper. Groups of up to five larvae of each female were placed in separate bowls and allowed to acclimate overnight. Watching each larva, the number of 5 mm grids passed by the animal within 5 min was counted. Afterwards, the larva was removed to prevent counting the same animal twice. Swimming activity was determined always in the middle of the moulting cycle. Zoea I larvae raised in NSW at 9, 4 or 1 °C were observed on day 2, 5 or 8 after hatching. Animals reared in NSW + Mg^{2+} at 9 or 1 °C were studied on day 2 or 8 after hatching. Zoea II larvae raised in NSW at 9, 4 or 1°C were investigated on day 3, 10 or 20 after moulting. Individuals reared in NSW + Mg^{2+} at 9 or 1 °C were studied on day 3 or 20 after moulting. Developmental times in the respective treatments will be published elsewhere, but see also Anger et al. (2003) for values in NSW.

Activity of zoea I and crab I during acute cold exposure

Zoeae and first instar juveniles reared at 9 °C either in NSW or NSW + Mg^{2+} were fixed in a temperature-controlled microchamber underneath a stereomicroscope (Olympus SZX16) equipped with a digital video camera (Olympus DP71) and software Cell D (Soft Imaging System) similar to the method described in Storch et al. (2009). The animals were superglued to a pointed glass microcapillary attached to a small glass table, which was placed to the centre of the mircrochamber filled with the respective culture sea water. Care was taken to fix the individuals to the capillary at the dorsal side of the cephalothorax slightly anterior to the heart region, which allowed the animals to freely move their appendages. Underneath the stereomicroscope the animals were observed laterally with the heart, scaphognathite, first maxillipeds (zoea I) and antennules (crab I) being clearly visible. In the evening, the animals were placed in the chamber, which was covered with a piece of cloth to prevent visual disturbance of the animal, and left to acclimate to the experimental control conditions at 9 °C overnight (at least 12 h). The next day, the chamber was sequentially cooled to 6, 3, 1 and -1 °C, with each temperature change taking 30 min and each acclimation period taking

90 min at the respective temperature steps. At the end of each acclimation period, beginning at the control temperature of 9 °C a 2 min video was recorded at 30 frames s^{-1}. All heart, scaphognathite, maxilliped and antennule beats during the 2 min were counted manually and are given as beats s^{-1}. In zoeal stages the maxillipeds are the appendages, which are mainly used for swimming. To estimate activity of first instar juveniles, the antennules were observed, because the crabs did not perform regular locomotory movements (using their pereiopods) in this experimental setup.

Statistical analyses

Before calculating means ± standard deviation (SD), outliers were identified by use Nalimov's test. One-way ANOVA and *post hoc* Tukey's multiple comparison tests were run to identify significant ($p < 0.05$) changes of haemolymph ion composition during ontogeny and upon cold exposure of crab I. To allow statistical analyses of percentage data of haemolymph ion composition, the data were transformed using an arc sine function. Two-way ANOVA and *post hoc* Bonferroni tests were performed to analyse effects of temperature and magnesium concentration on spontaneous swimming speed of zoea I and II. Two-way ANOVA for repeated measures and *post hoc* Bonferroni tests were conducted to identify effects of temperature and magnesium concentration on heart rates, scaphognathite beat rates and maxilliped/antennule beat rates of zoea I and crab I, respectively. An F-test was used to identify significant effects of magnesium concentration on slopes and elevations of linear regressions fitted to the heart rates of zoea I and crab I. Statistical and regression analyses were performed using Prism 4.0a.

Results

Ontogeny of haemolymph ion composition

Significant hyporegulation of haemolymph magnesium compared to the sea water magnesium fraction (4.7 ± 0.1 % of sum, 51 ± 1 mmol L^{-1}, Table 1) was observed in the megalopa stage (4.1 ± 0.2 % of sum, Fig. 1). The megalopa exhibited a similar magnesium fraction as the first juvenile stage and adult male specimens (4.0 ± 0.2 % of sum, 40 ± 2 mmol L^{-1}). The second juvenile instar and the ovigerous females displayed the lowest haemolymph magnesium fractions of 3.7 ± 0.1 % of sum and 3.6 ± 0.2 % of sum (35 ± 4 mmol L^{-1}) respectively.

The extracellular calcium fraction was significantly higher than that in sea water (0.92 ± 0.01 % of sum, 9.9 ± 0.2 mmol L^{-1}, Fig. 1) in all the investigated groups except the second juvenile stage. Zoeal stage I exhibited the highest value of 1.32 ± 0.09 % of sum, whereas the second juvenile stage displayed a calcium fraction similar to that in sea water.

The Ca^{2+}:Mg^{2+} ratio of all groups was significantly higher than that of sea water (0.20 ± 0.01). It did not change significantly until the second juvenile instar was reached and comprised values of e.g. 0.29 ± 0.01 in zoea I and 0.26 ± 0.01 in crab II. Adult female *P. granulosa* exhibited a significantly higher Ca^{2+}:Mg^{2+} ratio of 0.31 ± 0.01 compared to adult male specimens (0.27 ± 0.02).

Haemolymph sulphate fractions of all groups were significantly lower than that in sea water (2.5 ± 0.1 % of sum, 27 ± 1 mmol L^{-1}). During larval development the sulphate fraction increased significantly from 1.0 ± 0.1 % of sum in the zoea I to 1.8 ± 0.2 % of sum in the megalopa. There was no further change in the subsequent stages.

Sodium fractions in the haemolymph of the larval stages (e.g. zoea I: 46.8 ± 2.3 % of sum) were significantly higher than that in sea water (42.0 ± 0.6 % of sum, 453 ± 10 mmol L^{-1}) and those of the later stages (except adult (f), e.g. crab I: 43.3 ± 0.4 % of sum).

Chloride fractions in the haemolymph of the larval stages (e.g. zoea I: 46.0 ± 2.5 % of sum) were significantly lower than that in sea water (49.9 ± 0.3 % of sum, 538 ± 4 mmol L^{-1}) and those of the more advanced stages (except adult (f), e.g. crab I: 50.2 ± 0.7 % of sum).

Effect of cold exposure on haemolymph ion composition of crab I

The extracellular magnesium fraction of the first juvenile instar was significantly lower than that in sea water at all experimental temperatures. Incubation at 1 °C (3.6 ± 0.5 % of sum) resulted in a significant decrease of the magnesium fraction compared to 9 °C (3.9 ± 0.2 % of sum, Fig. 2).

The calcium levels at 4 °C and 9 °C were equal and were significantly higher than that at 1 °C (0.8 ± 0.2 % of sum). None of the values differed significantly from that in sea water in this comparison.

The Ca^{2+}:Mg^{2+} ratio of the juveniles kept at 1 °C (0.23 ± 0.04) was significantly reduced compared to that of the animals incubated at 4 °C and 9 °C (0.31 ± 0.06 and 0.29 ± 0.02 respectively) and was not significantly different from the sea water ratio.

The sulphate fractions were equally low in juveniles incubated at 4 °C or 9 °C (both 1.8 ± 0.2 % of sum) but was significantly increased in individuals at 1 °C (2.1 ± 0.2 % of sum) compared to the other temperatures. Nevertheless, all groups maintained sulphate values significantly below that of sea water.

Haemolymph sodium was significantly increased in the individuals at 4 °C (44 ± 4 % of sum) compared to the values at 9 °C and that in sea water.

The chloride level was significantly lower in the group at 4 °C (49 ± 1 % of sum) compared to the groups at 1 °C and 9 °C. None of the haemolymph chloride fractions were significantly different from that of sea water.

Spontaneous swimming activity of zoeal stages at culture temperatures

Swimming activity of the zoeal stages was characterized by a high degree of variability (Table 3). Amongst the larvae cultured in NSW, the zoeal stage I swam significantly less at 1 and 4 °C than at 9 °C. The zoea II only showed a tendency for lower activity at lower temperatures. There was also a tendency for lower swimming activity with proceeding development. At 9 °C the zoea I swam 1.5 ± 1.6 mm s^{-1} and the zoea II only 0.6 ± 0.5 mm s^{-1}. The maximal speed of a zoea I observed at 9 °C was 4.2 mm s^{-1}, which corresponds to 0.7 body lengths s^{-1}. Larvae raised in NSW + Mg^{2+} did not swim at all at 1 °C. At 9 °C they exhibited slightly lower speeds at the elevated magnesium level compared to NSW (zoea I: 0.4 ± 0.2 mm s^{-1}, zoea II: 0.3 ± 0.3 mm s^{-1}; not significant).

Activity of zoea I and crab I during acute cold exposure

Culture of the animals in NSW + Mg^{2+} resulted in a significant increase in the haemolymph magnesium fraction in both zoea I (7.8 ± 0.5 % of sum) and crab I (7.4 ± 0.2 % of sum) compared to those raised in NSW (zoea I: 4.6 ± 0.2 % of sum, crab I: 3.8 ± 0.2 % of sum).

Heart rate, scaphognathite beat rate, maxilliped beat rate (zoea I) or antennule beat rate (crab I) significantly decreased with temperature (9 to -1°C) in both stages and both incubation media (Fig. 3, for results of statistical analyses see Tables 4 and 5).

Two-way ANOVA for repeated measures detected a significant effect of magnesium concentration on heart rate in the first juvenile instar, but not in the zoea I (Fig. 3A and D, Table 4). Despite this, linear regression analysis revealed that the *y*-intercept of the linear fit of heart rate by temperature of both stages was significantly lower in NSW + Mg^{2+} than in NSW (Table 5).

Scaphognathite beat rate was not influenced by the culture medium (Table 4). However, there was a trend for a steeper decrease upon cooling in NSW + Mg^{2+} in both zoea I and crab I (Fig. 3B and E). Zoea I in NSW + Mg^{2+} did not ventilate anymore at 1 and -1 °C, whereas the larvae in NSW as well as the juveniles in both incubation media did not cease ventilation completely even at the lowest temperature of -1 °C. Data of the ventilation rates

of the first juvenile instar were best fitted by a Gaussian curve for the NSW and a linear slope for the NSW + Mg^{2+} treatments respectively (see Table 5 for goodness of fits).

Maxilliped beat rate of zoea I was significantly lower in NSW + Mg^{2+} than in NSW at 1, 3 and 6 °C (Fig. 3C). The larvae in NSW + Mg^{2+} stopped swimming movements at 1 °C whereas the larvae in NSW were still performing some swimming movements even at -1 °C. Gaussian curves fitted the data with maxima of 9.6 beats s^{-1} at 6.6 °C in NSW and of 7.7 beats s^{-1} at 7.6 °C in NSW + Mg^{2+} respectively. Two-way ANOVA for repeated measures revealed a significant interaction between the effect of temperature and that of magnesium (Table 4).

Antennule beat rate of the first juvenile stage was not significantly affected by magnesium concentration (Fig. 3F). However, no antennule beats were observed at -1 °C in animals in NSW + Mg^{2+}, whereas individuals in NSW exhibited still some movements at this temperature.

Discussion

Ontogeny of haemolymph ion regulation

Haemolymph magnesium fractions decreased during larval development of *P. granulosa.* This implies that active magnesium hyporegulation improved successively. The high haemolymph magnesium fractions in the zoeal stages may indicate that the antennal gland was not yet entirely functional during early ontogeny (Khodabandeh et al., 2006). The megalopa stage already exhibited the same capacity for magnesium extrusion as the first juvenile stage and adult males. This suggests that antennal gland function was developed in this last larval stage. Similarly, hyporegulation of magnesium improved during ontogeny of the lobster *Homarus gammarus* (Newton and Potts, 1993) and of *Cancer magister* (Brown and Terwilliger, 1992). Whereas in these species larvae and juveniles did not reach haemolymph magnesium levels of the strongly hyporegulating adults, juveniles of the weak hyporegulator *Chionoecetes opilio*, comparable to our results, regulated magnesium to the same extent as the adults (Charmantier and Charmantier-Daures, 1995).

We observed a difference in haemolymph magnesium concentration dependent on the gender of crabs. The significantly lower extracellular magnesium concentration in ovigerous females compared to that of the males might be linked to their enhanced activity during brooding and release of larvae. However, even the significantly lower Ca^{2+}:Mg^{2+} ratios for juveniles subjected to cold exposure and significantly higher ratios of the adult female individuals are within Robertson's range of values for "semi-narcotized" species

(Robertson, 1953). Neither the magnesium fraction nor the Ca^{2+}:Mg^{2+} ratio show patterns that parallel the activity level of the ontogenetic stages. Following Robertson's (1953) rationale, the determined Ca^{2+}:Mg^{2+} ratios imply that the activity level is the same in larvae, the first juvenile instar and adult female *P. granulosa*. In contrast, zoeal stages are more active than the megalopa and juveniles should forage more actively than the adults, due to their higher weight-specific energy requirements (Zanders and Rodriguez, 1992; Anger, 2001).

All larval stages as well as the first juvenile instar kept the haemolymph calcium fraction above that of sea water. This observation is similar to earlier results on megalopa, juvenile and adult stages of brachyuran species. They hyperregulate calcium concentration or keep it close to the level of the surrounding medium (Brown and Terwilliger, 1992; Charmantier and Charmantier-Daures, 1995). The carapace of adult crabs is heavily calcified whereas that of zoeae exhibits only a small degree of calcification and therefore is lightweight, rather soft and transparent (Anger, 1996; Spicer and Eriksson, 2003). Higher haemolymph calcium fractions in the early stages may result from an existent ability for calcium uptake without or low incorporation of the ion into the exoskeleton during relatively short moulting cycles.

The lobster *Homarus gammarus* equally enhances the capacity for magnesium and sulphate excretion during ontogeny and has a higher capacity for the regulation of these ions during the whole life cycle than *P. granulosa* zoeae (Newton and Potts, 1993). It is astonishing that the first zoeal stage of *P. granulosa* manages to reduce sulphate to a level half of that of adults. First, the surface to volume ratio is higher in smaller animals. Therefore it will be energetically more costly for the larvae to maintain a large gradient between the external sea water and the haemolymph. Second, one might expect that the capacity for magnesium and sulphate regulation is similar in a stage, given that both ions are excreted by the antennal glands in adult crabs (Robertson, 1949; Robertson, 1953; Zanders, 1980; Freire et al., 2008), although by independent transport mechanisms (Zanders and Martelo, 1984). This suggests that the antennal glands are not yet fully functional and sulphate is excreted at a different site, e.g. the hepatopancreas (Gerencser et al., 2001). Alternatively, differential expression or activation of ion transporters could cause these results. The digestive system, including the hepatopancreas, undergoes morphological and functional changes during ontogeny depending on the utilisation of external food versus endogenous reserves (Nishida et al., 1995; Abrunhosa and Kittaka, 1997; Anger, 2001; Saborowski et al., 2006). This may also be accompanied by changes in the ion regulatory capacity. The benefit of enhanced sulphate hyporegulation in the zoeae could be a reduction of density (Newton and Potts, 1993). Replacement of the heavy anion sulphate with a lighter anion will increase buoyancy and reduce the amount of energy required for swimming. Consistent with this is the increased

haemolymph concentration of sulphate in the preferentially benthic megalopa stage and in the benthic juveniles and adults.

Interestingly, haemolymph sodium and chloride fractions of the larval stages were respectively higher and lower than in the juvenile stages and the adult male specimens. This indicates that haemolymph composition changes substantially at the transition from the non-feeding and developing larval to the feeding juvenile phases. Sodium and chloride are the inorganic ions, which mainly determine the osmolarity of the haemolymph (Charmantier et al. 2009). Adult specimens of this family are isosmotic to sea water (Mackay and Prosser, 1970; Thomas and Rice, 1992; Wittmann et al., 2010) and at least 2-year-old juvenile and adult *Paralithodes camtschatica* exhibit a low capacity for osmoregulation (Mackay and Prosser, 1970; Thomas and Rice 1992). We therefore expect the larvae of *P. granulosa* also to be isosmotic to sea water. Future studies should include measurement of other charged compounds (e.g. bicarbonate, free amino and fatty acids, protein) as well as osmolarity and actual ion concentrations to better understand the significance of these differences in sodium and chloride.

Effect of temperature on ionic regulation in the first juvenile instar

Interestingly, three weeks of exposure to 1 °C, which is a temperature below the naturally encountered thermal range of this species (Hoggarth, 1993; Lovrich and Vinuesa, 1993), did not affect the extracellular concentration of all investigated ions in the same way. Whereas haemolymph magnesium excretion was affected only slightly, sulphate excretion and calcium uptake were hampered upon cold exposure. If only passive processes (water and ion permeability) were affected by temperature, we expect that all considered ions are affected similarly and would reach equilibrium with sea water. We therefore suggest that differential effects of temperature on energy-consuming active ion transport processes caused the present results.

The hypothesis can be rejected that a thermally induced breakdown of magnesium regulation is responsible for the slow-down of cardiovascular functions and locomotory movements in the cold, because magnesium levels were maintained at a low level during cold exposure. However, more extreme lethal conditions like exposure to temperatures far below natural conditions or extended emersion/hypoxia may induce an increase in haemolymph magnesium concentration (Tentori and Lockwood, 1990; Whiteley and Taylor, 1992).

The significance of haemolymph sulphate regulation is not well understood. Intracellularly, sulphate plays a role in the sequestration of xenobiotics in crustaceans

(Ahearn, 1996; Gerencser et al., 2001). In the extracellular compartment, sulphate in the form of sulphonated compounds is involved in cell-cell adhesion (Sugahara et al., 1996), cell-cell communication and modification of second-messenger cascades (Bowman and Bertozzi, 1999; Strott, 2002). It is remarkable, that similar to our results during cold exposure, stress induced by high salinity first led to a breakdown of sulphate regulation and only at extreme salinities and temperatures to an impairment of magnesium regulation in decapods (Campbell and Jones, 1989; Zanders and Martelo, 1984) and amphipods (Kiko et al., 2009). We may therefore conclude that increased haemolymph sulphate concentration indicates sublethal thermal constraints.

Altered regulation of ions in the cold, especially with respect to calcium uptake, may render successful moulting more difficult, because a number of ion transport processes are involved (Robertson, 1960; Glynn, 1968; Greenaway, 1985; Mercaldo-Allen, 1991; Chang, 1995; Ahearn et al., 2004). Whereas the megalopa will not moult to the first juvenile stage at 1 °C (Anger et al., 2003), it is not known whether the juveniles are able to grow and moult at temperatures as low as this. We observed that all experimental animals survived the three-weeks exposure to cold. They were feeding on *Artemia* sp. nauplii, as evidenced by the orange colouration of the stomach that was visible through the carapace.

Effects of temperature and magnesium on activity

When comparing the physiological data on zoea I and crab I it should be kept in mind that the zoea I was constantly swimming at most temperatures investigated, whereas the first juvenile instar was not performing regular locomotory movements. Similar to adults (Cumberlidge and Uglow, 1977; De Wachter and Wilkens, 1996), larvae and juveniles will exhibit higher heart and ventilation rates during locomotion than during rest due to the higher oxygen requirement. Heart rate was only marginally affected by a change of external magnesium concentration in the resting adult spider crab *Maja squinado*. However, stroke volume and cardiac output increased significantly, when magnesium concentration was reduced (Frederich et al., 2000a). This might be similar in larval and juvenile crabs, but unfortunately we were not able to derive these data from our recordings because resolution was too low. Cardiac output can be modulated by both heart rate and stroke volume independently. The extent to which these two factors are modulated may change during development and in response to abiotic factors (McMahon and Burnett, 1990; McGaw et al., 1994; Reiber and Harper, 2001; Harper and Reiber, 2004). For example, temperature affected stroke volume and cardiac output of zoea I larvae of the majid crab *Taliepus dentatus*

differently, depending on the origin of the specimens. But the effect of temperature on heart rate did not differ between the two populations (Storch et al., 2009).

An effect of magnesium on the thermal response of ventilation rate, as reported here, has not previously been investigated, neither in early life-history stages nor in adult crabs. The trend for larger slopes at higher magnesium concentration in both the zoea I and the first juvenile instar may reflect a change in the function of the respiratory pigment haemocyanin. The pigment is already present in oocytes and capable to bind oxygen in zoeae of *Cancer magister* (Terwilliger and Dumler, 2001). High magnesium concentrations increase the oxygen affinity of the pigment (Terwilliger, 1998; Bridges, 2001). This might impede unloading of oxygen in the tissues and might be compensated for by an increased ventilation rate to maintain oxygen supply. The scaphognathite beat rate of first stage zoeae was generally more variable than that of the juveniles. This may indicate that the gills are not yet fully developed and that respiratory gas exchange via the thin and transparent cuticle plays a role in the zoea I (Spicer and Eriksson, 2003).

Our results suggest that the zoeal stages of *P. granulosa* are strong swimmers despite naturally high haemolymph magnesium levels and will perform sustained swimming (maxilliped beating) when experimentally kept in the water column. When spontaneous activity was monitored in the culture bowls, however, the larvae spent most of the time sitting on the bottom. This may either be due to the confinement to a small stagnant water body, or it may reflect the demersal character of these lecithotrophic larvae, which so far have not been found in plankton samples (Lovrich, 1999; Anger et al., 2003). In any case, very low mean spontaneous swimming speeds were observed, which even at the highest temperature (9 °C, 1.5 ± 1.6 mm s^{-1}) were at least ten times slower than maximal swimming speeds previously reported for crustacean larvae (see review by Chia et al., 1984). For example planktotrophic zoeae of the red king crab *Paralithodes camtschatica* (CL 1.2 mm, Zaklan, 2002 and references therein), which are only half the body size of the zoea I of *P. granulosa*, swim 17.0 mm s^{-1} at 4 - 6 °C (Shirley and Shirley, 1988; recorded during sustained swimming in response to a light stimulus). It is striking that the maximally observed maxilliped beat rate of *P. granulosa* zoea I is just as high as that of the planktotrophic zoea I of *Taliepus dentatus* at a comparable temperature (10 s^{-1} at 7 °C, Storch et al., 2009). This illustrates, that on the one hand, *P. granulosa* zoeae may swim just as actively as planktotrophic larvae, if they are forced. On the other hand, their disposition to move is very low as indicated by their low mean spontaneous swimming speed.

The anaesthetizing effect of a magnesium concentration twice as high as in natural sea water on spontaneous swimming is visible only as a weak trend, but is clearly observed during sustained swimming, when maxilliped beats of zoea I are considered. The significant

interaction between the effects of temperature and magnesium indicates that both factors influence the maxilliped beat rate synergistically (Quinn and Keough, 2006). Increased magnesium concentration caused not just a shift of the curve by about 2 °C towards warmer temperatures, but also a reduction of the maximal speed and likely the optimal temperature range. Cold tolerance of sustained swimming activity in zoea I was thus impaired by a supranaturally high magnesium concentration.

An increase in magnesium concentration did not influence antennule beat rate of the juveniles, but as the antennule is a neuromuscular system, it should also be affected. Conspicuously, movements with a relatively low frequency (heart, scaphognathite and antennule beats) of both stages were hardly affected whereas sustained high-frequency swimming movements of the zoea were significantly decreased by the elevated magnesium concentration. The fact that the high-frequency locomotory system is more susceptible to increased magnesium concentration compared to the low-frequency systems may be due to their structural and biochemical differences. Generally, muscle fibre composition as well as innervation is related to functional properties in crustacean motor complexes (Silverman and Charlton 1980; Atwood and Lnenicka 1987; Stephens 1990; Millar and Atwood 2004). Different types of motor complexes may be differentially affected by magnesium and low temperature.

We may conclude from the present findings, that extracellular magnesium concentration is a factor, which influences cold tolerance of actively swimming zoea larvae. The biogeography of species with planktotrophic larvae exhibiting a low capacity for magnesium regulation may therefore be constrained by low temperature. Future studies should investigate ionic regulation of subpolar and polar brachyuran and caridean planktotrophic larvae. This should show whether the cold tolerance and biogeography of Antarctic caridean shrimps is linked to enhanced magnesium excretion of their larvae. For the lecithotrophic demersal larvae of *P. granulosa* it is important to save energy until metamorphosis to the first feeding stage, the first juvenile instar. This is most easily done by reducing the disposition for activity and the cost of activity. In the more active zoeal stages naturally high haemolymph magnesium, which may reduce swimming activity, and low sulphate concentrations, which may affect buoyancy, may serve to save energy. During cold exposure, not only activity is slowed down, but also ionic regulation is affected (as shown for the first juvenile stage). This may indicate a mismatch between energy supply and demand, or a limitation in the capacity of ion pumps. In order to develop and grow at low temperatures it is not enough to maintain activity, but maintenance of ion regulation is crucial especially during moulting to the next stage. Therefore, not just magnesium

excretion, but adaptation of the entire ion regulatory capacity to low temperature is a prerequisite for crustacean species to inhabit polar regions.

Acknowledgements

We greatly appreciate the help of the crew of RV Polarstern and Fredy Véliz Moraleda during the transport of live animals. We would like to thank Uwe Nettelmann and Dana Pargmann for their assistance with culturing larvae and juveniles and Charlyn Völker and Marc Bullwinkel for their help with the analysis of the videos. The experiments comply with the current laws of the country in which they were performed. This study was supported by Deutsche Forschungsgemeinschaft grants no. SA 1713/1-1 and 1-2 and by the Alexander von Humboldt Foundation, Germany (Feodor Lynen fellowship to DS). The authors declare that there is no conflict of interest.

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Table 1: Ion composition (mmol L^{-1}, % of sum) of culture media and haemolymph of adult specimens of *Paralomis granulosa*. NSW: natural sea water, salinity 32.5 PSU, NSW + Mg^{2+}: natural sea water to which $MgCl_2$ * 6 H_2O was added and which was subsequently diluted down to 32.5 PSU, n = number of samples. Data of adult (m) from Wittmann et al. (2010).

	n	Unit	Na^+	Cl^-	Mg^{2+}	Ca^{2+}	SO_4^{2-}	Sum
NSW	4	mmol L^{-1}	453 ± 10	538 ± 4	51 ± 1	9.9 ± 0.2	27 ± 1	1078 ± 10
		% of sum	42.0 ± 0.6	49.9 ± 0.3	4.7 ± 0.1	0.92 ± 0.01	2.5 ± 0.1	100
NSW + Mg^{2+}	1	mmol L^{-1}	383	580	97	7.8	24	1092
		% of sum	35.1	53.1	8.9	0.7	2.2	100
Adult (m)	7-8	mmol L^{-1}	430 ± 10	491 ± 15	40 ± 2	10.7 ± 0.4	17 ± 2	994 ± 16
		% of sum	43.5 ± 1.1	49.6 ± 1.0	4.0 ± 0.2	1.07 ± 0.04	1.8 ± 0.2	100
Adult (f)	4-5	mmol L^{-1}	442 ± 22	464 ± 18	35 ± 4	10.7 ± 0.5	17 ± 2	970 ± 46
		% of sum	45.6 ± 0.3	47.9 ± 0.5	3.6 ± 0.2	1.08 ± 0.05	1.8 ± 0.2	100

Table 2: Overview of experiments and parameters determined at the respective developmental stages. NSW: natural sea water, NSW + Mg^{2+}: sea water of increased magnesium concentration. T: temperature, ZI: zoea I, ZII: zoea II, M: megalopa, CI: crab I, CII: crab II, A: adult, frq: frequency

Determined parameters	Conditions		ZI	ZII	M	CI	CII	A
	T (°C)	Culture media						
Haemolymph ion composition	4	NSW						x
	9	NSW	x	x	x	x	x	
Haemolymph [Mg^{2+}]	9	NSW + Mg^{2+}	x			x		
Haemolymph ion composition after 3 wk cold exposure	1	NSW				x		
	4	NSW				x		
	9	NSW				x		
Spontaneous swimming activity at culture temperatures	1	NSW & NSW + Mg^{2+}	x	x				
	4	NSW	x	x				
	4	NSW + Mg^{2+}	x					
	9	NSW & NSW + Mg^{2+}	x	x				
Activity rates during acute cold exposure:								
Heart frq	-1, 1, 3, 6, 9	NSW & NSW + Mg^{2+}	x			x		
Scaphognathite frq	-1, 1, 3, 6, 9	NSW & NSW + Mg^{2+}	x			x		
Maxilliped frq	-1, 1, 3, 6, 9	NSW & NSW + Mg^{2+}	x					
Antennule frq	-1, 1, 3, 6, 9	NSW & NSW + Mg^{2+}				x		

Table 3: Spontaneous swimming activity (mm s^{-1}) of zoeal stages at the respective culture temperatures and media. Number in brackets are numbers of observed animals. The culture medium did not have a significant effect on swimming speed in neither zoeal stage. NSW: natural sea water, NSW + Mg^{2+}: sea water of increased magnesium concentration. * significantly different from value at 9 °C. n.d. not determined.

Culture temperature (°C)	**NSW**		**NSW + Mg^{2+}**	
	zoea I	zoea II	zoea I	zoea II
1	0.07 ± 0.14 (9)*	0.02 ± 0.02 (9)	0 ± 0 (5)*	0 ± 0 (5)*
4	0.4 ± 0.2 (4)*	0.1 ± 0.2 (9)	n.d.	n.d.
9	1.5 ± 1.6 (9)	0.6 ± 0.5 (9)	0.4 ± 0.2 (5)	0.3 ± 0.3 (5)

Table 4: Results of two-way ANOVA for repeated measures to assess the effects of temperature and magnesium on heart rate, scaphognathite beat rate, maxilliped beat rate and antennule beat rate of zoea I and crab I during acute tempeature reduction (Fig. 3). Frq frequency.

Stage	**Response variable** (beats s^{-1})	**Magnesium effect**			**Temperature effect**			**Interaction**		
		F	df	p	F	df	p	F	df	p
Zoea I	Heart frq	1.94	1	0.20	82.18	4	**<0.0001**	0.57	4	0.68
	Scaphognathite frq	0.005	1	0.95	19.61	4	**<0.0001**	1.74	4	0.17
	Maxilliped frq	70.85	1	**<0.0001**	59.47	4	**<0.0001**	2.79	4	**0.04**
Crab I	Heart frq	6.23	1	**0.04**	388.67	4	**<0.0001**	1.35	4	0.27
	Scaphognathite frq	4.94	1	0.06	38.81	4	**<0.0001**	3.25	4	**0.02**
	Antennule frq	0.42	1	0.27	91.09	4	**<0.0001**	1.35	4	0.27

Table 5: Best-fit values (r^2) of linear or nonlinear regression analyses and results of an F-test, which detected significant differences dependent on the culture medium between the elevations of the linear fits of the heart rates of both zoea I and crab I during acute temperature reduction (Fig. 3). Frq frequency.

Stage	**Response variable** (beats s^{-1})	**Type of fit**	**NSW** r^2	**NSW + Mg^{2+}** r^2	**Slopes** F	df	p	**Elevations** F	df	p
Zoea I	Heart frq	Linear	0.7090	0.9242	0.17	1	0.68	5.11	1	**0.03**
	Maxilliped frq	Gaussian	0.8638	0.8956	-	-	-	-	-	-
Crab I	Heart frq	Linear	0.9170	0.9385	2.05	1	0.16	11.79	1	**0.001**
	Scaphognathite	Linear		0.7320	-	-	-	-	-	-
	frq	Gaussian	-0.8266	-	-	-	-	-	-	-

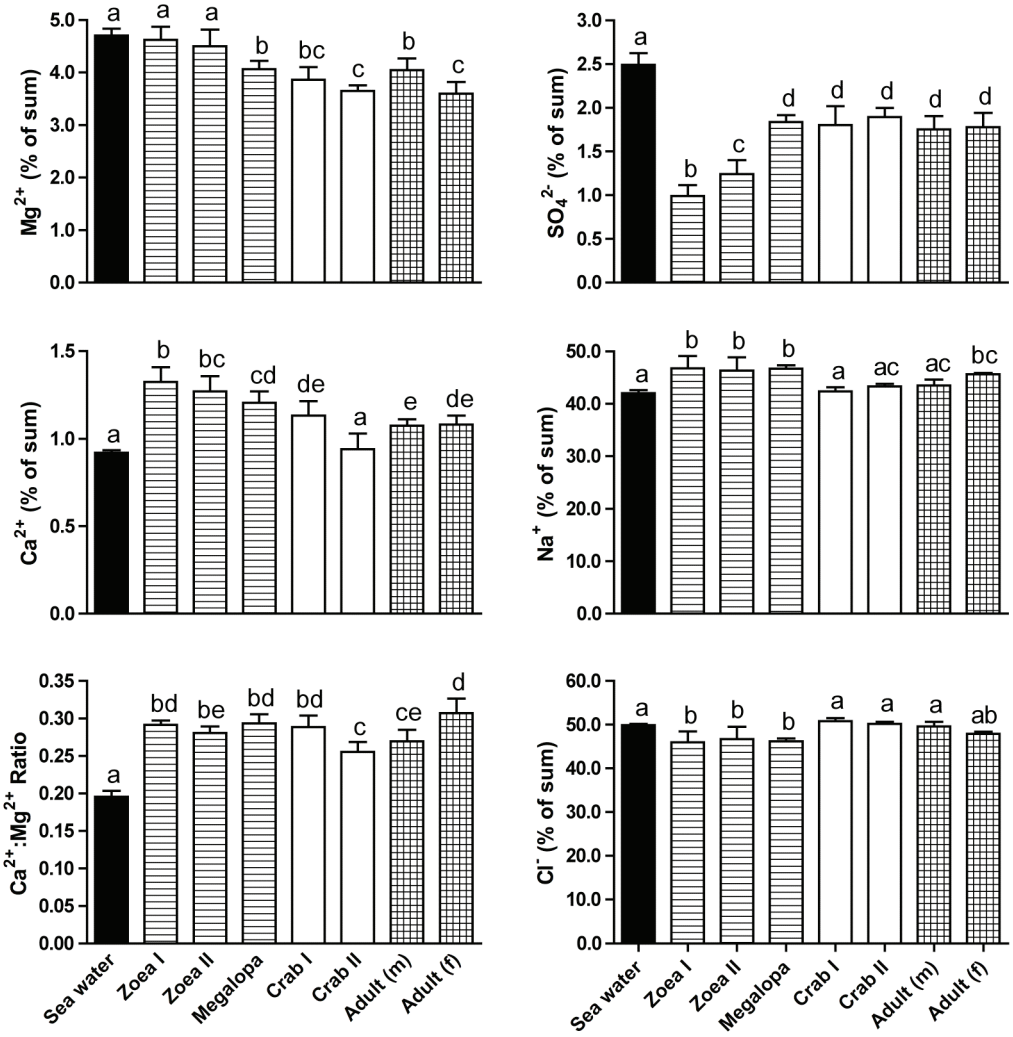

Figure 1: Sea water and haemolymph magnesium, calcium and sulphate, sodium and chloride fractions given in percent of the sum of ion concentrations as well as Ca^{2+}:Mg^{2+} ratio during complete larval development, in the first and second juvenile instars (crab I and II) and in adult males (m) and ovigerous females (f) of *Paralomis granulosa*. Acclimation temperatures zoea I – crab II: 9 °C, adult (m) and (f): 4 °C. Values are means ± SD. Sea water: n = 4, larvae and juveniles: n = 8-10, adult (m): n = 8, adult (f): n = 4-5. Different letters denote groups which are significantly different from each other ($p < 0.05$). Data for adult (m) from Wittmann et al. (2010).

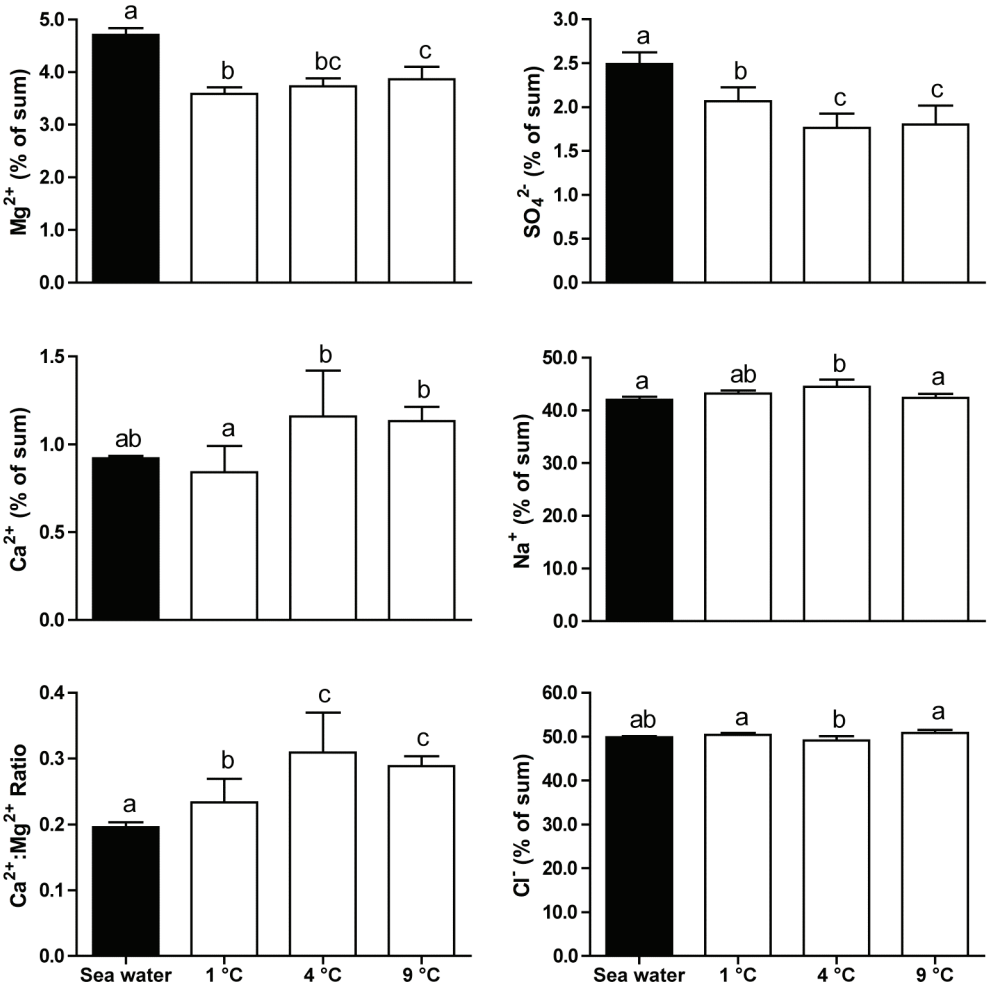

Figure 2: Change of haemolymph ion composition (% of the sum of ion concentrations) and Ca^{2+}:Mg^{2+} ratio in first instar juveniles of *Paralomis granulosa* raised at 9 °C after three weeks of cold exposure at 1 or 4 °C. Values are means ± SD. Sea water: n = 4, 1 °C and 4 °C: n = 6-7, 9 °C: n = 9. Different letters denote groups which are significantly different from each other ($p < 0.05$).

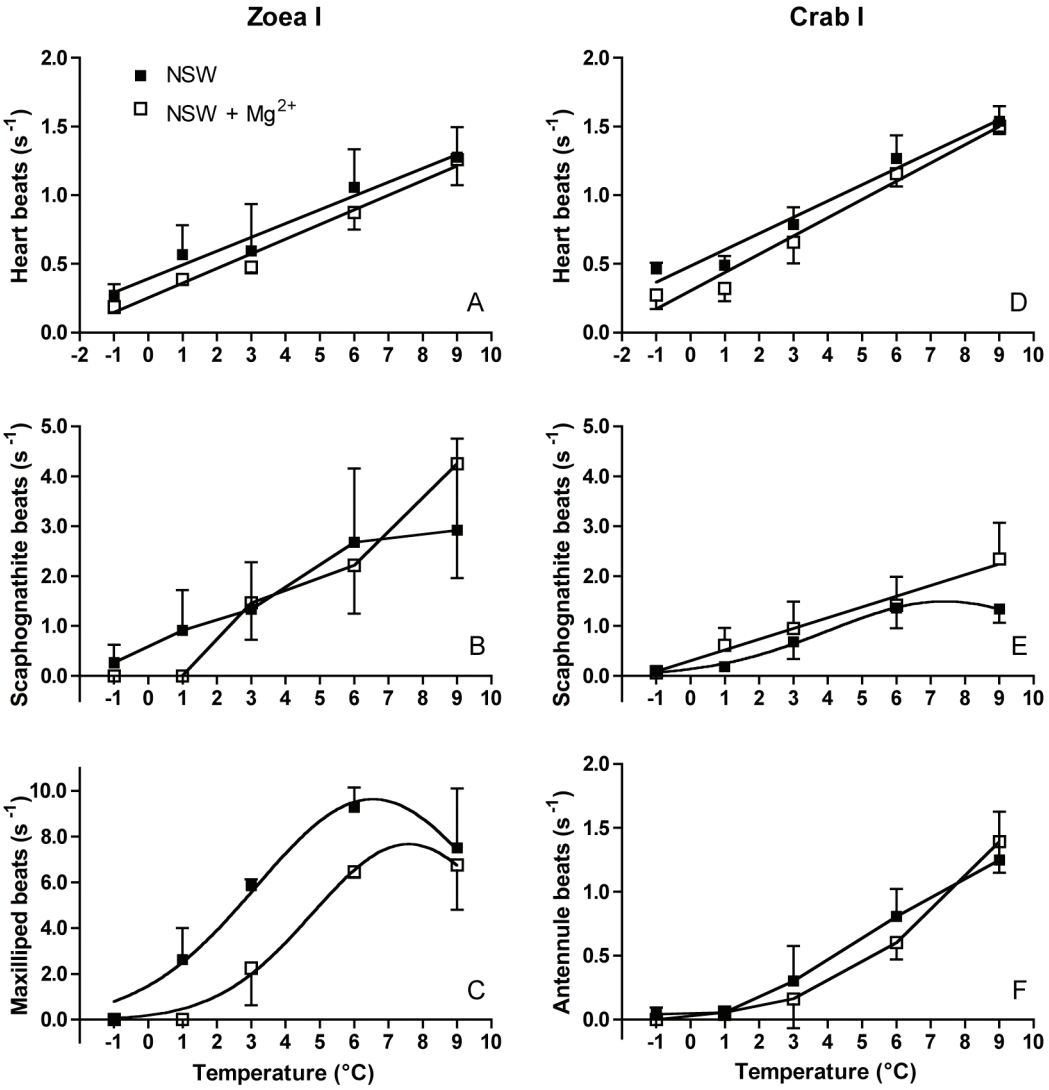

Figure 3: Effect of temperature and increased magnesium concentration on heart rate (A), scaphognathite beat rate (B) and maxilliped beat rate (C) in zoea I and on heart rate (D), scaphognathite beat rate (E) and antennule beat rate (F, s^{-1}) of crab I of *Paralomis granulosa* during successive cooling, starting at the acclimation temperature of 9 °C. Note the different scales of maxilliped and antennule beat rate. NSW: natural sea water, $[Mg^{2+}]$ = 51 mmol L^{-1}, 4.7 ± 0.1 % of sum, zoea I: $[Mg^{2+}]_{HL}$ = 4.6 ± 0.2 % of sum, crab I: $[Mg^{2+}]_{HL}$ = 3.8 ± 0.2 % of sum, NSW + Mg^{2+}: natural sea water plus $MgCl_2$ * 6 H_2O, $[Mg^{2+}]$ = 97 mmol L^{-1}, 8.9 % of sum, zoea I: $[Mg^{2+}]_{HL}$ = 7.8 ± 0.5 % of sum, crab I: $[Mg^{2+}]_{HL}$ = 7.4 ± 0.2 % of sum. Means ± SD, zoea I: n = 4-6, crab I: n = 5, some error bars are not visible as they are smaller than the plot symbols.

Publication III

The role of oxygen delivery and extracellular magnesium in thermal tolerance of the sub-Antarctic stone crab *Paralomis granulosa* (Jacquinot)

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(Submitted)

The role of oxygen delivery and extracellular magnesium in thermal tolerance of the sub-Antarctic stone crab *Paralomis granulosa* (Jacquinot)

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Keywords: Antarctic, *Paralomis granulosa*, activity, thermal tolerance, magnesium

Summary

A low capacity for extracellular Mg^{2+} regulation has been proposed to constrain marine decapod crustaceans to waterbodies of above 0°C of the sub-Antarctic. Thermal tolerance was determined in the sub-Antarctic lithodid crab *Paralomis granulosa* using an acute stepwise temperature protocol (-1, 1, 4, 7, 10, 13°C). Arterial and venous haemolymph oxygen partial pressures ($HLPO_2$), heart and ventilation frequency and haemolymph cation composition were measured at rest and after a forced activity (righting) trial. Scopes for heart and ventilation were calculated and intermittent heart and scaphognathite beating at rest was evaluated. Haemolymph [Mg^{2+}] was experimentally reduced from 30 mmol L^{-1} to a level naturally observed in Antarctic caridean shrimps (12 mmol L^{-1}) to investigate whether the animals remain more active and tolerant to cold (-1, 1, 4°C). In natural sea water, righting speed was significantly reduced at -1 and 13°C compared to acclimation temperature (4°C). Arterial and venous $HLPO_2$ increased in response to cooling even though heart and ventilation rates as well as scopes decreased. At rest ionic composition of the haemolymph was not affected by temperature. Activity induced a significant rise in haemolymph [K^+] at -1 and 1°C. Reduction of haemolymph [Mg^{2+}] did not result in an increase in activity, heart and ventilation rates or a shift in thermal tolerance to lower temperatures. In conclusion, oxygen delivery in this cold-water crustacean was not acutely limiting cold tolerance and animals may have been constrained more by their functional capacity and motility. These constraints remained insensitive to changing Mg^{2+} levels.

Introduction

The concept of oxygen- and capacity-limited thermal tolerance has been supported by work on specimens from various marine ectothermic animal phyla from various climate zones (for review see e.g. Pörtner, 2002, 2010, and addressing the Antarctic: Pörtner, 2006). It states, that tolerance to low and high temperatures is set at the highest organisational level, the whole organism. Limiting processes include the capacities of cardiovascular and ventilatory systems to deliver oxygen to the tissues. Within limits these capacities are subject to adaptive adjustments at the molecular, cellular and tissue levels. These should support resting conditions as well as routine rates of activity, growth and reproduction within the temperature range of optimal performance, which defines the biogeographic range of a species (Pörtner and Knust, 2007). Aerobic scope or aerobic functional flexibility, the increment from a rate at rest to an active rate, is thought to be the first process to experience thermal limitation. It may be determined as the increase in metabolic rate during forced activity (metabolic scope for activity, Bennett, 1978; Crear and Forteath, 2000; Fry and Hart, 1948; Rutledge and Pritchard, 1981) or after feeding (specific dynamic action, Robertson et al., 2002), but is also thought to support temperature-dependent growth. Aerobic scope has not been investigated in an array of crustaceans from different latitudes. To date, the hypothesis has mainly been based on investigations of systemic variables at rest in temperate species. In fish the thermal specialization of aerobic scope has also been investigated during exercise (e.g. Farrell et al., 2008). In the face of ocean warming, studies on cold-adapted species have focussed on thresholds of heat tolerance. But to understand the implications of thermal tolerance for biogeography, it is also important to consider the lower temperature thresholds, especially if we want to apprehend the distribution of decapod crustaceans in the Southern Ocean.

Decapod crustacean diversity is low in the Antarctic compared to sub-Antarctic, temperate and tropical regions (Gorny, 1999; Astorga et al., 2003). Brachyuran crabs occur in the warmer sub-Antarctic at water temperatures above 0°C but not in the high Antarctic at temperatures as low as –1.8°C (Gorny, 1999). The biogeography of the anomuran lithodid crabs is probably also constrained by low temperature, as this group has only been found in waters warmer than 0°C (Hall and Thatje, 2009). Their southernmost habitat is the continental slope of the western Antarctic Peninsula in the Bellingshausen Sea (García Raso et al., 2005; Thatje et al., 2008). In fact, lithodid crabs are the only group of reptant decapod crustaceans found in warmer water bodies of the Antarctic. They are expected to be amongst the first to expand their distribution in the Antarctic in response to global warming (Thatje et

al., 2005). In contrast, caridean decapod shrimps are frequently found in shallow waters of the continental shelf of Antarctica (Gutt et al., 1991).

In the temperate spider crab *Maja squinado*, optimal arterial haemolymph PO_2 at rest correlated well with the thermal range naturally encountered by the species (9 – 19°C, Frederich and Pörtner, 2000). Furthermore, discontinuities in temperature-dependent arterial haemolymph PO_2 and ventilation frequency were used to define upper and lower pejus thresholds (pejus = getting worse). At the lower pejus temperature the temperature-dependent slope of ventilation frequency increased to the steep slope of the optimal range. Ventilation rate was maximal at the upper pejus temperature, where haemolymph PO_2 started to drop. In previous studies on the brachyuran crab *Cancer magister* changes in ventilation frequency as well as cardiac output related to changes in metabolic rate in response to temperature and activity (McMahon et al., 1978; McMahon et al., 1979). However, ventilation frequency rather than heart frequency or cardiac output limited active oxygen consumption and metabolic aerobic scope at both low and high temperatures in the temperate freshwater crayfish *Pacifastacus leniusculus* (Rutledge, 1981).

Processes at the tissue and cellular levels influence these systemic variables. Concerning the nervous system, results from several phyla suggest that thermal sensitivity rises in the following order: nervous conduction < direct stimulation of muscle < neuromuscular transmission at the synapse (Prosser and Nelson, 1981). Temperature-dependent neuromuscular function in leg muscle tightly relates to locomotory behaviour in the warm-stenothermal ghost crab *Ocypode ceratophthalma* (Florey and Hoyle, 1976). The temperature range of optimal neuromuscular transmission was much broader in eurythermal freshwater crayfish than in the stenothermal ghost crab and was shifted after thermal acclimation (Harri and Florey, 1977; White, 1983). Transmitter release at the synapse is an important parameter related to the speed of neuromuscular transmission (Millar and Atwood, 2004). Low temperature reduces quantal content in crayfish axons, which is thought to be the result of declined Ca^{2+} conductivity of the presynaptic membrane, and causes a reduction of signal transduction velocity (Dunn and Mercier, 2003).

Interestingly, the ion Mg^{2+} has a similar effect as it slows down neuromuscular transmission and reduces the contraction of muscle fibres probably by blocking Ca^{2+} influx at both the pre- and postsynaptic membranes (Dudel et al., 1982; Hagiwara and Takahashi, 1967; Parnas et al., 1994; Ushio et al., 1993). It has long been known, that Mg^{2+} can be used to paralyse vertebrates and invertebrates (Iseri and French, 1984; Katz, 1936; Lee et al., 1996; Pantin, 1948; Waterman, 1941). The capability to hyporegulate extracellular [Mg^{2+}] below ambient (50 mmol L^{-1}) has been suggested to influence the biogeography of decapod crustaceans in the Southern Ocean. Sub-Antarctic brachyuran and lithodid crabs are weak

Mg^{2+} hyporegulators (haemolymph [Mg^{2+}]: 20 – 50 mmol L^{-1}) and Antarctic caridean shrimps are strong Mg^{2+} hyporegulators (haemolymph [Mg^{2+}]: 5 – 12 mmol L^{-1}; Frederich, 1999; Frederich et al., 2000b; Wittmann et al., 2010). Low temperature and high [Mg^{2+}] may interact to reduce the scope for activity and affect ventilation and circulation. Referring to the concept of oxygen- and capacity-limited thermal tolerance, anomuran and brachyuran crabs may therefore be restricted to the subpolar temperature regime (Frederich, 1999; Frederich et al., 2000a; Frederich and Pörtner, 2000; Frederich et al., 2000b; Frederich et al., 2001; Pörtner, 2002; Sartoris et al., 1997).

In support of this hypothesis, it was shown for the temperate brachyuran *Maja squinado* that the experimental reduction of haemolymph [Mg^{2+}] to that in caridean shrimp species induces a downwards shift of the lower pejus threshold by 2°C (Frederich et al., 2000a). This was concluded from an increase in resting cardiac output and stroke volume, and from the fact that redirection of haemolymph flow to supply locomotion occurred at a lower temperature when haemolymph [Mg^{2+}] was reduced. Heart beat frequency, however, was not significantly affected. Reduced haemolymph [Mg^{2+}] significantly accelerated oxygen consumption, walking and righting activity and heart rate of 5°C-acclimated temperate and sub-Antarctic brachyuran crabs (Frederich et al., 2000b). The thermal tolerance window of these crabs was extended towards lower temperatures and even below 0°C.

The goal of this study was to characterise the thermal tolerance window of the sub-Antarctic lithodid crab *Paralomis granulosa*. Furthermore, the hypothesis was tested that extracellular [Mg^{2+}] plays a role in cold tolerance and therefore the biogeography of this species. Haemolymph PO_2, heart and ventilation frequency as well as haemolymph cation composition at rest and after a righting trial were determined at naturally high (30 mmol L^{-1}) and reduced (12 mmol L^{-1}) haemolymph [Mg^{2+}]. This is the first study to address the effect of temperature on the systemic physiology of a lithodid crab.

Materials and Methods

Animal maintenance

Male specimens of *Paralomis granulosa* (FW 500 ± 63 g, CL 9.6 ± 0.2 cm) were obtained from local fishermen in Punta Arenas, Chile in April 2008. The animals were transported to the Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, Germany on board RV Polarstern (ANT-XXIV/4) and thereafter kept in a recirculated aquarium system at 4°C, in natural sea water (NSW) of 32.5‰ salinity and an artificial 12:12 h light:dark cycle. The

crabs were fed *ad libitum* with frozen mussels, but remained unfed six days prior to experimentation.

Preparation of animals and experimental protocol

For repeated measurements of arterial haemolymph PO_2, a small hole was drilled through the carapace above the pericardial sinus without injuring the hypodermis. The hole was covered with a piece of latex dam. After a recovery period of 3 days one animal at a time was examined in a 100-L-experimental chamber. The individuals were fixed to a plastic grid by two pereiopods and the chelipeds to immobilize them. A plethysmograph infrared sensor (iSiTec, Bremerhaven, Germany; Depledge and Andersen, 1990) was superglued to the carapace posterior to the latex dam cover to monitor heart frequencies. Additional sensors were placed above each scaphognathite to record ventilation frequencies. After an acclimation period of 24 h at 4°C in the experimental chamber filled with NSW, haemolymph was withdrawn for the determination of arterial and venous haemolymph PO_2 and cation composition at rest. The animal was released from the plastic grid and a righting trial was carried out. Subsequently another set of haemolymph samples was taken. The animal was again fixed to the plastic grid and given 60 min to recover from handling stress, before the ramp to the next temperature step was started.

Water temperature was controlled with a thermostat (Lauda T1200) and Wintherm software (version 2.2). The temperature was reduced at a rate of 1°C h^{-1} to 1°C. After an acclimation period of 20 h measurements and sampling were repeated as stated above. The same procedure was used to collect data at -1°C after 20 h of acclimation. Then temperature was returned by 1°C h^{-1} to 4°C. The animal was released from the experimental chamber for two days and water was exchanged. In the second week the same animal was reintroduced into the chamber filled with NSW at 4°C and the protocol was repeated for the successive temperature steps 7, 10 and 13°C.

For experiments in artificial sea water with reduced [Mg^{2+}] (ASW –Mg^{2+}, NaCl was added to adjust for salinity, recipe modified after Langer et al., 2006) each animal was first transferred to a separate recirculated system at 4°C filled with ASW –Mg^{2+} for 6 days, until haemolymph [Mg^{2+}] had reached a new and constant value (see Table 1 for water and haemolymph cation concentrations). Subsequently, each animal was again examined at 4, 1 and -1°C with 20 h intervals for acclimation in between using the same protocol as above kept in ASW –Mg^{2+}. All animals survived experimentation.

Righting

The animals were detached from the plastic grid and allowed to recover for 10 min. They were turned to the dorsal side of the carapace and placed on the bottom of the experimental chamber. The time (s) was recorded until the animals had returned to an upright position and all of the pereiopods touched the ground. This was done seven times with 30 s intervals between trials, unless the animals did not right themselves twice consecutively within 180 s. The mean time-to-right was calculated as the mean of all seven bouts recorded in each animal.

Haemolymph PO_2

Haemolymph PO_2 was determined using oxygen optodes (sensor type IMP-PSt1-L5-LIC0-BFG3-TF-YOP), connected to TX3-AOT instruments with Oxyview TX3-v6.02 Software (PreSens, Regensburg, Germany). Temperature-compensated two-point calibration was carried out daily in water vapour (100% air saturation) and saturated sodium sulphite solution (0% air saturation) prior to measurements. Drawn out Pasteur pipettes equipped with oxygen sensors were used to remove haemolymph from the pericardial sinus (arterial haemolymph) or from the ventral sinus at the base of a pereiopod (venous haemolymph). After haemolymph collection (up to 10 µl), the tip of the pipette was immediately plugged with dental wax and submerged in the experimental chamber to keep the temperature stable during the measurement. Data were recorded within 5 min after haemolymph collection, before clotting or air diffusion could have obscured the results.

Analysis of heart and scaphognathite recordings

A PowerLab system with Chart v5.5.6 Software (AD Instruments, Spechbach, Germany) was used for the continuous recording of heart and ventilation frequencies. The peak detection routine of the programme was employed to determine heart and ventilation rate (bpm). Average heart and scaphognathite frequency at rest was determined over 30 min immediately prior to taking the first set of haemolymph samples. Uninterrupted heart and scaphognathite frequencies at rest, excluding pauses in ventilation and circulation, were determined over 20-60 s intervals within the 30 min period prior to haemolymph sampling in resting animals. Pauses are periods in which no heart or scaphognathite beats were detected. Because the animals did not exhibit pauses after the righting trial, heart and scaphognathite frequencies were determined over only 10 min immediately after the animals

had been reintroduced into the experimental chamber after taking the second set of haemolymph samples. The mean of the left and right scaphognathite beat frequencies was calculated to provide the ventilation rate of each animal. The frequencies of the pauses in heart beating and ventilation at rest (bpm) were calculated by subtracting the rates at rest from the rates at rest excluding the pauses. The scopes for heart and ventilation rates (bpm) were calculated by subtracting the minimal rates at rest from the maximal rates recorded after the righting trial.

Ion chromatography

Up to 10 µl of haemolymph were collected from the base of the last walking leg using a drawn out glass Pasteur pipette after animals had been blotted dry. A known volume of haemolymph was immediately added to 50 µl of deionised water. Sea water samples were collected for comparison and all samples were stored at -20°C. Cation composition of haemolymph and sea water was determined by ion chromatography (ICS-2000, Dionex®, Idstein, Germany) after the respective dilution of the samples with deionised water. A conductivity cell and a self-regenerating suppressor were used to reduce background conductivity. Na^+, K^+, Mg^{2+} and Ca^{2+} were separated on an IonPac® CS16 column with methane sulphonic acid (30 mmol L^{-1}) as an eluent at a flow rate of 0.36 mL min^{-1} at 40°C. Ion concentrations were calculated in mmol L^{-1} relative to the Dionex® Six Cation-II Standard.

Statistical analyses

Before calculating means ± standard deviation (s.d.), outliers were identified by use of Nalimov's test. One-way ANOVA and post-hoc Tukey's multiple comparison tests were run to identify significant ($p < 0.05$) changes in response to temperature. In case the data did not fulfil the prerequisites for this test, the nonparametric Kruskal-Wallis and a subsequent Dunn's multiple comparison tests were used. Two-way ANOVA and Bonferroni post-hoc tests were performed to analyse for effects of temperature, $[Mg^{2+}]$ and righting activity. An F-test was used to identify significant effects of righting activity on slopes and elevations of linear regressions fitted to the heart and ventilation rates. Discontinuities in heart and ventilation rates were identified according to Yeager and Ultsch (1989). Resulting regressions were tested for significant differences using an F-test (Nickerson et al., 1989). Statistical and regression analyses were performed using Graph Pad Prism 4.0a.

Results

Mean time-to-right

The mean time-to-right was lowest at the acclimation temperature of 4°C in both NSW (11 ± 5 s) and ASW –Mg^{2+} (19 ± 16 s, Fig. 1). In NSW it was significantly higher at -1°C and 13°C than at 4°C with 68 ± 44 s and 54 ± 42 s, respectively. In ASW –Mg^{2+} righting speed was significantly slower at -1°C (82 ± 62 s) compared to acclimation temperature. No significant differences were observed dependent on the [Mg^{2+}] ($F = 1.24$, $p = 0.272$).

Haemolymph PO_2

In NSW arterial haemolymph PO_2 declined significantly with increasing temperature in a sigmoidal manner both at rest and after the righting trial, whereas the decline in venous haemolymph PO_2 was significant only at rest (Fig. 2A, B, Table 2). Values at rest ranged from 14.1 ± 1.7 kPa at -1°C to 3.5 ± 0.8 kPa at 13°C in arterial haemolymph and from 10.1 ± 5.0 kPa at -1°C to 2.8 ± 0.6 kPa at 13°C in venous haemolymph (Fig. 2A). At rest arterial PO_2 was significantly higher than venous PO_2 at -1, 1 and 4°C (-1°C: $t = 3.63$, $p < 0.01$, 1°C: $t = 4.00$, $p < 0.001$, 4°C: $t = 5.54$, $p < 0.001$).

After the righting trial, both arterial and venous PO_2 were significantly decreased compared to values at rest at -1, 1, 4 and 7°C (arterial) and at -1, 1 and 4°C (venous, Fig. 2B). Arterial PO_2 comprised values from 8.5 ± 2.7 kPa at -1°C to 2.8 ± 0.7 kPa at 13°C. Venous PO_2 ranged from 3.3 ± 1.9 kPa at -1°C to 1.7 ± 0.3 kPa at 13°C. Arterial PO_2 differed significantly from venous PO_2 at -1°C ($t = 7.31$, $p < 0.001$) and 1°C ($t = 6.95$, $p < 0.001$).

The difference between arterial and venous PO_2 decreased significantly with increasing temperature both at rest and after the righting trial (Fig. 2C, Table 2). It was highest at rest at 4°C comprising 6.7 ± 1.0 kPa. Only at this temperature it was significantly reduced after the righting trial ($t = 4.78$, $p < 0.001$).

Arterial and venous haemolymph PO_2 of animals in ASW –Mg^{2+} were significantly reduced at -1°C compared to NSW both at rest and after righting (compare Fig. 2A and D, B and E, rest: $t = 3.65$, $p < 0.01$, after righting: $t = 5.08$, $p < 0.001$). The difference between arterial and venous PO_2 was significantly lower in ASW –Mg^{2+} than in NSW in resting crabs at -1°C ($t = 3.19$, $p < 0.01$) and 1°C ($t = 2.75$, $p < 0.05$, Fig. 2C, F).

Heart and ventilation frequencies

Heart rate increased significantly with temperature following linear slopes in both NSW and ASW –Mg^{2+} (Fig. 3A, C, Tables 2, 3, 4 and 5). The best-fit model for the heart rates of the animals at rest in NSW was a continuous two-phase model (Fig. 3A). The first segment ranged from -1 to 7°C, the second from 7 to 13°C. The slope of the first segment was not significantly different from zero. Values at rest were 12 ± 4 bpm at -1°C, 18 ± 5 bpm at 7°C and 43 ± 10 bpm at 13°C. Resting heart rates were significantly lower than resting rates excluding pauses only at 4°C ($t = 3.30$, $p < 0.01$) and 7°C ($t = 3.85$, $p < 0.01$). After the righting trial the heart rates were significantly increased compared to rest at all temperatures in both NSW and ASW –Mg^{2+}. In NSW values ranged from 27 ± 2 bpm at -1°C to 63 ± 8 bpm at 13°C after the righting trial. No significant differences were identified in relation to [Mg^{2+}]. Temperature-dependent relationships were similar in NSW and ASW –Mg^{2+} (Tables 4 and 5).

Ventilation rates at rest did not increase significantly with temperature as the slopes in both NSW and ASW –Mg^{2+} did not differ significantly from zero (Fig. 3B, D, Tables 4 and 5). Values ranged from 21 ± 7 bpm at -1°C to 48 ± 36 bpm at 13°C in NSW. A continuous two-phase model fitted best the resting ventilation rate excluding pauses in NSW (Fig. 3B). The slope of the first segment from -1 to 4°C differed significantly from zero, whereas there was no further increase of ventilation rate with temperature in the second segment from 4 to 13°C. Values comprised 40 ± 15 bpm at -1°C, 86 ± 21 bpm at 4°C and 74 ± 30 bpm at 13°C. In NSW at 4, 7 and 10°C the ventilation rate at rest excluding pauses was significantly higher than the rate including pauses (4°C: $t = 4.38$, $p < 0.001$, 7°C: $t = 5.31$, $p < 0.001$, 10°C: $t = 4.08$, $p < 0.001$). After the righting trial ventilation rates were significantly elevated at all temperatures compared to the resting rates. Values increased significantly with temperature from 74 ± 8 bpm at -1°C to 172 ± 17 bpm at 13°C following a linear slope. Similar relationships were observed in ventilation rates in ASW –Mg^{2+} (Fig. 3D, Table 5). There was no significant increase with temperature at rest, but values were significantly elevated after the righting trial and then were positively correlated to temperature. Only at 4°C ventilation rates at rest with and without pauses were significantly lower in ASW –Mg^{2+} than in NSW (rest: $t = 2.87$, $p < 0.05$, rest excluding pauses: $t = 3.40$, $p < 0.01$).

In NSW the frequency of pauses in heart beating increased significantly from -1 to 7°C and subsequently declined to a minimum at 13°C (Fig. 4A). The frequency of pauses in ventilation showed a similar picture with a maximum at 7°C (Fig. 4B). Scopes for heart and ventilation rates were dependent on temperature and exhibited maxima at 7°C (Fig. 4C and D). Both scopes increased significantly from 4 to 7°C, but subsequently did not rise further

with temperature. Neither the frequency of the pauses nor the scopes were significantly affected by [Mg^{2+}] (data not shown).

Cation regulation

In both NSW and ASW –Mg^{2+}, haemolymph Na^+, Ca^{2+} and Mg^{2+} concentrations neither changed dependent on temperature nor dependent on activity (Tables 1, 2 and 3). Therefore means were calculated over all data collected from each animal in the respective incubation media. Incubation in ASW –Mg^{2+} resulted in a significant increase in haemolymph [Na^+] from 423 ± 10 to 458 ± 18 mmol L^{-1}, which was still significantly lower than the ambient [Na^+] (516 ± 4 mmol L^{-1}). Haemolymph [Mg^{2+}] was reduced from 30 ± 2 to 12 ± 2 mmol L^{-1} upon incubation in ASW –Mg^{2+}. In both NSW and ASW –Mg^{2+}, the animals maintained [Mg^{2+}] significantly different from the ambient concentrations (47.3 ± 0.9 and 5.9 ± 0.1 mmol L^{-1}, respectively). Because [Ca^{2+}] and [K^+] did not differ between incubation media, haemolymph concentrations of these ions also remained the same (Table 1, Fig. 5).

Haemolymph [K^+] was not affected by temperature change, but was increased after the righting trial (Fig. 5, Tables 2 and 3). The effect of activity was statistically significant at -1 and 1°C in NSW (-1°C: $t = 3.066$, $p < 0.01$, 1°C: $t = 2.489$, $p < 0.05$) and at -1 and 4°C in ASW –Mg^{2+} (-1°C: $t = 2.681$, $p < 0.05$, 4°C: $t = 2.492$, $p < 0.05$).

Discussion

We present the first study of thermal tolerance of a sub-Antarctic anomuran lithodid crab in the adult stage. Lithodids are the southernmost distributed reptant decapods and therefore should display well-expressed cold tolerance. We studied the parameters righting speed, haemolymph PO_2, heart and ventilation frequencies, scopes for heart and ventilation rates and extracellular cation composition in *Paralomis granulosa*. We reduced extracellular [Mg^{2+}] of *P. granulosa* to a level of that of caridean shrimps to test whether Mg^{2+} plays a role in limiting cold tolerance.

In the warm-eurythermal spider crab *Maja squinado* acclimated to 10°C oxygen supply to tissues during progressive cooling was constrained by insufficient ventilation of the gills and a reduced heart rate (Bock et al., 2001; Frederich et al., 2000a; Frederich and Pörtner, 2000). This led to the onset of a decrease in haemolymph PO_2 at about 9°C and was defined as the lower pejus temperature of these animals (pejus = getting worse) where oxygen demand starts to exceed oxygen delivery. In contrast, *P. granulosa* exposed to low

temperatures maintained high arterial and venous haemolymph PO_2s despite reduced heart and ventilation rates. This suggests, that cold tolerance of this species is not acutely limited by oxygen supply to tissues. Similarly, the cold-eurythermal *Hyas araneus* acclimated to 10°C maintained high arterial PO_2 at decreasing heart rates between 0 and 4°C (Walther et al., 2009). The high haemolymph PO_2 in both *H. araneus* and *P. granulosa* at low temperatures might result from high oxygen solubilities in water and haemolymph combined with low metabolic rates. This likely reflects the general alleviation of oxygen supply constraints by cold temperatures, which was postulated to shape the evolution of Antarctic marine fauna (Pörtner, 2006).

The heart rate of *P. granulosa* at 13°C compares well to that of another lithodid crab species, *Lopholithodes mandtii*, (45-55 bpm, 12°C, 300-500 g animals, McGaw and Duff, 2008). Furthermore, heart rates were about the same as those in *M. squinado* for the same temperature range (mean weight was also similar, *M. squinado*: 595 ± 151 g, Frederich and Pörtner, 2000). In contrast, the temperature-dependent curves of ventilation rate (at rest excluding pauses) and arterial haemolymph PO_2 at rest are shifted towards lower temperatures by about 13°C compared to *M. squinado*: in *P. granulosa*, a discontinuity in ventilation rate was found at 4°C, whereas in *M. squinado* it was located at about 17°C (90 bpm). Interestingly, above these temperatures ventilation frequencies did not rise further and this discontinuity coincides with the onset of a decrease in arterial PO_2 in both *M. squinado* and *P. granulosa*, respectively. Constant ventilation frequencies at rising temperatures as well as the intermittency of ventilation may represent regulatory mechanisms to repress metabolic rate and delay heat limitation. The metabolic cost of ventilation in marine crabs is high and e.g. comprises 30% of total metabolic rate in resting *Carcinus maenas* (Wilkens et al., 1984). The data indicate that there is a cold-compensation of the ventilation rate in *P. granulosa* at the expense of a downward shift in upper thermal limits.

Pejus thresholds of decapod crabs have previously been determined by use of the temperature-dependent curve of resting arterial PO_2 and ventilation (Frederich and Pörtner, 2000) or heart rate (Walther et al., 2009). Neither of the respective characteristics are as clearly applicable to our results as (1) it is highly unlikely that the upper pejus temperature of our experimental animals is at or even below 4°C, where resting arterial PO_2 starts to drop and (2) heart rate does not rise exponentially. This may be explained at least in part by a role of the respiratory pigment haemocyanin, which possibly comes into action at intermediate high exposure temperatures and supports oxygen delivery at declining $HLPO_2$s (see e.g. McMahon et al. 1978; McMahon et al. 1979, where both haemolymph oxygen contents and PO_2s were determined). We suggest that the optimal temperature of our experimental

animals may be found between 4 and 7°C. At these temperatures they exhibited the fastest righting speeds, with the caveat that the righting response may also be fuelled by anaerobic metabolism. The difference between arterial and venous PO_2 was largest at 4°C and at 7°C the animals were able to „afford" pauses in heart and scaphognathite beating. Furthermore, the scopes for heart and ventilation rates were highest at 7°C. With some uncertainty pejus thresholds may thus be found between 1 and 4°C as well as between 7 and 10°C. Roughly, they correspond with the natural temperature regime of the Magellan region, which is characterized by mean temperatures of 2 to 4°C in winter and 9 to 11°C in summer (Hoggarth, 1993; Lovrich and Vinuesa, 1993).

Righting speed was significantly slower at -1°C and 13°C compared to the acclimation temperature of 4°C. Similarly, specimens of the subpolar brachyuran crabs *Eurypodius latreillei*, *Halicarcinus planatus* and *Hyas araneus* were three to ten times slower at subzero temperatures compared to 4°C (Frederich, 1999; Frederich et al., 2000b). In *H. araneus* and *E. latreillei* the experimental reduction of Mg^{2+} to levels in caridean shrimps (10 - 15 mmol L^{-1}) increased righting or walking speed at subzero temperatures (Frederich et al., 2000b). The fact that *P. granulosa* did not react in the same manner may imply that an influence of Mg^{2+} regulation on cold tolerance in the adult stage of this species is unlikely.

While oxygen supply was likely not limiting the righting response in the cold, low temperature reduces functional capacity and causes a slowing of righting velocity, possibly involving reduced transmission velocity at the neuromuscular junctions (Dunn and Mercier, 2003; Macdonald, 1981). Furthermore, both low temperature and high Mg^{2+} concentration may synergistically act to block Ca^{2+} influx at the synapse (Dudel et al., 1982; Dunn and Mercier, 2003; Parnas et al., 1994). However, in our study, a reduction of extracellular Mg^{2+} from 30 to 12 mmol L^{-1} did not suffice to remove a possible block by Mg^{2+}. *P. granulosa* did not even get more active by trend, in contrast to observations in the sub-Antarctic brachyuran *Eurypodius latreillei* (Frederich et al., 2000b). This suggests that limitation by other processes in the complex temperature-dependent mechanism of synaptic transmission may take precedence, either individually or in combination. These include effects of temperature on enzyme synthesis, protein-protein interactions, exocytosis, phosphorylation state of channels and transmitter-receptor interaction at pre- and postsynaptic locations (Dunn and Mercier, 2003; Macdonald, 1981 and references therein; White, 1983). While cold compensation of nerve conduction velocity has been shown for Antarctic fish and crustaceans (Macdonald, 1981; Young et al., 2006), temperature-compensation of synaptic function has to date only been investigated in Antarctic fish (MacDonald and Montgomery, 2005). Furthermore, it is not understood how synaptic function contributes to defining thermal tolerance of subpolar crustaceans. In the light of a whole organism to molecular

hierarchy of thermal limitation we expect that synaptic transmission alone functions in a wider thermal range than the complex whole organism, as observed in tropical and Antarctic fish. However, the lower recording limit for miniature endplate currents, a measure of synaptic function, parallels normal ambient temperature of the temperate notothenioid fish *Pseudoaphritis urvillii* (MacDonald and Montgomery, 2005). It would therefore be worthwhile to compare thermal effects on synaptic properties in crustacean species from different latitudes and relate the results to thermal tolerance of the entire organisms.

The righting trial caused a reduction of $HLPO_2$ at temperatures higher than 1°C. At these temperatures the animals probably switched from aerobic to anaerobic metabolism. At 13°C, anaerobic capacity was most likely exhausted. This may have resulted in a significant slowing of the animals. Similarly, *Limulus polyphemus* was challenged with an activity trial and responded with a decrease of arterial PO_2, an accumulation of lactate and a depletion in the phosphagen phospho-L-arginine, which both indicate anaerobiosis (Carlsson and Gäde, 1986). At -1°C the time-to-right increased significantly in *P. granulosa*, as expected from kinetic constraints in the cold. This may coincide with a limitation in the capacity to enhance metabolism for locomotory activity at these low temperatures. The predominance of a capacity limitation might be reflected in high levels of haemolymph oxygenation and unchanged differences between arterial and venous PO_2 before and after the righting trial and also by the low scopes for heart and ventilation rates in the cold.

Besides a limitation in aerobic scope for activity, we may also hypothesise that anaerobic metabolism was limiting. In crustaceans exercise is also fuelled by non-oxidative metabolism as evidenced by the production of lactate despite maintained haemolymph PO_2 (Booth et al., 1984; Burke, 1979; Hamilton and Houlihan, 1992; Houlihan and Innes, 1984; Houlihan et al., 1984). Furthermore, the relative portion of non-oxidative metabolism in the total metabolic cost of exercise was greater in the cold (15°C) than in the warmth (24°C) in the ghost crab *Ocypode quadrata* (Weinstein and Full, 1998) and the intertidal crab *Pachygrapsus crassipes* (Burke 1979).

The significant rise in haemolymph $[K^+]$ may indicate a shortcoming of the cells to control ion homeostasis during activity at -1 and 1°C. K^+ loss to haemolymph or blood during exercise has also been observed in the crab *Callinectes sapidus* (Booth et al., 1984), in fish (Holk and Lykkeboe, 1998), in humans and in other vertebrates (Allen et al., 2008; Lindinger, 1995; Sjogaard, 1996). In fish, an increase in blood $[K^+]$ indicated limited swimming endurance and was attributed to an insufficient activity of Na^+/K^+-ATPase (Holk and Lykkeboe, 1998). Increased external $[K^+]$ causes membrane depolarisation, failure of excitation and a reduction in force responses in musculature (Allen et al., 2008). Therefore,

the significant cellular loss of K^+ to the haemolymph may have contributed to the slowing of the righting response in *P. granulosa*.

In conclusion, the temperature range in which arterial PO_2 at rest is highest does not coincide with the temperature range of maximal activity in this lithodid crab. We therefore suggest that parameters other than resting arterial PO_2 should be used to characterise thermal tolerance in this sub-Antarctic species. The difference between arterial and venous PO_2, pauses in ventilation and heart rates as well as scopes and activity patterns seem to be more suitable. The dataset illustrates, that on short to intermediate time scales, oxygen delivery is not the limiting factor in cold tolerance in this cold-adapted crustacean. As this finding resembles that of another cold-adapted species, *Hyas araneus* (Walther et al., 2009), future studies should investigate how kinetic constraints at tissue and cellular levels contribute to the capacity limitation of cold tolerance, which apparently takes precedence over oxygen limitation in cold adapted species. A reduction of extracellular $[Mg^{2+}]$ did not have an activating effect on the animals. Because cold-compensation of ventilation was observed in animals kept in natural sea water, we reject the hypothesis that naturally high $[Mg^{2+}]$ limits the capacity of oxygen delivery at low temperatures in *P. granulosa*. A role of Mg^{2+} regulation in the biogeography of this species is thus questionable.

Abbreviations

A acclimation temperature
Art arterial
ASW –Mg^{2+} artificial sea water with reduced $[Mg^{2+}]$
bpm beats per minute
CL carapace length
Frq frequency
FW fresh weight
$HLPO_2$ haemolymph oxygen partial pressure
NSW natural sea water
PO_2 oxygen partial pressure
Seg segment
Ven venous

Acknowledgements

We would like to thank Daniela Storch and Fredy Véliz Moraleda for invaluable support in the acquisition of experimental animals. We greatly appreciate the help of the crew of RV Polarstern during the transport of the animals. The experiments comply with the current laws of the country in which they were performed. This study was supported by Deutsche Forschungsgemeinschaft grant no. SA 1713/1-1 and 1-2. The authors declare that there is no conflict of interest.

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Table 1: Cation composition (mmol L^{-1}) of incubation media and haemolymph of *Paralomis granulosa* kept in NSW and ASW –Mg^{2+}. Because Na^+, Mg^{2+} and Ca^{2+} concentrations did not change dependent on temperature or activity, a mean was calculated over all values collected from each animal. K^+ values see Fig. 5. NSW: natural sea water, ASW –Mg^{2+}: artificial sea water with reduced [Mg^{2+}], NaCl was added to adjust it to the same salinity as NSW (32.5 ppt). Values are means ± s.d., n = 8, * significantly different from sea water, # significantly different from the value in NSW.

	NSW		ASW –Mg^{2+}	
	Sea water	**Haemolymph**	**Sea water**	**Haemolymph**
Na^+	439 ± 6	423 ± 10*	516 ± 4 #	458 ± 18 *#
Mg^{2+}	47.3 ± 0.9	30 ± 2*	5.9 ± 0.1 #	12 ± 2 *#
Ca^{2+}	9.8 ± 0.9	10.1 ± 0.6	9.6 ± 0.6	9.6 ± 0.5
K^+	10.4 ± 0.2	See Fig. 5	10.5 ± 0.1	See Fig. 5

Table 2: Results of two-way ANOVA carried out on data collected in NSW to test for overall effects of temperature and righting activity. Art: arterial, Ven: venous, frq: frequency

Response variable	**Temperature**		**Activity**		**Interaction**	
	F	**p**	**F**	**p**	**F**	**p**
Haemolymph PO_2 (kPa)	63.68	**<0.0001**	110.53	**<0.0001**	9.81	**<0.0001**
Art – Ven PO_2 (kPa)	10.13	**<0.0001**	10.78	**0.002**	3.81	**0.004**
Heart frq (bpm)	69.46	**<0.0001**	83.98	**<0.0001**	1.25	0.268
Ventilation frq (bpm)	21.85	**<0.0001**	232.86	**<0.0001**	5.11	**<0.0001**
Na^+ (mmol L^{-1})	0.44	0.821	1.14	0.289	2.11	0.074
Mg^{2+} (mmol L^{-1})	1.87	0.111	0.11	0.736	0.38	0.859
Ca^{2+} (mmol L^{-1})	1.83	0.119	0.99	0.324	0.64	0.674
K^+ (mmol L^{-1})	1.56	0.184	34.35	**<0.0001**	0.58	0.714

Table 3: Results of two-way ANOVA carried out on data collected in ASW –Mg^{2+} to test for overall effects of temperature and righting activity. Art: arterial, Ven: venous, frq: frequency

Response variable	**Temperature**		**Activity**		**Interaction**	
	F	**p**	**F**	**p**	**F**	**p**
Haemolymph PO_2 (kPa)	4.85	**0.010**	18.59	**<0.0001**	1.32	0.260
Art – Ven PO_2 (kPa)	2.65	0.086	0.55	0.463	6.63	**0.004**
Heart frq (bpm)	10.36	**0.0001**	23.03	**<0.0001**	0.68	0.610
Ventilation frq (bpm)	35.79	**<0.0001**	246.35	**<0.0001**	12.24	**<0.0001**
Na^+ (mmol L^{-1})	1.64	0.207	1.46	0.235	1.47	0.243
Mg^{2+} (mmol L^{-1})	3.21	0.052	1.58	0.217	0.25	0.781
Ca^{2+} (mmol L^{-1})	0.86	0.434	0.06	0.806	2.85	0.071
K^+ (mmol L^{-1})	2.12	0.1361	10.68	**0.003**	0.59	0.558

Table 4: Linear regressions of heart and ventilation rates dependent on temperature in NSW. frq: frequency, Seg: segment

Response variable (bpm)	**R^2**	**Type of fit**		**Slopes non-zero?**	
				F	**p**
Heart frq					
- Rest	0.6570	Two-phase linear	Seg 1 Seg 2	4.082 25.00	0.054 **<0.0001**
- Rest w/o pauses	0.6662	Linear		83.81	**<0.0001**
- After righting trial	0.8813	Linear		319.2	**<0.0001**
Ventilation frq					
- Rest	0.0818	Linear		3.82	0.057
- Rest w/o pauses	0.4148	Two-phase linear	Seg 1 Seg 2	33.06 0.72	**<0.0001** 0.404
- After righting trial	0.8609	Linear		253.9	**<0.0001**

Table 5: Linear regressions of heart and ventilation rates dependent on temperature in ASW –Mg^{2+}. frq: frequency

Response variable (bpm)	**R^2**	**Type of fit**	**Slopes non-zero?**	
			F	**p**
Heart frq				
- Rest	0.0480	Linear	1.11	0.304
- Rest w/o pauses	0.4955	Linear	17.68	**0.0005**
- After righting trial	0.6789	Linear	44.40	**<0.0001**
Ventilation frq				
- Rest	0.0003	Linear	0.06	0.802
- Rest w/o pauses	0.5642	Linear	24.60	**<0.0001**
- After righting trial	0.8448	Linear	103.4	**<0.0001**

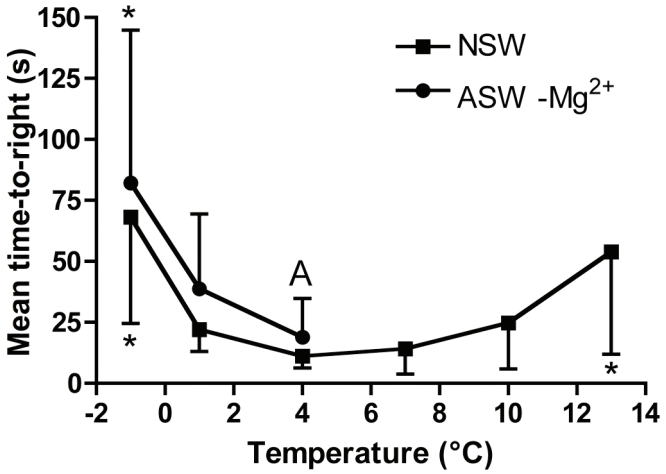

Figure 1: Mean time-to-right (s) of *Paralomis granulosa* dependent on temperature and ambient [Mg^{2+}]. NSW: [Mg^{2+}] = 47 mmol L^{-1}, ASW –Mg^{2+}: [Mg^{2+}] = 6 mmol L^{-1}.Values are means ± s.d., n = 5-8. * significantly different from value at the acclimation temperature of 4°C (A). [Mg^{2+}] did not affect the mean time-to-right.

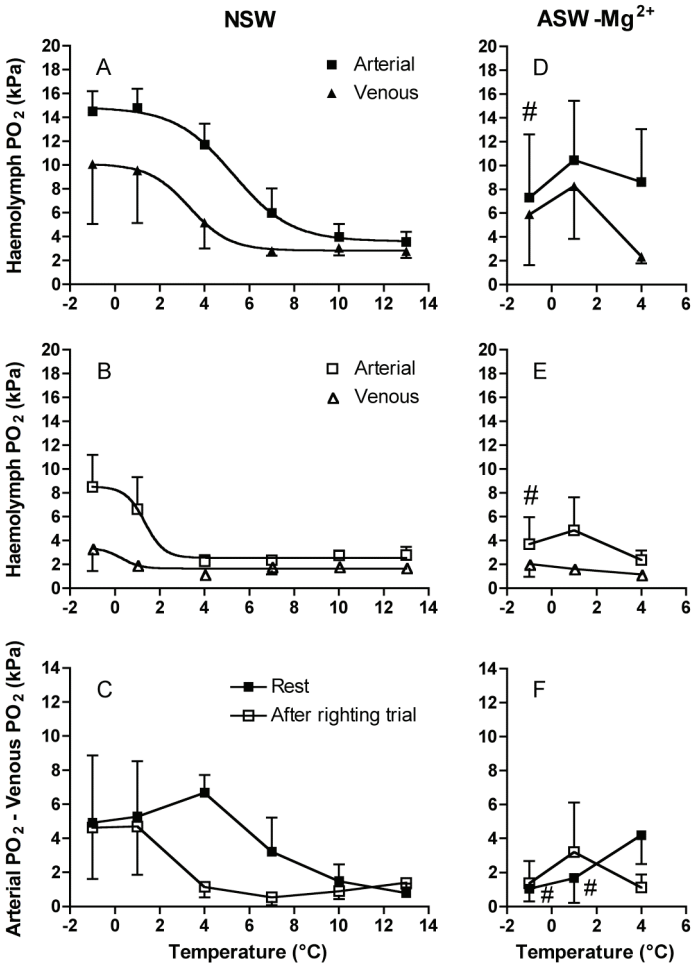

Figure 2: Arterial and venous haemolymph oxygen partial pressure (PO_2, kPa) of *Paralomis granulosa* acclimated to 4°C dependent on temperature and [Mg^{2+}] at rest (A and D, closed symbols) and after the righting trial (B and E, open symbols), as well as the difference between arterial and venous PO_2 (C and F). NSW: [Mg^{2+}] = 47 mmol L^{-1}, ASW –Mg^{2+}: [Mg^{2+}] = 6 mmol L^{-1}.Values are means ± s.d., n = 5-8, some error bars are not visible as they are smaller than the plot symbols. # significantly different from value in NSW.

NSW rest (A): arterial PO_2, r^2 = 0.9093; venous PO_2, r^2 = 0.5797

NSW after righting trial (B): arterial PO_2, r^2 = 0.7402; venous PO_2, r^2 = 0.3521.

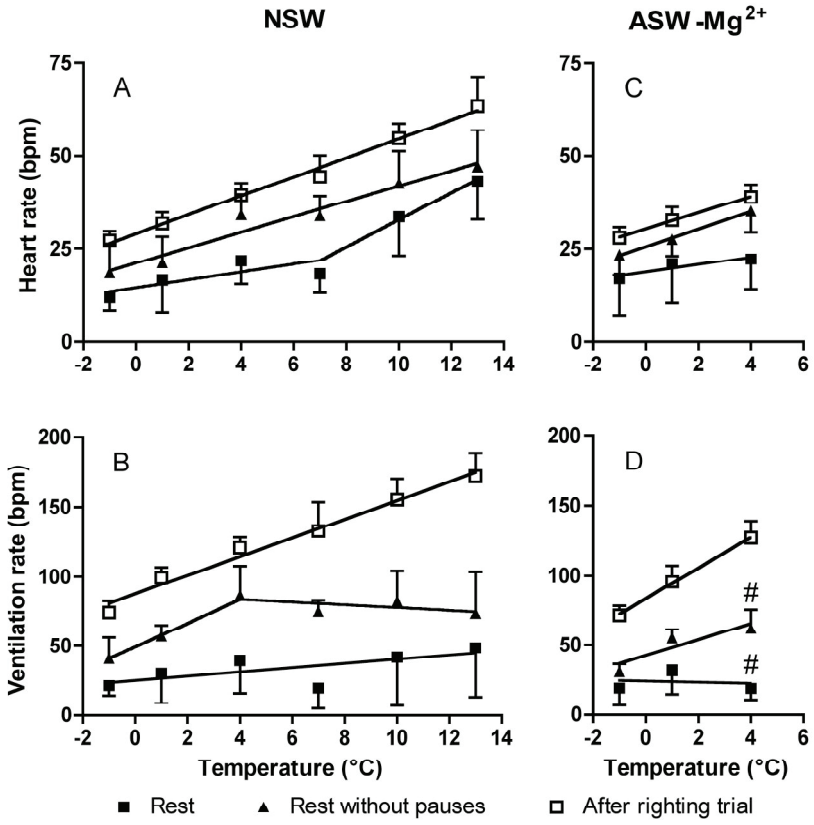

Figure 3: Heart (A and C) and ventilation rate (B and D, bpm) of *Paralomis granulosa* acclimated to 4°C dependent on temperature and $[Mg^{2+}]$ at rest and after the righting trial. NSW: $[Mg^{2+}]$ = 47 mmol L^{-1}, ASW –Mg^{2+}: $[Mg^{2+}]$ = 6 mmol L^{-1}.Values are means ± s.d., n = 6-8, # significantly different from value in NSW. r^2 see Tables 4 and 5.

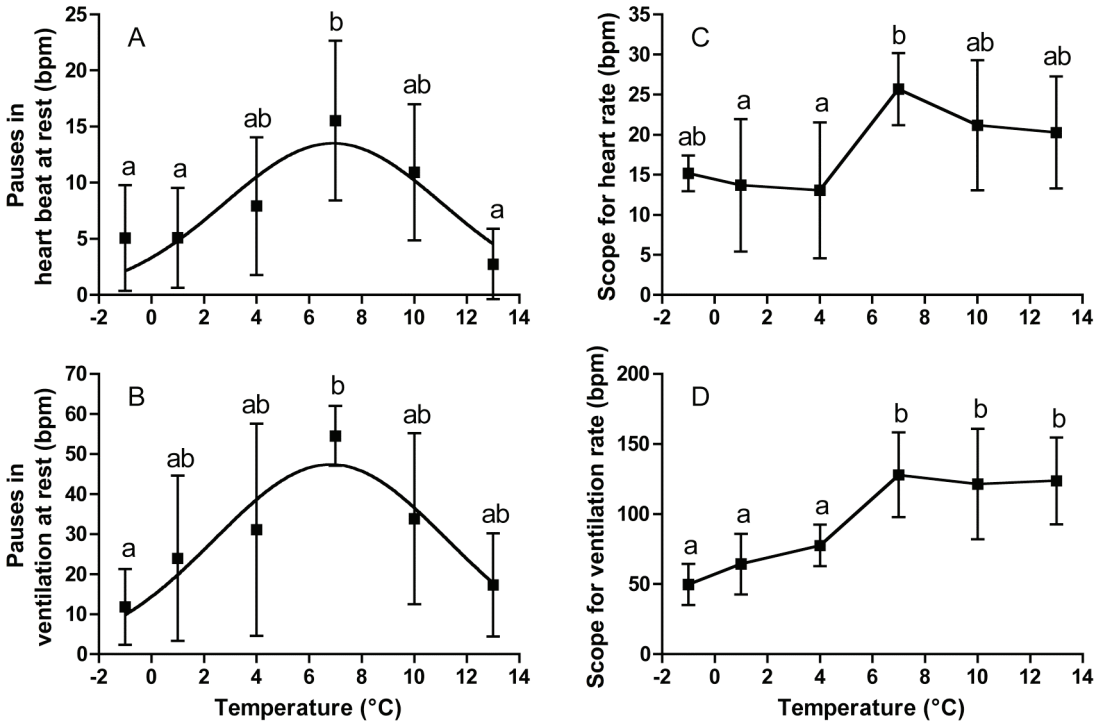

Figure 4: Pauses in heart beat (A) and ventilation (B) at rest and scopes for heart (C) and ventilation (D) rates of *Paralomis granulosa* in NSW acclimated to 4°C. Values are means ± s.d., n = 6-8. Different letters denote significant differences, A, C, D: ANOVA and post hoc Tukey's multiple comparison test, B: Kruskal-Wallis test and post hoc Dunn's multiple comparison test. A: Gaussian curve $r^2 = 0.3021$, B: Gaussian curve $r^2 = 0.3467$.

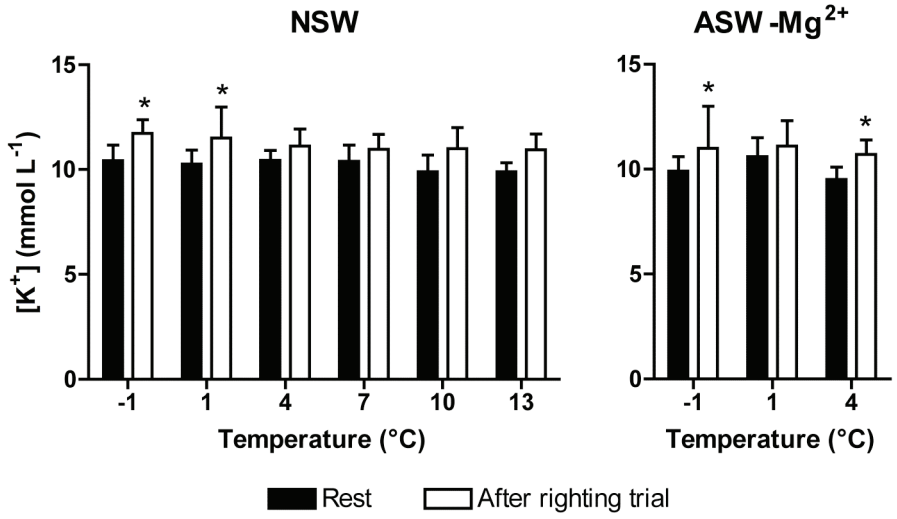

Figure 5: Haemolymph $[K^+]$ (mmol L^{-1}) of *Paralomis granulosa* dependent on temperature and $[Mg^{2+}]$ at rest and after the righting trial. NSW: $[Mg^{2+}]$ = 47 mmol L^{-1}, ASW –Mg^{2+}: $[Mg^{2+}]$ = 6 mmol L^{-1}.Values are means ± s.d., n = 6-8, * significantly different from value at rest. Temperature and $[Mg^{2+}]$ did not affect haemolymph $[K^+]$.

Publication IV

Effects of temperature and magnesium on metabolism and elemental composition (CHN) during early development of the sub-Antarctic crab *Paralomis granulosa* (Jaquinot)

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(In preparation)

Effects of temperature and magnesium on metabolism and elemental composition (CHN) during early development of the sub-Antarctic crab *Paralomis granulosa* (Jaquinot)

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Keywords: Temperature, magnesium, survival, oxygen consumption, CHN, Decapoda, Lithodidae, larval development

Running head: Effects of temperature and magnesium on *Paralomis granulosa*

Abstract

The capacity for magnesium excretion is believed to be related to cold tolerance of decapod crustaceans, and therefore may be a major determinant of their biogeography in the Southern Ocean. Since the early developmental stages generally represent a physiological bottleneck within the life cycle, effects of increased magnesium concentration (97 mmol L^{-1}) in combination with an exposure to low temperatures (1, 4 and 9°C) on survival, development, dry weight (W), elemental composition (C, H, N) and respiration were studied in the larval stages of a sub-Antarctic species of lithodid crab, *Paralomis granulosa*. Lecithotrophic larval development resulted in the consumption of stored C and in a gradual decrease of the C:N ratio, which indicates a preferential utilization of lipid reserves. These patterns were more pronounced at 1 and 4°C, where larval development was prolonged and survival reduced as compared to 9°C. High magnesium concentration did not affect W, C, H and N contents of the larval stages, but it impaired their survival and development at 1°C causing complete mortality in the zoea II stage. Oxygen consumption decreased throughout larval development but subsequently increased after metamorphosis to the first juvenile stage. In all investigated stages it increased with increasing temperature. An elevated magnesium concentration reduced at both 1 and 9°C (4°C not tested) the oxygen consumption of the zoea I stage. As the effects of increased magnesium levels were in general most pronounced at the lowest temperature, a high capacity for magnesium excretion in the larval stages should be advantageous for crabs living in cold regions.

Introduction

Anomuran crabs of the family Lithodidae, commonly referred to as "stone crabs", are the only group of reptant decapod crustaceans found in the Antarctic (Gorny 1999). While several species of this family occur in deeper waters of the Antarctic continental slope areas of the Bellingshausen Sea at temperatures above 0°C, they are absent in shallower areas of the Weddell and Ross Seas at -1.8°C (Klages et al. 1995, García Raso et al. 2005, Thatje et al. 2008). The enigma of low decapod crustacean diversity at high southern latitudes (Gorny 1999, Astorga et al. 2003, Thatje et al. 2005) has been attributed, at least in part, to a physiological trait, namely the capacity for magnesium excretion. Behavioural studies as well as measurements of respiration and cardiorespiratory parameters suggested that cold tolerance of brachyuran crabs is linked to haemolymph magnesium levels (Frederich et al. 2000a, Frederich et al. 2000b, Frederich et al. 2001). Thus, it has been concluded that the inability to excrete major amounts of magnesium (haemolymph $[Mg^{2+}]$ = 20 – 50 mmol L^{-1}) excludes reptant (anomuran and brachyuran) crabs from the coldest areas of the Antarctic continental shelf. In contrast, caridean decapod shrimps, which exhibit a high capacity for magnesium extrusion (haemolymph $[Mg^{2+}]$ = 5 – 20 mmol L^{-1}) are abundant in these regions (Gutt et al. 1991, Frederich 1999, Frederich et al. 2000b).

For over 50 years magnesium is known for its paralysing effect on vertebrates and invertebrates (Katz 1936, Waterman 1941, Pantin 1948, Iseri & French 1984, Lee et al. 1996). A 1.5- to 2-fold elevation of external magnesium concentration above the natural concentration in sea water (50 mmol L^{-1}) has been shown to diminish the response of decapod locomotor nerve-muscle preparations to stimulation. In contrast, submaximal tension and facilitation of neuromuscular transmission increased when magnesium concentration was reduced (Katz 1936, Waterman 1941, Boardman & Collier 1946). With these results in mind, Robertson (Robertson 1949, 1953) noted that the inactive and sluggish behaviour of majid and lithodid crabs might be related to their high haemolymph magnesium concentrations. In the temperate amphipod sandhopper *Talitrus saltator*, a decline in temperature induced not only a reduction in oxygen consumption but also an increase in haemolymph magnesium concentration, which may explain the inactivity of these animals in winter (Spicer et al. 1994).

To understand distribution patterns in marine species, it is important to consider the various life-history stages, because each ontogenetic stage may exhibit differential tolerance limits to variations in environmental factors (Anger 2001, Anger et al. 2003, Pörtner & Farrell 2008, Storch et al. 2009). As temperature is a key determinant for biogeography and biodiversity (Parsons & Lear 2001, Astorga et al. 2003, Tittensor et al. 2010), cold tolerance of

the larval stages is crucial for understanding the distribution of decapod crustaceans in the Southern Ocean.

Our model species, the sub-Antarctic stone crab *Paralomis granulosa*, is distributed in the Magellanic Province. At the southern tip of South America, this species is exposed to water temperatures of 2 – 4°C in winter and 9 – 11°C in summer. It is the only species of this genus, which occurs in shallow depths and even in the intertidal (0 – 100 m; Boschi 1979, Hoggarth 1993, Lovrich & Vinuesa 1993, Boschi 2000, Macpherson 2004). Its larval development is abbreviated comprising only two zoeal stages and a megalopa (Campodonico & Guzman 1981). These stages are entirely lecithotrophic, implying that larvae do not take up food but rely on the utilization of internal lipid stores as energy source, as evidenced by a gradual decrease of the carbon content and the carbon:nitrogen (C:N) ratio throughout the larval development (Calcagno et al. 2003, 2004, Kattner et al. 2003).

The larvae hatch during the austral winter (July – September) at water temperatures of about 4 – 5°C. The first juvenile crab instar, which is the first ontogenetic stage that depends on food uptake, is probably reached in September – November, when the spring phytoplankton bloom begins and temperatures have slightly increased to about 5 – 7°C (Lovrich & Vinuesa 1993, Anger et al. 2003, Thatje et al. 2003a). Larval survival is maximal at 6 – 9°C, while development time decreases with increasing temperature (investigated range: 1 – 15°C). At a temperature as low as 1°C, the larvae of *P. granulosa* survive for about two months and develop to the megalopa stage, but no successful development through metamorphosis has been observed (Anger et al. 2003).

The zoeal stages of this species do not significantly downregulate the magnesium concentration of the haemolymph, while the megalopa stage already reaches levels of downregulation that are similar to those in the conspecific adults (Wittmann et al. 2010), Wittmann et al. submitted). While the knowledge on the developmental biology of lithodid crabs has grown throughout the past 15 years, effects of temperature on larval biomass, biochemical composition and oxygen consumption have scarcely been studied.

The aim of this study was to investigate effects of low temperature in combination with an elevated magnesium concentration on larval *Paralomis granulosa* to gain an understanding on how the capacity for extracellular magnesium regulation may be linked to distribution patterns of lithodid species in the Southern Ocean. It was not possible to mimic reduced haemolymph magnesium levels similar to those in caridean shrimps, because it was unfeasible to raise the larvae in artificial sea water, irrespective of the magnesium concentration. Studying the effects of an increase in external magnesium concentration may give a first insight into the sensitivity of the larvae to this ion at various temperatures and

may allow to assess putative advantages for such species, which are able to downregulate this ion.

Materials and Methods

Animals

Egg-carrying specimens of *Paralomis granulosa* were obtained from local fishermen in Punta Arenas, Chile, in April 2008. The animals were transported to the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven, Germany on board RV Polarstern (ANT-XXIV/4) and thereafter kept in a recirculated aquarium system at 4°C, 32.5 PSU and an artificial 12:12 h light:dark cycle. Two females were transferred in June to the Marine Biological Laboratory Helgoland, Germany, and placed in individual flow-through tanks (20 L) kept at 6°C, 33 PSU and an artificial 12:12 h light:dark cycle. Beginning one week after the arrival of the animals on Helgoland, water temperature was raised at 1°C day^{-1} to 9°C. This temperature was subsequently kept constant. The crabs were fed *ad libitum* with pieces of mussels, shrimps or isopods.

Every morning, newly hatched larvae were collected from sieves (300 µm mesh size) receiving the overflowing water from the aquaria in which the females were maintained individually. Actively swimming larvae were randomly selected, and groups of 10 individuals were placed in 400 mL glass bowls (diameter 10.5 cm). These rearing containers were filled with either filtered natural sea water from the North Sea (NSW, 32.5 PSU; control condition) or with NSW to which $MgCl_2*6H_2O$ was added and which was subsequently diluted with deionised water to a total salinity of 32.5 PSU (NSW +Mg^{2+}; treatment; for ionic composition see Table 1) at 9°C (control temperature). The bowls were transferred to incubators kept at 1 or 4°C or remained in a cold room at 9°C. The larvae were raised without food, and water was changed every other day. The bowls were checked daily for deaths and moults to record survival and developmental time. When the larvae had reached the megalopa stage, they were provided with pieces of nylon mesh as a substrate. After metamorphosis to the first juvenile stage, the animals were fed with newly hatched *Artemia* sp. nauplii (Sanders Brine Shrimp Company) and water was changed daily.

In the middle of each moult-stage (intermoult; Drach & Tchernigovtzeff 1967) samples were collected for later measurements of biomass (dry weight, elemental composition) and for measurements of larval oxygen consumption (Table 2). After metamorphosis to the first crab instar, juveniles were reared for two weeks at 9°C, before

they were transferred to the experimental temperatures (1 and 4°C). Their oxygen consumption rates were determined two weeks later, i.e. 30 days after moulting.

CHN and dry mass

Individual larvae and juveniles were rinsed with deionised water over a 300 µm mesh sieve, blotted dry and stored at -20°C in preweighed tin cartridges (HEKAtech GmbH, Wegberg, Germany). The samples were freeze-dried for 24 h at 0.05-0.12 mbar using a Christ Alpha 1-4 LDC-1M vacuum drier before dry weight (W) was determined to the nearest 0.1 µg on a Sartorius 4504MP8 supermicro balance. Contents of carbon (C), hydrogen (H) and nitrogen (N) (collectively CHN) were measured in a Euro EA 3000 elemental analyser (HEKAtech GmbH, Wegberg, Germany) using acetanilide as a standard. C:N mass ratios were calculated as a proxy for changes in the lipid:protein ratio (Anger 2001) occurring during development or dependent on temperature and magnesium concentration. The sum of CHN was subtracted from W to estimate the build-up of other, mainly inorganic compounds.

Oxygen consumption

Oxygen consumption measurements of individual larvae and juveniles were carried out in 5-mL Hamilton syringes (Hamilton Bonaduz AG, Bonaduz, Switzerland) similar to the method established by Thatje et al. (2003a). Air saturation was recorded using needle-type fibre-optic oxygen micro-optodes connected to a Microx TX3 unit (Presens GmbH, Regensburg, Germany). The temperature-compensated calibration of the optodes was carried out prior to measurements using a saturated ascorbic acid solution (0% air saturation) and water vapour (100% air saturation). Single individuals were introduced into each syringe and the water volume was adjusted to 300 µl by carefully moving the plunger. Keeping the syringe submerged in sea water, the side of the cannula was sealed with a rubber septum. The needle of the oxygen sensor was inserted through this septum and the tip of the sensor was positioned in the middle of the chamber. Syringes were placed upside down in Erlenmeyer flasks filled with air-saturated filtered sea water. The flasks were kept in a temperature-controlled bath containing cooling fluid. Oxygen depletion in the chamber was recorded down to 80% air saturation to keep stress and CO_2 accumulation at a minimum. Blanks were run before and after the experiments to evaluate microbial respiration.

Statistics

Before calculating means and standard deviation (SD) outliers were identified at the 95% significance level using Nalimov's test, and removed. Statistical analyses at the $p < 0.05$ level were carried out using Graph Pad Prism 4.0a. Differences in larval survival were identified by use of a Chi-square test on the raw data. Survival (number of individuals which moulted to the next stage) is given as the percentage of the number of individuals at the beginning of the experiment. Two-way ANOVA and subsequent Bonferroni tests were carried out on dry weight (W), elemental (CHN) composition, C:N ratios, oxygen consumption and duration of development dependent on temperature and magnesium concentration. Prior to statistical analyses C:N ratios were transformed using an arc sine function.

Results

Elevated magnesium concentration (97 mmol L^{-1}) did not affect larval survival at 9°C so that 74 and 79% of the larvae successfully moulted in NSW and NSW +Mg^{2+}, respectively, to the first juvenile instar (Table 3). Exposure to 1 and 4°C significantly reduced the survival in NSW compared to 9°C ($p < 0.0001$), with only 38% and 60% of the larvae reaching the megalopa stage, respectively. At the end of the observation period only 23% of the animals had survived in NSW at 1°C 16 days after moulting to the megalopa stage. The increased magnesium concentration strongly reduced the rate of survival already in the first larval stage (to 19%), and none of the larvae survived at 1°C through the second zoeal stage. The effect of magnesium on larval survival at 1°C was highly significant ($p < 0.0001$ and $p = 0.0005$ in the zoea I and II, respectively).

Exposure to 1°C prolonged the duration of larval development drastically, and an elevated magnesium concentration potentiated this effect significantly ($p < 0.0001$, Table 4). However, there was no significant effect of magnesium on the duration of development at 9°C. In the NSW control, the duration of the zoea I was four times longer at 1°C than at 9°C, while the few surviving larvae in the NSW +Mg^{2+} treatment took even six times longer.

Oxygen consumption decreased at all temperatures during the development from the zoea I to the megalopa stage, but then it increased significantly after metamorphosis to the first juvenile instar (Figure 1A). Besides ontogeny, also temperature significantly affected the rate of oxygen consumption of larvae and juveniles reared in NSW, so that respiration was three- to fourfold higher at 9°C than at 1°C. Both at 1 and at 9°C, an elevated magnesium concentration significantly depressed the oxygen consumption in the first zoeal stage (Figure 1B). The subsequent stages were not affected by magnesium at 9°C (data not shown). As

survival in NSW +Mg^{2+} was low at 1°C no respiration measurements were undertaken in the subsequent stages.

In the NSW control, larval rearing at 1 and 4°C caused in the megalopa stage significantly reduced W, C and H contents, as well as lower C:N ratios compared to 9°C (Figure 2). Other, mainly inorganic compounds were also significantly lower in megalopae that had developed at low temperatures. During larval development at 1°C (data available only for the zoea I; not shown) and at 9°C (Figure 3), there was no significant effect of magnesium on W and CHN. In the first juvenile instar, W was significantly lower in NSW +Mg^{2+} than in NSW mainly due to significant differences in inorganic compounds.

Discussion

Survival of the lecithotrophic larvae of *Paralomis granulosa* from hatching through metamorphosis to the first juvenile crab stage is very high (74% at 9°C; cf. Anger et al. 2003) compared to planktotrophic larvae reared under optimal conditions, e.g. those of the temperate hermit crab *Pagurus bernhardus*, where only 20% reached the first juvenile instar at 12°C (Dawirs 1979). This is at least partly due to an abbreviated development (*P. granulosa* ca. 30 days, *P. bernhardus* ca. 50 days, both at 12°C) with a reduced number of moults until metamorphosis to the first juvenile stage (two vs. four zoeal stages, respectively). This may compensate for the lower number of offspring in the subpolar species with lecithotrophic larval development compared to the temperate species with planktotrophic larvae. These reproductive traits seem to be selected by harsh environments, which are characterized low temperatures and a pronounced seasonality of plankton production (Thorson 1950, Mileikovsky 1971, Clarke 1988, Thatje et al. 2003b). The evolution of these life-history traits does, however, not seem to be a prerequisite for the establishment of a species in the high Antarctic. In Antarctic caridean shrimps, for instance, completely lecithotrophic developments have not been described to date (Thatje et al. 2003b). However, only few but abundant caridean shrimp species are found in the Antarctic today, while the brooding peracarid crustaceans exhibit a high diverstiy and abundance (Brandt 1999; Gutt et al. 2004). Similarly, the number of brooding echinoderm species is large, whereas there are also few, but highly abundant species with planktotrophic development (Poulin et al. 2002, Pearse & Lockhart 2004).

Ontogenetic changes in oxygen consumption observed in the early developmental stages of *P. granulosa* are similar in magnitude to those previously found in fully lecithotrophic larvae of the northern stone crab *Lithodes maja*, which show also comparable W and CHN (Anger 1996). The temperature-dependent oxygen consumption of the zoea I of *P.*

granulosa determined by Thatje et al. (2003a) is however higher than that observed in the present study, especially at the low temperatures, where the values differ by a factor of four. These differences may be due to unknown differences in methodology.

Newly hatched zoea I larvae were in the present study about 25% heavier than those previously studied by Calcagno et al. (2003). On the other hand, developmental changes in dry weight, elemental composition and C:N ratios determined at 9°C were in our study similar to those observed by Calcagno et al. (2003) at 6°C. Carbon and hydrogen levels decreased significantly, whereas the nitrogen level remained fairly constant throughout the period of lecithotrophic larval development at 9°C. This pattern is typical for non-feeding larvae indicating a preferential consumption of internal lipid stores (Anger 1996, Anger 2001, Kattner et al. 2003). The same drop in the C:N ratio observed at 6 and 9°C, from 7.0 at hatching to 6.0 in the megalopa stage indicates that both of these temperatures allow for optimal development, which is also reflected by high survival rates (Calcagno et al. 2003, Anger et al. 2003).

Rearing at 1 and 4°C resulted in the megalopa stage in significantly lower values of dry weight, content of inorganic compounds, and C:N ratio compared to those obtained at 9°C. Hence, our data suggest that low temperatures caused a faster developmental depletion of lipid reserves as well as a disturbed uptake of inorganic compounds, which are necessary to build up the exoskeleton. Reduced C:N ratios alone, however, may not explain the poor survival observed at 1°C (23% on day 16 after moulting to the megalopa stage; Table 2). Calcagno et al. (2003) measured (at 6°C) near the end of the lecithotrophic phase of development, i.e. prior to metamorphosis to the first juvenile instar, a C:N ratio as low as 4.2 and a C content of only 300 µg. In the present study, by contrast, the lowest observed C:N value in the megalopa stage was still as high as 5.2, and the minimum C content was ca. 400 µg. This suggests that further mechanisms such as a failure of enzymatic processes involved in metabolism, the moulting cycle, or larval development may reduce the survival at low temperatures. Also, it must be considered that high mortality at 1°C did not allow for sampling megalopae older that 16 days for biomass determinations, which reduces the comparability of megalopal biomass data measured at 1°C with previously published data, and even with those measured by us at higher temperatures. The final C and C:N values prior to metamorphosis (not measured in the present study at this temperature) might actually have been similar to those measured by Calcagno et al. (2003), or perhaps even lower.

At 1 and 4°C, the rate of oxygen consumption did not significantly decrease after the moult to the megalopa (remaining at 75 and 81%, respectively, of the values measured in the zoea II). At 9°C, by contrast, it dropped significantly to 66% of the zoea II level. Together

with the prolonged development, this may explain the more pronounced lipid depletion (indicated by decreasing carbon) at 1 and 4°C (to 69 and 63%, respectively, of the values in zoea II) compared to the larvae reared at 9°C (82%).

Starvation experiments with planktotrophic larvae showed that the larvae lose their capability to recover from irreversible damage caused by nutritional stress, so that they cannot survive and moult when food deprivation exceeds a certain limit ("Point of no Return") (Anger 2001). This damage includes ultrastructural changes in the hepatopancreas (cell shrinkage, swollen mitochondria, loss of lipid vacuoles, deeply folded basal membranes) and other organs like the midgut epithelia, antennal glands and the gills. In first-stage zoeae of *Carcinus maeans* and *Hyas araneus* this critical point was reached when about 25-30% of the initial carbon pool was lost (Anger & Dawirs 1982, Dawirs 1984, 1987). However, relatively little is known about effects of temperature on critical points such as the PNR. In P. granulosa reared at 9°C 26% of the initial C was lost during the time of development from hatching to the megalopa stage, whereas larvae reared at 1 and 4°C lost during the study period (up to 85 days) ca. 40%. This suggests that the utilization of internal energy stores may become less efficient at unfavourably low temperatures. A similar effect seems to occur also in lecithotrophic krill larvae, where lower temperatures caused more rapid C utilization (Ross & Quetin 1989). Furthermore, the survival of larval stone crabs declined at low temperatures already in the second zoeal stage, when C content was still high. Significantly shorter times of survival under starvation conditions were observed also when larvae of temperate or subtropical crab species were exposed to lower than normal temperatures (Anger et al. 1981). These observations suggest that metabolic disorder offsets the retarding effect of low temperatures on rates of metabolism and reserve degradation. Besides differential climatic adaptation associated with the geographic range of different species, there is also intraspecific seasonal variation in the nutritional vulnerability of larval decapods (Gebauer et al. 2010), and there are significant maternal or genetic differences among hatches produced by different mothers (Anger et al. 2007). Therefore, future basic research must deepen our understanding of biological and environmentally induced variability, which may sometimes interact and mask the effects of single effectors like magnesium and other ions.

Most deaths during larval development occurred during moulting to the following stage. Prior to dying the animals exhibited a swelling of the carapace, suggesting that the animals were not able to maintain ion and water homeostasis. Reduced calcium concentrations in the haemolymph of juveniles exposed to 1°C for three weeks compared to control conditions at 9°C indicated temperature-dependent changes in calcium uptake (Wittmann et al., submitted). If ionic regulation is also altered in the larval stages, this may

explain difficulties during moulting and reduced amounts of inorganic compounds in the megalopa stage at low temperatures. In agreement with this, Anger (1987) suggested that increased mortality of *Hyas araneus* megalopae during exposure to an unfavourably high temperature was not caused by problems due to an impaired assimilation or conversion of organic matter, but because processes regulating the moulting cycle were disturbed.

Experimental doubling of the external magnesium concentration prolonged the larval development and reduced the rate of survival at 1°C, whereas no such effects were observed at 9°C. Furthermore, magnesium concentration did not affect dry weight and organic composition during larval development at 1 and 9°C. This suggests that the consumption of energy stores was not significantly changed by an elevated magnesium concentration. The dry weight of the first juvenile stage was in the NSW $+Mg^{2+}$ treatment at 9°C reduced compared to the control group in NSW. This seems to be mainly due to difficulties in the uptake of inorganic compounds, as no differences in the CHN fraction was observed, which is predominantly bound in organic compounds. An explanation might be that high external magnesium concentration may hamper the uptake of calcium in this stage, as it is known that magnesium can occupy calcium binding sites of structural, regulatory and ion transport proteins (Hagiwara & Takahashi 1967, Iseri & French 1984, Richmond et al. 1995).

Unaltered oxygen consumption at 9°C in the more advanced stages implies that a putative paralysing effect of magnesium was hardly visible at this temperature and in these stages. This is supported by only minor effects on heart and antennule beat rates at this temperature (Wittmann et al., submitted). Oxygen consumption of the most active larval stage, the zoea I, observed at both 1 and 9°C was significantly lower when the animals were exposed to a high magnesium concentration. In this case, magnesium probably anaesthetized the animals and lead to a reduced metabolic rate. Low temperature and a high magnesium concentration may thus have worked jointly hampering the movements needed for swimming as well as for shedding the exuvia at moulting. Moulting was prolonged or failed completely. Future studies should therefore test if a reduction of the magnesium concentration facilitates exuviation at low temperatures.

In conclusion, stone crab larvae tolerate for extended periods temperatures below their natural temperature regime. While our study suggests that differential utilization rates of internal energy stores were not the primary cause for increased mortality at very low temperature (1°C), we suggest that a more detailed study with a better temporal resolution should scrutinize this tentative conclusion. Doubling of the external magnesium concentration affected most strongly the first larval stage, which may reflect its dampening effect on the activity of crustaceans. As the effect of magnesium was stronger at a low temperature, an enhanced ability for magnesium excretion in larval stages should have an

adaptive value in cold regions, especially for planktotrophic larvae that actively forage for food.

Acknowledgements

We would like to thank captain and crew of RV Polarstern and Fredy Véliz Moraleda for indispensable help during acquisition, transport and maintenance of the animals. Dana Pargmann is acknowledged for her dedicated assistance with larval culture. Furthermore, we appreciate the help of Barbara Niehoff and Nadine Knüppel with CHN measurements. This study was funded by Deutsche Forschungsgemeinschaft grants no. SA 1713/1-1 and 1-2 and by the Alexander von Humboldt Foundation (Feodor Lynen fellowship to DS).

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Table 1: Ion composition (mmol L^{-1}) of culture media. NSW: natural sea water, salinity 32.5 PSU, NSW + Mg^{2+}: natural sea water to which $MgCl_2$ * 6 H_2O was added and which was subsequently diluted down to 32.5 PSU.

Medium	**Na^+**	**Cl^-**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**SO_4^{2-}**
NSW	473	538	9.9	51	9.9	27
NSW + Mg^{2+}	383	580	7.7	97	7.8	24

Table 2: Measurement and sampling days for the respective stages at the culture temperatures of 1, 4 and 9°C in NSW and NSW +Mg^{2+}. Days on which oxygen consumption measurements were carried out and samples for CHN analysis were collected, were estimated using duration of development of Anger et al. (2003) to preferably sample in the middle of the moult cycle of each stage. This however was not always exactly achieved (compare Table 4). ZI: zoea I, ZII: zoea II, M: megalopa, CI: first juvenile stage.

T (°C)	Stage	Days after last moult	Days after hatching
1	ZI	-	8
	ZII	21	~35
	M	16	~75
4	ZI	-	5
	ZII	10-11	~23
	M	34-36	~85
9	T0	-	0
	ZI	-	2
	ZII	3	~7
	M	15	~26
	CI	15-20	~57

Table 3: *Paralomis granulosa.* Larval survival (%) dependent on temperature and magnesium concentration. Values are means for larvae of the two females investigated as survival did not differ depending on the female. n: number of individuals at the beginning of the observation period. n. d.: not determined. *significantly different from value in NSW at 1°C.

Medium	T (°C)	n (= 100%)	% survival to ZII	% survival to M	% survival to CI
NSW	1	64	91	38	n. d.
NSW	4	47	92	60	n. d.
NSW	9	96	93	88	74
NSW +Mg^{2+}	1	106	19*	0*	n. d.
NSW +Mg^{2+}	9	112	94	80	79

Table 4: *Paralomis granulosa.* Duration of development (days) of larval stages. Values are means ± SD and are given separately for each female (1 and 2) as developmental times differ especially in the megalopa stage. *significantly different from value in NSW at 1°C.

Medium	T (°C)	ZI		ZII		M	
		1	2	1	2	1	2
NSW	1	13.5± 2.1	14.2± 2.9	44.0± 4.7	46.1± 5.9	n.d.	n.d.
NSW	4	13.0± 2.8	13.4± 2.9	36.4± 5.9	38.6± 5.3	n.d.	n.d.
NSW	9	3.5± 0.6	3.5± 0.7	7.1± 0.6	7.8± 0.8	25.3± 2.0	32.6± 2.3
NSW +Mg^{2+}	1	22.2± 6.0*	26.7± 8.2*	n.d.	n.d.	n.d.	n.d.
NSW +Mg^{2+}	9	3.9± 1.3	4.1± 1.1	8.0± 0.9	8.3± 0.7	24.5± 2.0	31.8± 2.6

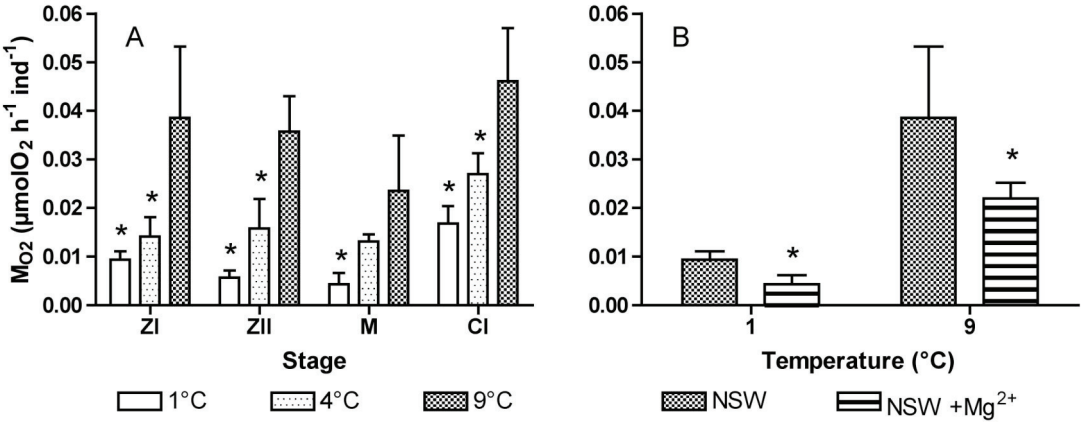

Figure 1: *Paralomis granulosa.* Oxygen consumption (M_{O2}, µmolO_2 h^{-1} ind^{-1}) of early developmental stages dependent on temperature and magnesium concentration. Temperature significantly affects respiration in NSW (A). Note that individuals in the first juvenile stage (CI) were raised at 9°C and acclimated to 1 and 4°C two weeks prior to measurements, whereas larval respiration was determined at the respective culture temperatures. *significantly different from value at 9°C. Magnesium significantly affects oxygen consumption of the zoea I (B). *significantly different from value in NSW. Values are means ± SD of 5 – 7 individuals.

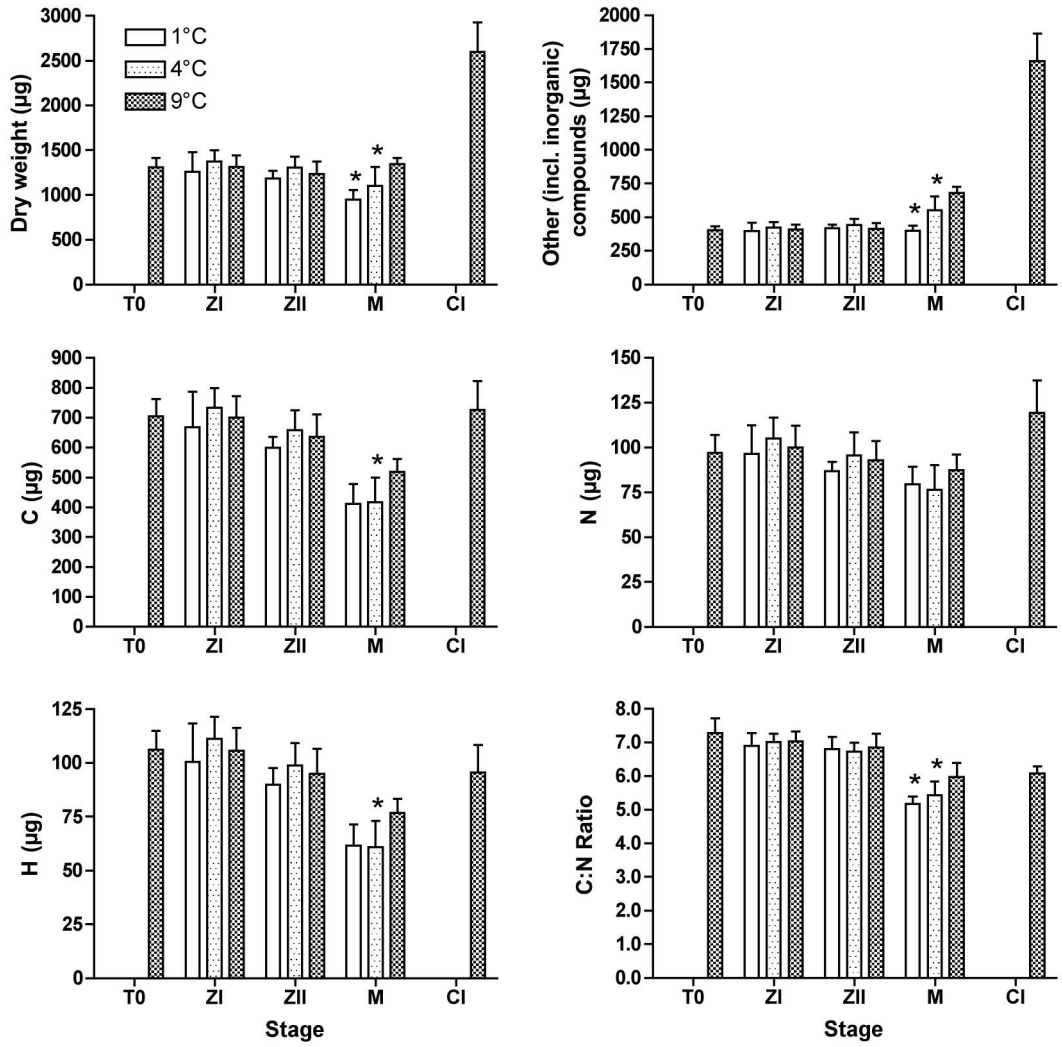

Figure 2: *Paralomis granulosa.* Effect of temperature on dry weight, C, H, N, remaining compounds (µg) and C:N ratio during early development in NSW. Samples were taken in the middle of the moulting cycle (see Table 2). Values changed significantly during development and dependent on temperature. *significantly different from value at 9°C. Values are means ± SD of 3 – 10 individuals.

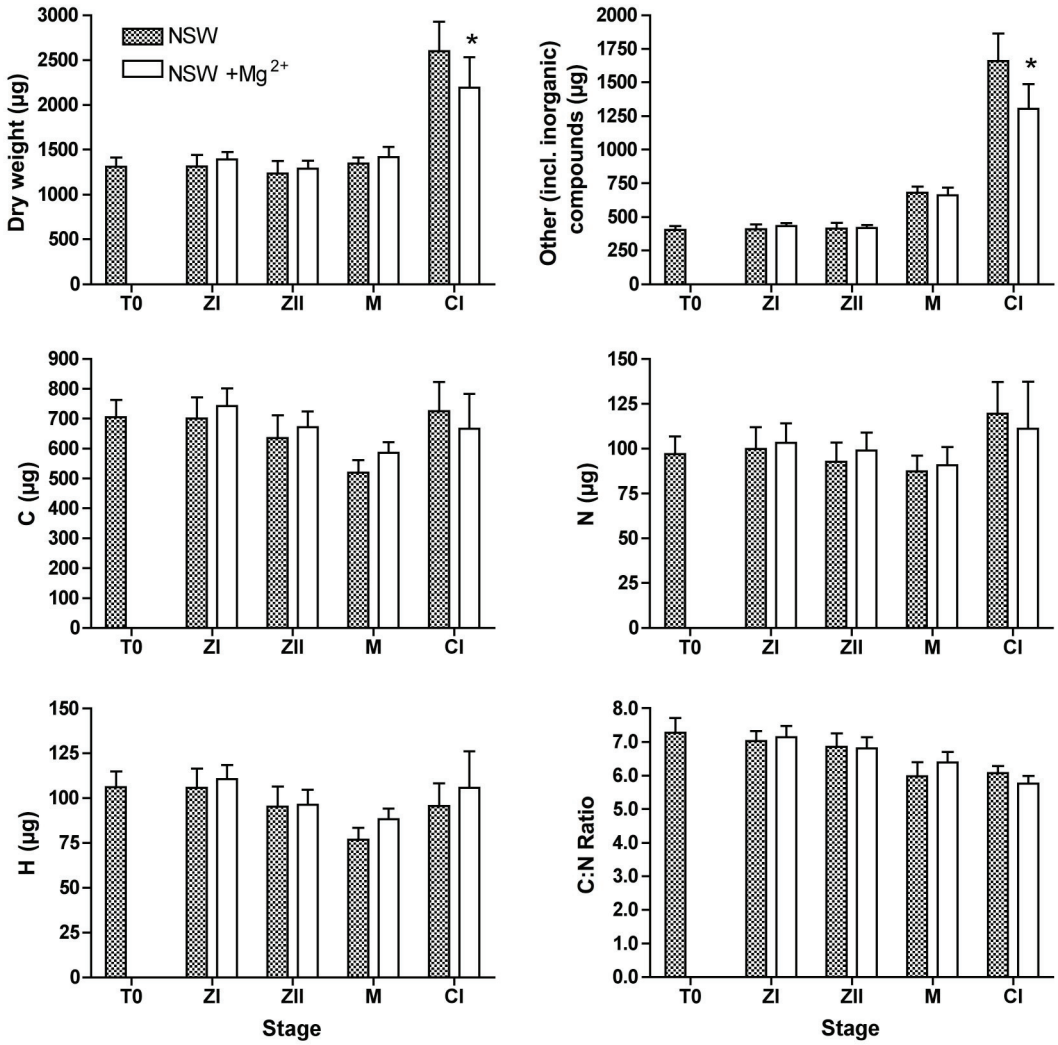

Figure 3: *Paralomis granulosa.* Dry weight, C, H, N, remaining compounds (µg) and C:N ratio of early stages raised in NSW or NSW +Mg^{2+} at 9°C. Samples were taken in the middle of the moulting cycle (see Table 2). Values changed significantly during development but dependent on magnesium concentration only in the first juvenile stage (CI). *significantly different from value in NSW. Values are means ± SD of 3 – 10 individuals.

4. DISCUSSION

4.1. How cold tolerant are sub-Antarctic lithodid crabs?

Temperature is an important determinant of the distribution of ectothermic species (Pörtner and Knust 2007; Calosi et al. 2010). Species of the family Lithodidae seem to be constrained in their distribution to habitat temperatures above 0°C (Hall and Thatje 2009). Short-term physiological studies may give insight into mechanisms shaping thermal tolerance of different life stages, but long-term studies are needed to be able to evaluate the relationship between thermal tolerance and the biogeography of a species (Anger et al. 2003; Pörtner and Knust 2007; Pörtner and Farrell 2008).

In this study, short-term (minutes to days) observations of effects of low temperature on cardiorespiratory variables as well as activity were carried out in adult and early life-history stages of the sub-Antarctic stone crab *Paralomis granulosa*. Long-term (days to months) effects of low temperature exposure were studied in the early developmental stages of *P. granulosa*, because physiological constraints in larval stages are thought to represent a bottleneck for species distribution (Anger et al. 2003; Pörtner and Farrell 2008; Hall and Thatje 2009; Storch et al. 2009a,b). Thus, it was attempted in this chapter to compare tolerances to cold between life stages (Figure 4.1) and to analyse whether physiological constraints relate to the southern distribution limit of this species, after addressing the question, which processes may determine cold tolerance.

Thermal tolerance thresholds of ectotherms in the adult stage have been determined by recording blood or haemolymph oxygenation and the onset of non-oxidative metabolism during acute temperature incubations (e.g. Frederich and Pörtner 2000; Lannig et al. 2004; Melzner et al. 2006b). Blood or haemolymph oxygen partial pressures are thought to reflect the aerobic scope (Frederich and Pörtner 2000; Pörtner 2002; Pörtner 2010). Oxygen delivery, facilitated by ventilation of the gills and circulation of the haemolymph by contractions of the heart, was reduced at temperatures below the pejus threshold temperature in the warm-eurythermal spider crab *Maja squinado*. This resulted in a drop in arterial haemolymph oxygen partial pressure (Frederich and Pörtner 2000). Such a reduction of arterial haemolymph P_{O_2} with declining temperature was also observed in 10°C-acclimated specimens of the warm-eurythermal *Carcinus maenas* in this study (Figure 6.3). In contrast, the cold-eurythermal spider crab *Hyas araneus* did not exhibit a decrease but an increase in arterial haemolymph P_{O_2} with decreasing temperature (Walther et al. 2009). This is similar to what was observed in this study on the cold-eurythermal *P. granulosa* (publication III). An emerging pattern therefore is, that cold tolerance of cold-eurythermal decapod crabs is, at

least on short time scales, not determined on the physiological level of oxygen delivery as previously hypothesized (Frederich et al. 2001). At least during short-term experiments, arterial haemolymph P_{O_2} does not seem to be a good proxy for thermal tolerance of these crabs (Figure 4.2, publication III). The following question arises: If oxygen delivery is not the limiting factor on a short time scale, what are the mechanisms that determine cold tolerance? Besides kinetic effects of temperature, putative implications of cold exposure on aerobic and anaerobic energy production, on membrane properties and associated ion regulation and muscle function, as well as on the hormonal system are considered in the following section. The role of extracellular magnesium regulation is discussed in the next chapter.

When temperature is reduced, standard metabolism of an ectotherm is also reduced (Q_{10} relationship, Eckert et al. 2000). This means that at low temperatures there is less energy available at the cellular level. Furthermore, aerobic metabolic scope, the ability to accelerate metabolism during activity or to digest a meal, is reduced in the cold (Rutledge and Pritchard 1981; Peck 2002). However, in *P. granulosa* the net scopes for heart and ventilation rates, which may translate into metabolic scope, were not significantly different from each other at -1 and 4°C even though righting speed of the animals was significantly slower at the lower temperature (publication III). In decapod crustaceans, bursts of activity, like during righting and running, as well as moderate activity levels are at least in part fuelled by anaerobic energy production (Burke 1979; Booth et al. 1982; Taylor 1982; Houlihan et al. 1984; Henry et al. 1994). In addition, the relative contribution of non-oxidative energy production during exercise is greater in the cold than in the warmth in intertidal and amphibious crabs (Burke 1979; Weinstein and Full 1998). Thus, not just the effect of temperature on aerobic metabolism may play a role in the slowing of activity in the cold, but also that on anaerobic metabolism.

Furthermore, temperature-dependent changes in membrane properties, which may affect intracellular and extracellular ion regulation, may become limiting. The Q_{10} for active ATP-dependent ion transport processes is greater than that for passive dissipative processes. In the cold the ion transport capacity may hence not suffice to counterbalance diffusion of ions (Hochachka 1988). Many cellular functions involve ion transport processes including energy production in the mitochondria, neuromuscular signal transduction and muscle contraction. In the present study, steady-state extracellular ion composition was not affected in *P. granulosa* exposed to -1°C. However, during activity the cellular potassium loss to the haemolymph was greater at -1 and 1°C than at 4°C, which may indicate a constraint in the function or capacity of the Na^+/K^+-ATPase (publication III). Increased extracellular potassium concentration causes membrane depolarisation, failure of excitation and a reduction in force responses in musculature (Allen et al. 2008). This may have contributed to

the slowing of movements of *P. granulosa* in the cold. The 10°C-acclimated shore crab *C. maenas* displayed an increase of haemolymph sodium, chloride and calcium concentrations when incubated at 1°C during resting conditions (Figure 6.1). It can only be speculated that this has detrimental effects and is linked to the low spontaneous walking activity in the cold (Figure 6.2). In the tropical prawn *Litopenaeus stylirostris* handling stress at low temperature (20 - 22°C) induced only a small increase in haemolymph osmotic pressure, which coincided with a reduction of haemolymph oxygen capacitance, and led to increased mortality (Wabete et al. 2008). This indicates that thermal limitation in this species may be based on constraints in osmoregulatory capacity as well as on oxygen delivery.

The extent to which the effect of low temperature on muscle function can be compensated for seems to be limited. Low speeds of locomotion have been reported for Antarctic invertebrates when compared to temperate species (Barnes and Peck 2008). Also, aerobic and anaerobic swimming capacities of Antarctic fish are limited, but a certain degree of compensation allows low to moderate aerobic activity (but see also Hardewig et al. 1998; Peck 2002; Pörtner 2006; and references therein). The Antarctic isopod *Glyptonotus antarcticus* exhibits a different fibre type composition and expresses a different myosin heavy chain isoform than temperate crustacean species. Sequence differences in the myosin heavy chain, which may compensate for temperature effects on binding properties, suggest that muscle function is to some extent adapted to the low temperatures found in the Southern Ocean (Whiteley et al. 1997; Holmes et al. 2002). Aronson et al. (2007) noted that the absence of skeleton-crushing fish and crabs in the Antarctic may be linked to insufficient muscle function and reduced force generation in the cold (Wakeling and Johnston 1998). A direct effect of temperature on myofilament function and insufficient mitochondrial capacity in muscle cells may therefore also have resulted in the reduced righting speed and maxilliped beat frequency in the cold in adult and larval *P. granulosa,* respectively.

In addition, temperature-dependent behavioural responses may be controlled by the hormonal system (Worden et al. 2006; Hamilton et al. 2007). These studies indicate that hormones function to widen the thermal window of systemic physiological functions. Future studies should investigate different levels of organismal complexity to identify the processes that determine cold tolerance of cold adapted species.

In the following section thermal tolerances of different life stages of *P. granulosa* determined during short-term experiments (publications II and III) will be compared with temperature-dependent larval survival rates (studied by Anger et al. 2003; Figure 4.1). Moreover, potential mechanisms that may influence temperature-dependent larval survival rate will be discussed. It is important to note that acclimation temperatures of larvae (9°C in this study, 6°C in Anger et al. 2003) and adults (4°C) were different, so direct comparisons

should be made cautiously. It is not known whether larval performance can be altered by changing acclimation temperatures and if the temperature history of the embryo or genetic predisposition determine thermal sensitivity during the larval phase. The differences in swimming performance of zoea I larvae of two populations of *Taliepus dentatus* from central and southern Chile with habitat temperatures differing by about 5°C were rather small (Storch et al. 2009a). This suggests that genetic predisposition plays a major role and might allow to compare temperature-dependent curves of larval survival and swimming performance of the zoea I.

Unlike the zoea I, adult *P. granulosa* were still performing locomotory movements at -1°C, although at a significantly reduced speed (Figure 4.1 B, C). This implies that the adults were more tolerant to cold than the zoeae. This is in line with what has been hypothesized with respect to changes in thermal sensitivity during the life cycle of fish (Pörtner and Farrell 2008). Short-term observations of cardiorespiratory physiology and activity of the various stages indicate that *P. granulosa* tolerates temperatures as low as -1°C (Figure 4.1 B, C). The inability to complete larval development at 1°C however implies that this species would not be able to establish itself on the continental shelf of Antarctica with extended periods at or even below -1°C (Figure 4.1 A; Anger et al. 2003; Peck et al. 2006; Clarke et al. 2009).

In this study, survival rates, dry weights, elemental composition and haemolymph ion composition of the early developmental stages provide some insight into the long-term effects of temperature on growth and moulting success. Fully lecithotrophic larvae like those of *P. granulosa* will not take up food but mainly consume their lipid reserves during ontogeny (Anger 1996; Anger 2001; Kattner et al. 2003). Developmental changes in dry weight, organic composition and C:N ratios at 9°C were similar to those observed at 6°C in a previous study (Calcagno et al. 2003). This suggests that both 6 and 9°C allow for optimal larval development, also reflected by equally high survival rates (ca. 74%, Anger et al. 2003; Calcagno et al. 2003, publication IV, Figure 4.1). The depletion of lipid stores was probably not the sole reason for the low survival rates at 1 and 4°C, as *P. granulosa* larvae likewise had consumed 40% of their initial carbon at 6°C prior to successful metamorphosis to the first juvenile stage (Calcagno et al. 2003; Kattner et al. 2003). Survival rates in the cold already declined during the second zoea, when carbon content was still high. Most larvae died during moulting to the next stage or after shedding the old exuvia. Prior to dying the animals displayed a swelling of the carapace. This indicates that the animals did not maintain ion and water homeostasis. In fact, exposure of juveniles to 1°C for three weeks resulted in a reduction of haemolymph calcium concentration. Therefore, temperature probably affected changes in the capacity for calcium uptake from the medium (publication II). If this also happened in the larval stages, it may explain the difficulties observed during

moulting at low temperatures. The reduced build-up of inorganic compounds in the megalopa stage in the cold (publication IV) probably resulted from reduced calcium uptake, as calcium carbonate is the major inorganic component of the carapace (Greenaway 1985). Thermal effects on the physiological mechanisms underlying the moulting cycle, like ionic regulation and hormonal control, remain to be examined and will give further insight into mechanisms of thermal limitation of larval stages.

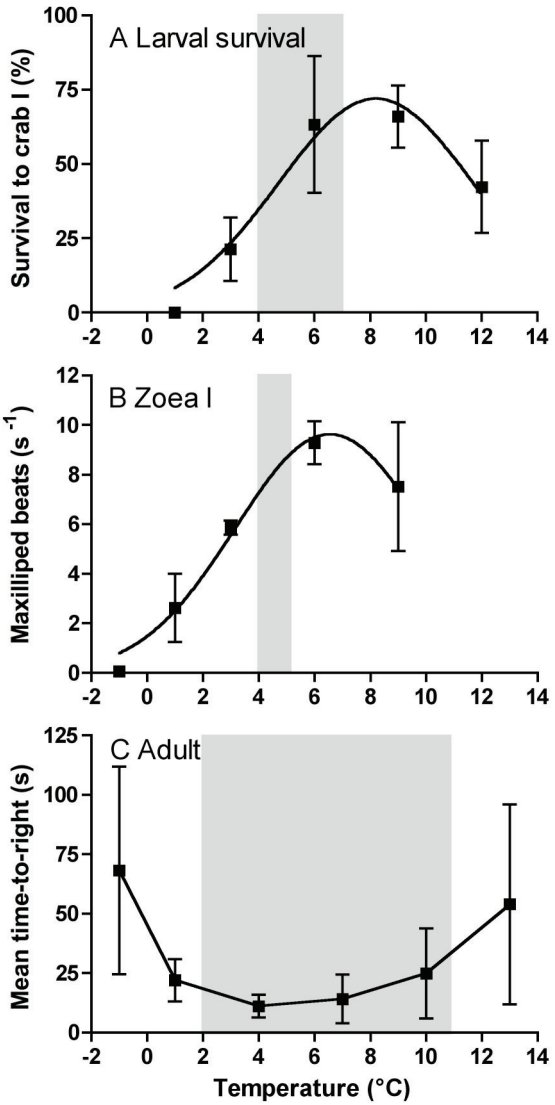

Figure 4.1: Thermal tolerance of *Paralomis granulosa*.
Tolerance curves of larval survival (A, adopted from Anger et al. 2003), acute changes of swimming performance in the zoea I stage (B) and acute changes of the mean time-to-right in adult male crabs (C). A: Means ± s.d. of larvae (hatched at 6°C) of 3 females, except at 1°C where larvae of only one female were considered (see Anger et al. 2003). B: Means ± s.d. of 6 individuals, larvae hatched at 9°C, see publication II. C: Means ± s.d. of 7-8 individuals acclimated to 4°C, see publication III. Grey areas: approximate habitat temperatures encountered by the respective stages in the Magellan region. Winter 2 – 4°C, summer 9 – 11°C (Anger et al. 2003, Hoggarth 1993; Lovrich and Vinuesa 1993).

To conclude, previous studies, together with the current work, show that none of the investigated life stages of *P. granulosa* would be able to sustain conditions of the high Antarctic. In their natural environment zoeae hatch in the austral winter (July–September) and are exposed to temperatures of 4 - 5°C (Figure 4.1). The first feeding stage, the first juvenile instar, is thought to be reached between September and November at 5 - 7°C with the onset of the phytoplankton bloom (Lovrich and Vinuesa 1993; Anger et al. 2003; Thatje et al. 2003a). In summer habitat temperatures are in the range of 9 - 11°C (Lovrich and Vinuesa 1993; Lovrich 1999; Arkhipkin et al. 2004). The temperatures of optimal systemic performance of the adults (4 - 7°C, publication II) match well with the temperatures the species encounters in its habitat (Figure 4.1). Larval survival and swimming activity of the zoea I however appear to be optimal at temperatures exceeding those of the natural environment. Thus, the species may extend, but not necessarily shift its distribution to the South if water temperatures increase further in the wake of global warming (Thatje et al. 2005a; Aronson et al. 2007; Clarke et al. 2009). For comparison, it would be interesting to study the thermal tolerance of lithodid crab species found on the continental slope of the Western Antarctic Peninsula in the Bellingshausen Sea, where the group encounters the lowest water temperatures of its distribution area. However, as Clarke et al. (2009) note, lithodid crabs are nearly absent in the shallowest waters at South Georgia, where bottom temperatures are warmest in the Southern Ocean (2 – 3°C). The authors suggest that other (unspecified) factors, apart from temperature, also play a role in the distribution of this group.

4.2. What is the role of magnesium in cold tolerance?

It is long standing practise for physiologists to use magnesium salts to paralyse marine invertebrates for experimentation (Pantin 1948). There is evidence that the effect of the ion is even more pronounced at low temperatures (Lagerspetz and Tiiska 1996). This, and observations on warm- and cold-eurythermal brachyuran crabs, have led Sartoris et al. (1997) and Frederich et al. (2001) to hypothesize that the inability of anomuran and brachyuran crabs to downregulate haemolymph magnesium concentration represents a major constraint for these groups to occur in the extremely cold high Antarctic latitudes.

The fact that Antarctic isopods exhibit the same haemolymph magnesium levels as lithodid crabs from the sub-Antarctic contrasts this hypothesis (publication I). The question arises whether crustaceans suffer from an anaesthetic effect at low temperatures and natural sea water magnesium concentration at all, or whether isopods and other marine invertebrates were able to overcome such an effect during evolution and decapod crabs were not. Studies on temperature-dependent activity rates of Antarctic isopods suggest that they

compensate for effects of temperature only imperfectly (Young et al. 2006; Barnes and Peck 2008). However, Antarctic isopod species engage in active predation (e.g. *Glyptonotus antarcticus*, Janssen and Hoese 1993) and are able to swim (Janecki et al. 2010). So even at extremely low temperatures their behaviour differs from a "semi-narcotized state" described for lithodid and majid decapod crustaceans (Robertson 1953).

This study addressed the question, whether the activity of decapod crustaceans is dependent on extracellular ion regulation. First, it was investigated if temperature affects haemolymph ion composition. Second, haemolymph magnesium concentration was altered to examine whether this affects temperature-dependent rates of activity. Experiments on the anomuran lithodid crab *P. granulosa* from the sub-Antarctic and the temperate brachyuran crab *Carcinus maenas* do not support the hypothesis that exposure to low temperature affects extracellular magnesium regulation and leads to an increase in haemolymph magnesium concentration, which may lead to anaesthesia. Haemolymph magnesium concentration was not influenced by temperature during both short-term exposure of adult *P. granulosa* and during long-term exposure of juveniles to cold (publications II and III). Also, during short-term cold exposure haemolymph magnesium concentration in *C. maenas* was not affected (Figure 6.1). The examination of a seasonal change in haemolymph composition in *C. maenas* did not reveal any differences between summer and winter months (Figure 6.4). Therefore, reduced activity of the animals in winter (Crothers 1968; Atkinson and Parsons 1973) may not be a result of increased magnesium concentration. The slowing of movements as well as the reduction of food consumption in the cold (Figure 6.2) may thus primarily result from a kinetic effect of temperature on metabolism, systemic physiology and muscle function of these crabs (Figure 6.3 and publication III).

It is interesting that there was a difference in the rate with which the animals' haemolymph ion concentrations equilibrated with the surrounding reduction in magnesium concentration when comparing the brachyurans used by Frederich et al. (2000b) and the anomuran investigated in this study (Figure 2.1). Frederich et al. mention that it took their experimental animals only three days at 5°C to reach the desired haemolymph magnesium concentration and attributed this to their low capacity for magnesium regulation. In the present study, the brachyuran *C. maenas* showed the same pattern at 10°C (Figure 2.2). In contrast, *P. granulosa* maintained a haemolymph magnesium concentration significantly above that of the surrounding sea water even after six days in ASW $-Mg^{2+}$ at 4°C. Furthermore, the animals maintained a sodium concentration significantly lower than that of the surrounding medium, but reached a new steady-state for this ion after three days already. One interpretation of these findings is, that the animals possess a capacity for both magnesium and sodium regulation, which may however differ between the ion species. A

set-point for sodium may exist, which the animals try to defend by active excretion. Their capacity to retain magnesium is higher than that of *C. maenas* and that of the brachyuran crabs investigated by Frederich et al. (2000b). The fact that the zoeal stages of *P. granulosa* downregulate sulphate concentration may further emphasize that this species does possess a high capacity for ion regulation (publication II). These are unexpected results, because other lithodid crab species have previously been described to have low capacities for magnesium excretion (Robertson 1949; McAllen et al. 2005) and osmoregulation (Mackay and Prosser 1970; Thomas and Rice 1992).

In marine invertebrates a high haemolymph magnesium concentration has been attributed to a reduced capability to excrete magnesium (Robertson 1949, 1953). However, it may as well be reasonable that *P. granulosa* maintains high magnesium levels intentionally. The divalent cations calcium and magnesium are effectors of the respiratory pigment haemocyanin and increase the pigment's affinity for oxygen (Truchot 1975; Mangum 1983). In the thalassinid *Callianassa*, magnesium is required to build functional aggregations of haemocyanin multimers (Morritt and Spicer 1993). Haemolymph magnesium concentration, protein and copper (a proxy for haemocyanin) levels covary seasonally in the same direction in the lithodid crab *Neolithodes grimaldii* (McAllen et al. 2005), indicating that magnesium may play a role in haemocyanin function of this crab. Furthermore, expression of low-affinity haemocyanin in early developmental stages of the crab *Cancer magister* have been interpreted to compensate for the high levels of haemolymph magnesium concentration in these stages, so that the oxygen carrying capacity of the whole haemolymph stays the same throughout development (Brown and Terwilliger 1998). However, only little is known about the properties of haemocyanin of cold-water lithodid crabs (Molon et al. 2000), its contribution to oxygen supply and a possible role of magnesium in the modulation of its function at low temperatures. In this study temperature-dependent haemolymph P_{O_2} has been determined, which does not give any insight into haemocyanin function, as only the oxygen in solution was detected, not that bound to the pigment.

In contrast to findings in the warm-eurythermal *Maja squinado* (Frederich and Pörtner 2000), haemolymph P_{O_2} did not fall in the cold-eurythermal *P. granulosa*, but increased with decreasing temperature (publication III, Figure 4.2). This was probably due to cold-compensation of ventilation frequency associated with a decrease in oxygen consumption at high oxygen solubilities in water and haemolymph. The difference between arterial and venous haemolymph P_{O_2} also remained high in the cold, which indicates functional circulation and that during the course of the experiment the animals did not suffer from oxygen deficiency. Thus, an oxygen limitation due to a paralysing effect of magnesium on ventilation and circulation as proposed by Frederich et al. (2001) was likely offset by high

oxygen solubilities in water and haemolymph as well as by low oxygen consumption rates at low temperatures.

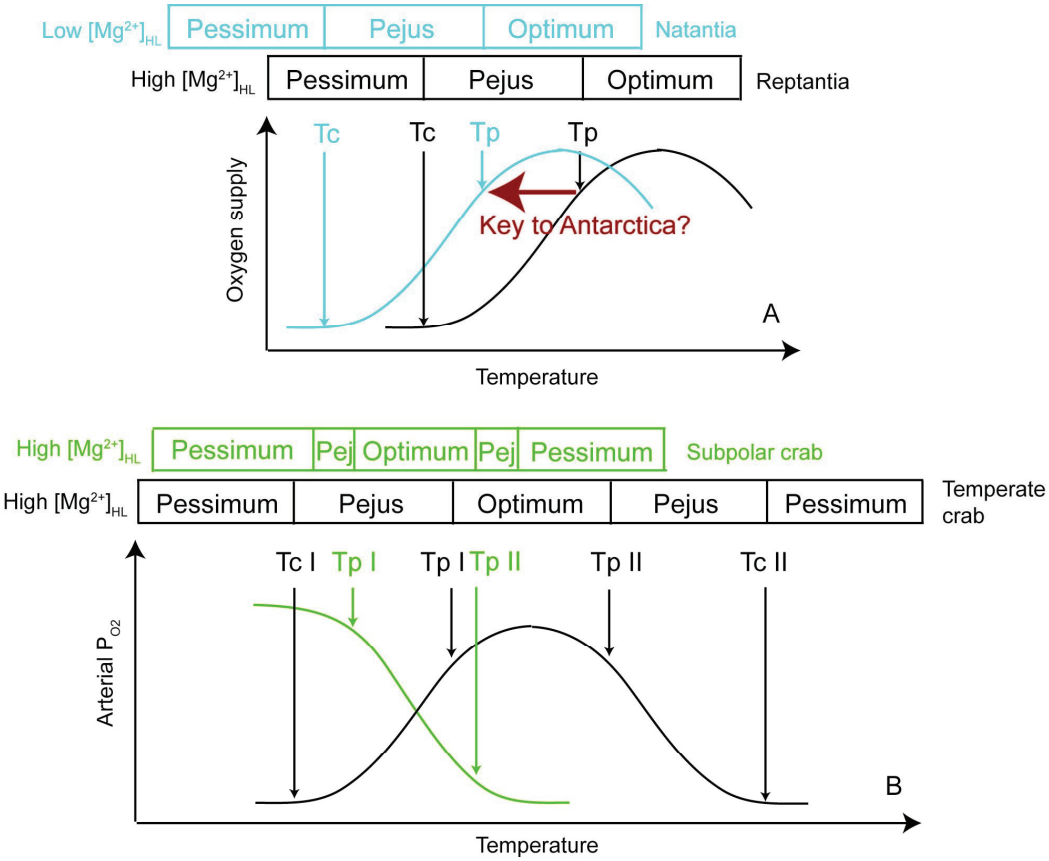

Figure 4.2: Schematic comparison of hypothesis (A) and results of current study (B). A: Anomuran and brachyuran crabs ("Reptantia") with their high haemolymph magnesium concentration ($[Mg^{2+}]_{HL}$) are missing in the high Antarctic. Oxygen supply (arterial haemolymph PO_2) curve shifts to colder temperatures when $[Mg^{2+}]_{HL}$ is reduced as expected in Antarctic caridean shrimps ("Natantia"). Redrawn after Frederich et al. (2001). B: Cold compensation of ventilation leads to high arterial haemolymph PO_2 at low temperatures despite high $[Mg^{2+}]_{HL}$ in the subpolar crab (*Paralomis granulosa*). While arterial haemolymph PO_2 is a good proxy for thermal tolerance of the temperate crab (*Maja squinado*, Frederich and Pörtner 2000), this is not the case for the subpolar crab (*P. granulosa*).

Nevertheless righting speed decreased with temperature to a greater extent than expected simply from kinetic constraints and was significantly lower at -1°C than at 4°C at natural haemolymph magnesium concentration (30 mmol L^{-1}). The experimental reduction of haemolymph magnesium concentration in adult *P. granulosa* from 30 to 12 mmol L^{-1} did not exert the same effect on righting speed as in the brachyuran *Hyas araneus* in a similar experiment (publication III; Frederich et al. 2000b). The latter, a cold-eurythermal species, which occurs in the sub-Arctic as well as in the North Sea (Christiansen 1969), accelerated righting speed at temperatures below 0°C when haemolymph magnesium concentration was reduced from the natural concentration of 46 to 8 mmol L^{-1}. The sub-Antarctic brachyuran *Eurypodius latreillei* showed a similar response in walking distance at a reduction from 50 to

15 mmol L^{-1} of haemolymph magnesium concentration and was more active in the cold at low magnesium concentrations compared to the natural condition (Frederich et al. 2000b).

Walters and Uglow (1980) compared haemolymph magnesium concentration and the scope for heart rate (as a parameter for the activity level) in an array of decapod crustacean species at 10°C and found that there is a negative correlation between the two factors. The reduction of magnesium concentration did not lead to an increase in the scopes for heart and ventilation rates in *P. granulosa*. This may indicate that other species-specific factors influence the scope for activity more strongly than magnesium. These may include properties of the musculature. Active pelagic fish exhibit for example higher mitochondrial densities in heart and skeletal muscle than more sluggish benthic fish, reflecting a greater aerobic scope in pelagic fish (Sänger et al. 2005). It is evident that the activity of *P. granulosa* should have increased when the sea water magnesium concentration was reduced, if the ion played a role in slowing the neuromuscular system in the cold in this species. Therefore, other mechanisms setting thermal thresholds are more prominent in this crab.

Frederich et al. (2000b) noted already that there are species-specific differences in the extent to which magnesium affected temperature-dependent heart rates, oxygen consumption and activity rates. The present results on *C. maenas* even suggest that inter-individual differences are quite prominent, which may explain the large error bars and the difficulties to detect significant differences dependent on magnesium concentration (Figure 6.2, Figure 6.3). In contrast, increased heart rates in the cold at reduced magnesium concentration have indicated a less pronounced thermal sensitivity and an improved cold tolerance of *C. maenas* in a previous study (Frederich et al. 2000b). Frederich et al. (2000b) found a more pronounced thermal sensitivity in heart rates in *C. maenas* kept in natural sea water than observed in this study. Similarly, the heart rate of *Hyas araneus* kept in natural sea water was affected more strongly by temperature in the study by Frederich et al. (2000b) than in the work by Walther at al. (2009). Differences in the experimental protocols used may explain these variations in thermal sensitivity. While in the present study and in the study by Walter et al. (2009) temperature was lowered continuously, Frederich et al. (2000b) may have used a step-wise temperature protocol giving the animals time to acclimate to the new conditions. Prolonged exposure time may reduce thermal tolerance of marine invertebrates (Peck et al. 2009) and may have resulted in lower heart rates at low temperatures in the control group compared to the results of the present study. Thus, the present results on food consumption and walking activity in *C. maenas* (Figure 6.2), which were investigated using a step-wise temperature protocol, may relate more closely to the data by Frederich et al. (2000b) than to those in this study (Figure 6.3). Significantly elevated food consumption at

reduced magnesium concentration compared to control conditions at low temperature may conform to the increased heart rates found by Frederich et al. (2000b).

In addition, there seem to be differential sensitivities to magnesium amongst amphipod crustaceans. On the one hand, a relationship between activity and haemolymph magnesium concentration was found in the supra-littoral amphipod sandhopper *Talitrus saltator*. These animals spend the winter in a dormant state, which coincides with high haemolymph magnesium concentrations, whereas during summer the animals are active and exhibit low haemolymph magnesium levels (Spicer et al. 1994). On the other hand, no correlation between activity and magnesium concentration was observed in a variety of pelagic mid-water amphipods. Sluggish neutrally buoyant forms displayed similar magnesium levels as species, which undertake vertical migrations (Tentori and Lockwood 1990).

Experiments on larval and juvenile stages of *P. granulosa* exposed to twice the magnesium concentration as in natural sea water showed that slowing effects are most prominent in high frequency swimming movements of the zoea I (publication II). The first larval stage is the most active with the highest metabolic rate (publication IV) despite exhibiting the highest haemolymph magnesium concentration (publication II). This contradicts the negative correlation between activity and haemolymph magnesium concentration postulated by Robertson (1949), a fact, which was previously also noted for the megalopae of *Cancer magister* (Brown and Terwilliger 1992). However, the first larval stage seems to be the most susceptible to magnesium anaesthesia as oxygen consumption was significantly reduced when the animals were exposed to a high magnesium concentration, whereas the later stages were not affected (publication IV).

Larval *P. granulosa* exclusively consume endotrophic reserves (Calcagno et al. 2003; Kattner et al. 2003; Calcagno et al. 2004; publication IV). Strategies, which save energy will therefore augment larval survival. The extremely low spontaneous swimming speeds compared to other crustacean larvae (Chia et al. 1984; Shirley and Shirley 1988; publication II) may reflect an energy-saving demersal lifestyle of the larvae, which have not been found in plankton samples so far (Lovrich 1999). In the zoea I spontaneous swimming behaviour was not affected by magnesium, but the high-frequency maxilliped beat was altered. The maxillipeds are used to propel the animals forward during swimming. When individuals in the first zoeal stage were fixed underneath the stereomicroscope, they were kept in the water column and swam continuously at most of the tested temperatures. We can infer two things from these behavioural observations: First, if their spontaneous behaviour in the laboratory reflects their natural behaviour, this means that they do not swim a lot and that magnesium concentration does not play a role for these animals. Second, because

maxilliped frequency was affected, cold tolerance of species with planktotrophic larvae, which rely on swimming for food uptake, may be affected by a high magnesium concentration in the haemolymph. Thus it would be interesting to study the ion composition of the haemolymph of larval caridean shrimps and brachyuran crabs from subpolar and polar regions.

An increased magnesium concentration prolonged larval development and reduced survival only at the incubation temperature of 1°C, whereas no effects were observed at the control temperature of 9°C (publication IV). Furthermore, no changes in dry weight and organic composition were observed during larval development at 1 and 9°C and depending on magnesium concentration. This may imply that low temperature and high magnesium concentration act synergistically at the neurophysiological level, paralyse the animals and impede movements to shed their exuviae during moulting. Therefore moulting took more time or failed completely, which may have resulted in suffocation of the animals in their old exuviae. It remains to be elaborated if a reduction of magnesium concentration facilitates moulting in the cold. This was not possible in the present study as raising the larvae in artificial sea water failed irrespective of the magnesium concentration or the temperature.

In conclusion, both early stages and adult *P. granulosa* displayed a low sensitivity to changes in magnesium concentration. It is thus rather unlikely that a high haemolymph magnesium concentration limits cold tolerance and the biogeography of *P. granulosa*. For a more comprehensive view, further experiments on thermally sensitive larval stages should include the application of media with reduced magnesium concentration. A comparison of the sensitivity of synaptic transmission to cold and magnesium between crustacean taxa and between populations from different latitudes may improve the understanding of the role of extracellular magnesium regulation in crustaceans and identify the mechanisms underlying its effect.

4.3. Why are “reptant” decapods not part of the Antarctic shelf fauna?

High magnesium concentration does not seem to influence cold tolerance, in *P. granulosa* and the investigated Antarctic isopods. The fact that not all crustaceans and even not all “reptant” decapod species are constrained by an insufficient magnesium excretion may be highlighted by the occurrence of the brachyuran majid crab *Chionoecetes opilio* in arctic-boreal regions of -1.5 - 4°C year round (Burmeister and Sainte-Marie 2010). Haemolymph magnesium concentration is high in *C. opilio* and the congeneric *C. tannerii* from the North Atlantic, comprising 35 - 45 mmol L^{-1} (Mackay and Prosser 1970; Charmantier and Charmantier-Daures 1995). Growth and reproduction are optimal in the narrow thermal range of the species’ natural environment, with most successful egg extrusion at 0 and 3°C

(Foyle et al. 1989; Webb et al. 2007). Remarkably, *C. opilio* is not only able to endure temperatures as low as -1°C for extended time periods, but eggs are extruded by females and embryos develop at this temperature (Webb et al. 2007). This exemplifies that adaptation to a stenothermal polar environment is possible for a brachyuran crab. But the question still remains open why brachyuran and anomuran decapods are absent from the coldest waters of the Antarctic.

A combination of circumstances may have led to the vast reduction of the diversity of decapods at the time when climatic cooling of the Antarctic started in the late Eocene (34 Mya, Figure 4.3) (Zachos et al. 2001; Feldmann and Schweitzer 2006). It is not clear whether a direct effect of temperature or associated changes in primary productivity caused the substantial ecosystem reorganization at this time (Clarke 1993; Crame 1999; Aronson et al. 2007; Ivany et al. 2008). Both temperature and primary productivity are important determinants of biogeography and diversity (Parsons and Lear 2001; Astorga et al. 2003; Tittensor et al. 2010). The fossil record suggests that at least some decapod species still occurred in a warmer phase of the glacial Miocene (23 Mya, a brachyuran, a nephroid lobster) and of the Pliocene (2 - 4 Mya, a palinurid lobster; Feldmann and Quilty 1997; Feldmann and Crame 1998; Uchman and Gazdzicki 2010). This may indicate on the one hand that decapods were able to adapt to cold-water conditions and recolonized the Antarctic even after the Antarctic Circumpolar Current had developed, and on the other hand that they were not eradicated all at once.

Besides the lower temperatures, Antarctic species face the problem that Antarctica is isolated from surrounding continents by the deep sea and the Antarctic Circumpolar Current (Clarke et al. 2005). Only one shallow land bridge connects the Antarctic Peninsula across the islands of the Scotia Arc with South America today. Hence, a northward emigration of benthic species in order to stay in favourable conditions was greatly impeded. Furthermore, periodic glaciations of the Antarctic continental shelf in the last 35 My not only reduced the space for benthic species, but probably also led to a reduction of primary productivity near the continental shelf due to the formation of a thick multiple-year sea ice layer adjacent to the shelf ice. This would have resulted in the differential extinction of species with planktotrophic larvae (Poulin et al. 2002). Current environmental conditions do allow, however, planktotrophic development. On the one hand there are relatively few, but highly abundant species in the Antarctic, which exhibit this mode of development (Pearse and Lockhart 2004). These species are found amongst echinoderms as well as caridean decapods. On the other hand the Antarctic shelf fauna is characterized by a great number of brooding echinoderm and peracarid crustacean species. Poulin et al. (2002) suggested that for the echinoderms this pattern is the result of both macro- and microevolutionary processes: On a

long time scale repeated glaciations led to a decline in the number of species with planktotrophic larvae while brooding groups radiated (high diversity, evolutionary success). The ecological success i.e. the high abundance of species with planktonic development may be due to their greater advantage to quickly recolonize disturbed habitats. Transferring this to crustacean species, repeated cooling and glaciation events might have led to the extinction of decapod species with planktonic development, whereas the brooding peracarid crustaceans were selected for. Caridean shrimps may be faster in the recolonization of the Antarctic shelf than "reptant" crabs due to their ability to swim.

Recolonization of the Antarctic by decapod crabs is possible (Thatje and Fuentes 2003; Clarke et al. 2005; Thatje et al. 2005a), but due to its greater isolation compared to the Arctic this probably takes much more time. The chances for survival of a decapod species during glaciation of the Arctic are likely greater because migration towards the South is possible. Glaciation of the Arctic has happened much more recently than in the Antarctic (Zachos et al. 2001), so fewer putative extinction events may have recently taken place. Moreover, recolonization of Arctic shelf areas is facilitated by their connection to boreal and temperate shelf regions.

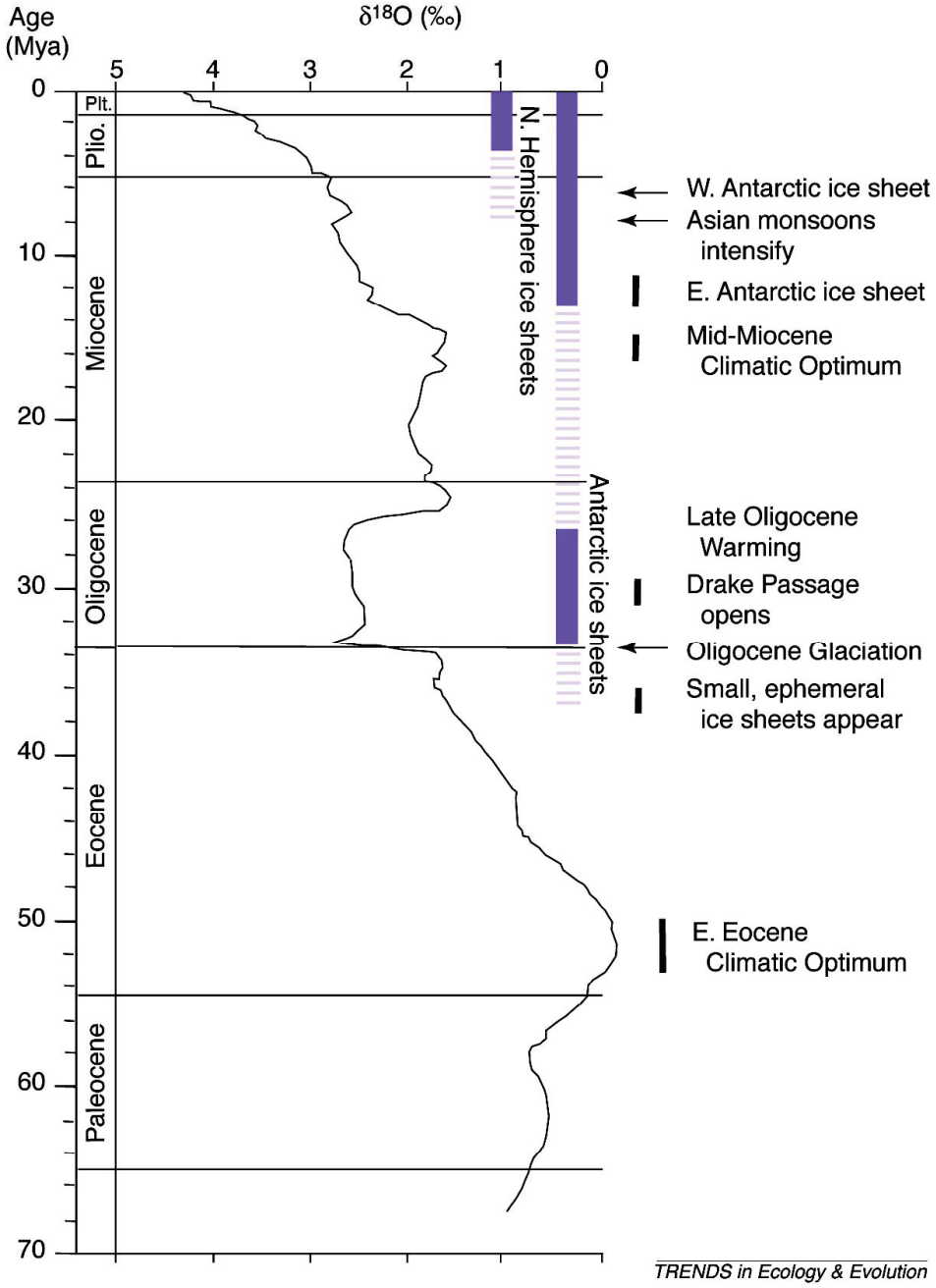

Figure 4.3: Change of deep-sea temperature and ice-sheet coverage in the northern and southern hemispheres in the last 70 My.
$\Delta^{18}O$ is a proxy for temperature and ice volume and can be used as a direct measure for temperature only during ice-free periods. Bottom temperatures declined in the Eocene from 12°C at the climatic optimum to 4.5°C at the end of the epoch. During the Oligocene glaciation temperature was estimated to be as low as 4°C and ice mass was up to 50% of that of present-day. During the early Miocene temperatures were slightly higher and ice volume was reduced. After the Mid-Miocene climatic optimum temperature gradually declined and a major ice-sheet re-established on Antarctica by 10 Mya. The early Pliocene was characterized by a subtle warming trend until ca. 3.2 Mya, when glaciation of the northern hemisphere began (Zachos et al. 2001 and references therein). Figure from Poulin et al. (2002), but see also (DeConto et al. 2008).

4.4. Conclusions

Cold exposure did not affect haemolymph magnesium concentration of various life stages of *Paralomis granulosa*, neither during short-term experiments nor during incubations that lasted up to three weeks. This implies that reduced activity levels as well as heart and ventilation rates at low temperatures are not associated with a relaxing effect caused by increased extracellular magnesium concentrations. However, the higher potassium loss during activity may have contributed to the slower righting speeds of adult specimens of *P. granulosa* in the cold. Therefore, thermal thresholds of locomotory performance may be related to the capacity for cellular potassium regulation.

Long-term effects of cold exposure on haemolymph calcium and sulphate concentrations in juvenile *P. granulosa*, as well as a lack of build-up of inorganic compounds in megalopae cultured at low temperature may indicate constraints in ion homeostasis and moulting, and may be related to the low survival rates of larvae at 1°C. Increased external magnesium concentration reduced larval survival of *P. granulosa* at low temperature to an even greater extent, and also prolonged developmental time. Low temperature and high magnesium concentration therefore worked synergistically to impede larval development. This was probably caused by a direct paralysing effect of the combined factors, which led to the reduction of movements for shedding the exuviae during moulting. A reduction in activity was evidenced by a significant effect of magnesium on oxygen consumption and maxilliped beat frequency (during forced swimming activity) of the zoea I. Whether a reduction of magnesium concentration will facilitate moulting and will make larvae more cold tolerant remains to be investigated. Moulting is a crucial process in crustaceans, because without it development and growth is impossible. Moulting is controlled by the hormonal system and involves periodic changes of ion transport processes and water permeability of the integument. However, it is not known, in which way these mechanisms are impacted by temperature or other abiotic factors that affect larval survival rates, and how they contribute to thermal limitation in crustaceans.

Ontogenetic changes in haemolymph magnesium concentration did not correlate with the activity levels of the various stages. The zoeal stages displayed the highest values, but are capable of performing high-frequency swimming movements, whereas the later stages are benthic and excreted magnesium to a greater extent. The first juvenile stage was less sensitive to an experimental increase in magnesium concentration than the zoea I. Furthermore, the reduction of magnesium to a level of that of caridean shrimps did not result in enhanced righting speed or scopes for heart and ventilation rates in adult specimens. This suggests that other life stage- and species-specific factors determine activity

rates and scopes, like mitochondrial capacities and other properties of the musculature. However, since the most active zoea I stage is the most sensitive to a change in magnesium concentration, planktotrophic larvae, which are capable of magnesium excretion may have an advantage over those that are not. This may have implications for the distribution of brachyuran crabs and caridean shrimps in the Southern Ocean. The fact that early stages as well as adult *P. granulosa* overall are rather insensitive to changes in sea water magnesium concentration suggests that haemolymph magnesium concentration plays only a minor role for thermal limitation and the biogeography of this species. Whereas this species is constrained to water temperatures of above 1°C by the temperature sensitivity of larval development, development of other lithodid crab species may be adapted to the low-temperature regime of the Antarctic Bellingshausen Sea.

This study shows that a low haemolymph magnesium concentration *per se* is not a prerequisite for a crustacean species to occur in high Antarctic marine habitats. High extracellular magnesium concentrations do not constrain the investigated isopods to water temperatures above 0°C. Thus, during evolution at least two solutions to handle a putative paralysing effect of magnesium may have developed in species, which exhibit a high tolerance to cold. First, an enhanced excretion of magnesium is observed in polar caridean decapod and amphipod crustacean species. And second, a reduced sensitivity of neuromuscular systems to magnesium may be hypothesized for e.g. isopod crustaceans, some "reptant" decapod crustaceans and cephalopod molluscs. Besides this, a high starvation tolerance and an efficient energy metabolism at low temperatures seem to be crucial adaptive traits for species thriving in the high Antarctic marine environment.

This study is the first to show that the sub-Antarctic *P. granulosa* was not limited by oxygen supply to the tissues at low temperature, at least on short time scales. Therefore, tolerance to cold may be determined on a different level of organismal complexity. In this short-term experiment, arterial haemolymph P_{O_2} turned out not to be indicative of thermal tolerance of this species. Other parameters like the difference between arterial and venous P_{O_2}, activity, intermittent ventilation and circulation, as well as the scopes for heart and ventilation rate may be better short-term indicators in a cold-eurythermal species. The current model of oxygen- and capacity-limited thermal tolerance (Figure 1.2) actually suggests that the performance near the lower pejus temperature is declining not due to a reduction of oxygen supply, but because of kinetic constraints. Therefore, at low temperature thresholds a capacity limitation may predominate over an oxygen limitation. This awaits however further investigation, since only few studies have addressed mechanistic relationships of thermal limitation at low threshold temperatures in marine ectotherms. In addition, no long-term studies exist on the role of oxygen delivery in thermal limitation.

There is a need for long-term experiments, which combine work on thermal effects on the systemic physiology and those on growth and reproduction of invertebrates to assess how these parameters relate to each other.

While the present study revealed that there is no simple relationship between the capacity for extracellular magnesium regulation and the biogeography of marine crustaceans, the enigma of low decapod crustacean diversity at high Southern latitudes remains unresolved. Furthermore, it emphasises that there is a need to improve the understanding of mechanisms, which determine low temperature thresholds. After all, species distribution shifts, expected as a consequence of ocean warming, are dependent on both upper and lower thermal constraints.

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6. APPENDIX

6.1. Additional results

6.1.1. Effects of season, temperature and magnesium on *Carcinus maenas*

6.1.1.1. Haemolymph ion composition, walking activity and food consumption

In artificial sea water with normal magnesium concentration (ASW, see Table 2.1 for ionic composition), haemolymph ion composition of animals changed significantly only after a 20 h–exposure to the lowest experimental temperature of 1°C (Figure 6.1). Na^+, Cl^- and Ca^{2+} concentrations were significantly increased, K^+ concentration significantly decreased at 1°C compared to values determined at the acclimation temperature of 10°C. Mg^{2+} and SO_4^{2-} concentrations were only slightly elevated in the cold. Accordingly, the sum of all ion concentrations, which is approximately equal to the osmolarity of the haemolymph, was significantly higher at 1°C than at 10°C. There were no significant changes observed in haemolymph ion composition of animals kept in ASW –Mg^{2+} (artificial sea water with reduced magnesium concentration Table 2.1) and exposed to a decline in temperature from 10 to 1°C. However, haemolymph Mg^{2+} concentration was significantly lower than in individuals kept in ASW.

Spontaneous walking activity and food consumption declined with temperature (Figure 6.2). At 1°C activity was slightly higher in ASW –Mg^{2+}, but the difference was not significant. However, the amount of food consumed at 1°C was significantly higher in ASW –Mg^{2+} than in ASW.

6.1.1.2. Haemolymph P_{O_2}, ventilation and heart frequency in response to acute cold exposure

Arterial haemolymph P_{O_2} of specimens of *C. maenas* subjected to an acute temperature ramp significantly declined with temperature both in ASW and ASW –Mg^{2+} (Figure 6.3). Oxygen partial pressures between 1 and 4°C in animals kept in ASW –Mg^{2+} were only slightly higher than in individuals kept in ASW ($p > 0.05$). This may be due to the significantly increased ventilation rate in these animals between 2 to 5°C compared to those incubated in ASW. The temperature-dependent slope of the heart rate was significantly greater in animals incubated in ASW –Mg^{2+} than that of the animals in ASW. In contrast to ventilation rate, the values differed most strongly at temperatures of 4 to 10°C.

6.1.1.3. Seasonal effects on extracellular ion composition of C. maenas*?*

Season did not affect ionic composition of the haemolymph of *C. maenas* (Figure 6.4). This was true for divalent as well as for monovalent ions (data not shown), even though water temperature differed by 8°C between late winter (7°C) and summer (15°C).

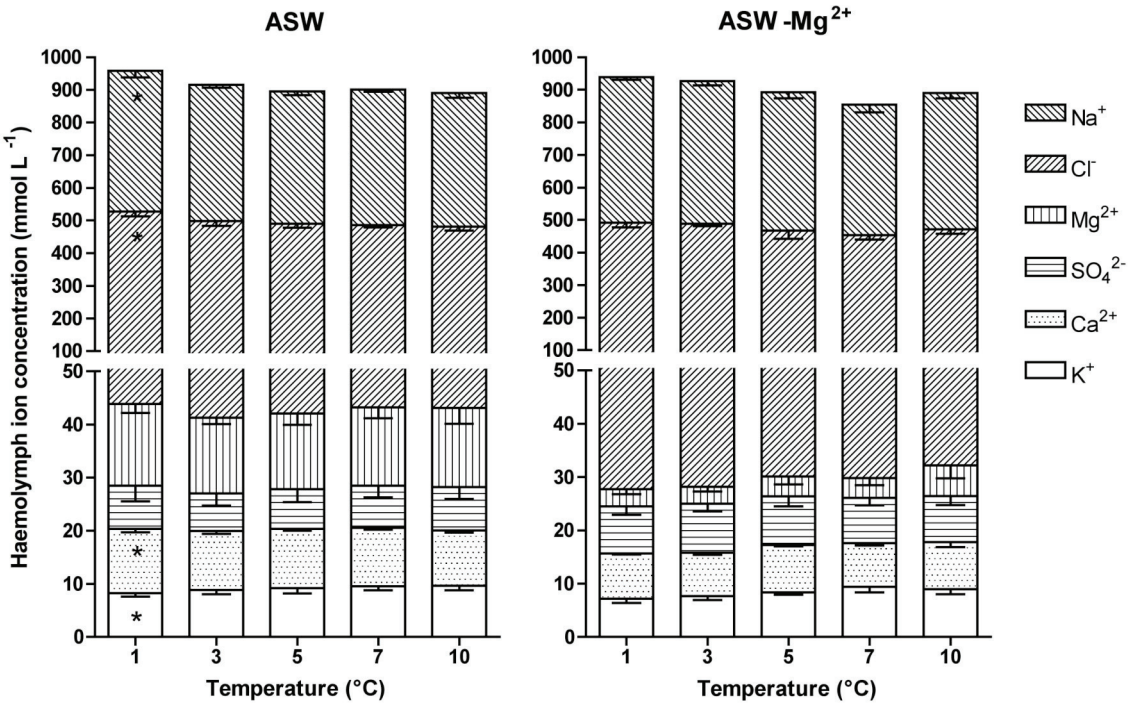

Figure 6.1: Haemolymph ion composition (mmol L^{-1}) of *Carcinus maenas*.
Animals were subjected to a step-wise temperature protocol with 20 h intervals for acclimation. Temperature only affected ionic composition of animals in ASW. *significantly different from values at 10°C. Values are means ± s.d. of 5 – 7 individuals.

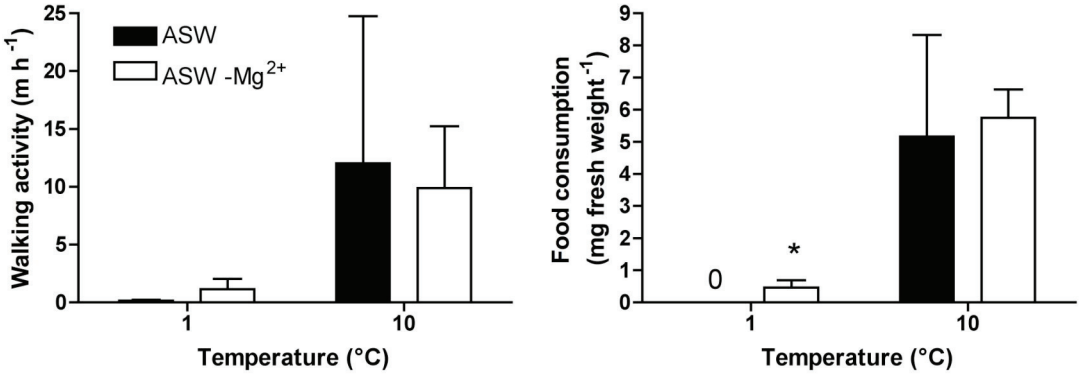

Figure 6.2: Spontaneous walking activity (m h^{-1}) and food consumption (mg fresh $weight^{-1}$) of *Carcinus maenas*.
Animals were subjected to a step-wise temperature protocol with 20 h intervals for acclimation at the same temperatures as in Figure 6.1. *significantly different from value in ASW. Values are means ± s.d. Walking activity: 5 – 7 individuals, Food consumption: 3 groups of 2 – 3 animals.

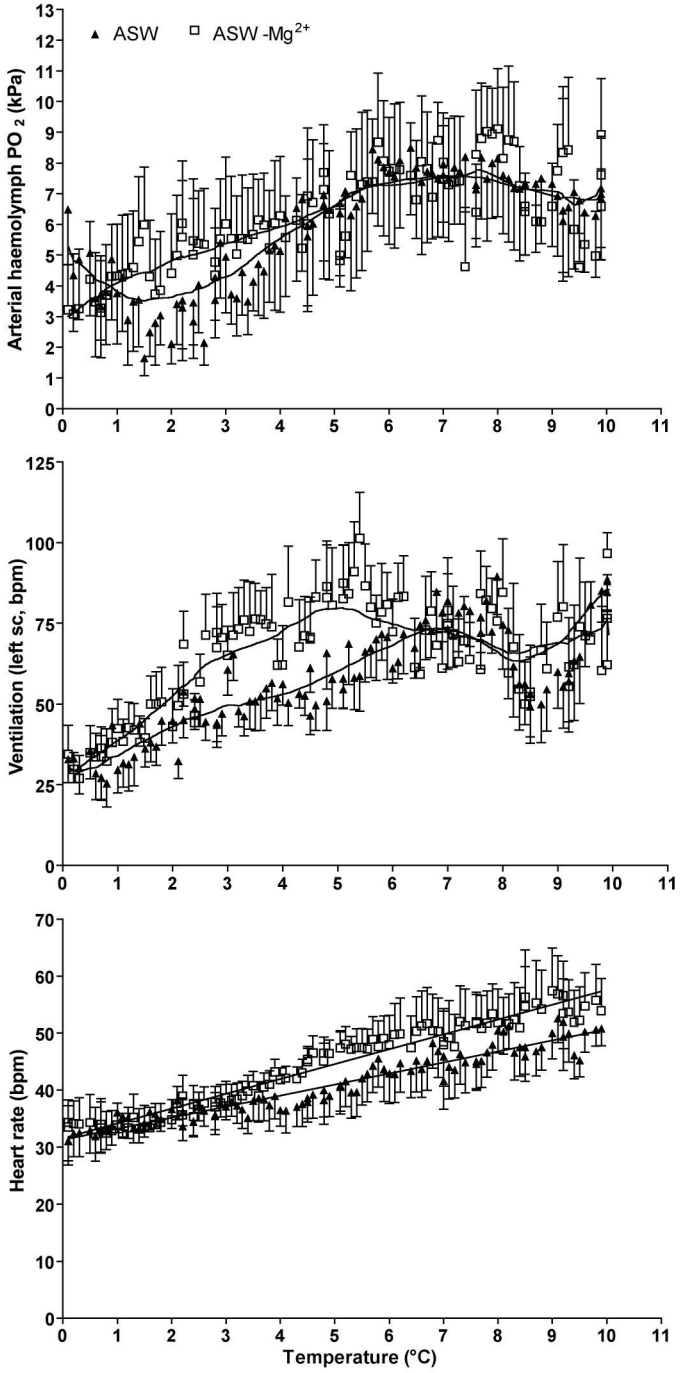

Figure 6.3: Arterial haemolymph PO_2 (kPa), ventilation and heart rates (bpm) of resting *Carcinus maenas* dependent on temperature and magnesium concentration.
Animals were subjected to continuous temperature decrease (1°C h^{-1}). Reduced magnesium concentration significantly affected ventilation and heart rates, but not arterial haemolymph PO_2. Temperature significantly affected all parameters. Values are means ± s.e. of 5 – 9 animals.

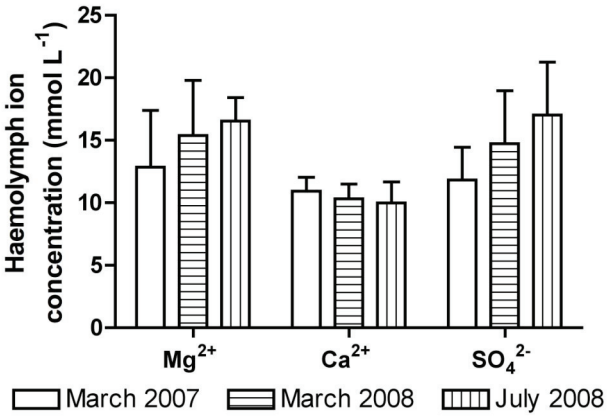

Figure 6.4: Haemolymph concentration of divalent ions (mmol L^{-1}) in *Carcinus maenas*.
Ion concentrations did not differ between seasons. Water temperature in March: 7°C, in July: 15°C. Values are means ± s.d. of 10 – 20 individuals.

6.2. Additional publication

Kiko R, Werner I, Wittmann A (2009) Osmotic and ionic regulation in response to salinity variations and cold resistance in the Arctic under-ice amphipod *Apherusa glacialis*, Polar Biology 32: 393-398

Danksagung

Prof. Dr. Hans Pörtner, in dessen Arbeitsgruppe am AWI diese Arbeit entstand, danke ich für die Diskussionsbereitschaft und die Motivation im Endspurt, und dafür dass ich die Arbeit weitgehend in Eigenregie durchführen konnte.

Bei Prof. Dr. Ulrich Saint-Paul möchte ich mich dafür bedanken, die Rolle des Zweitgutachters zu übernehmen. PD Dr. Holger Auel danke ich dafür, dass er sich dazu bereit erklärt hat, meinem Promotionskolloquium als Prüfer beizuwohnen.

Dr. Franz Sartoris, ohne den es diese Arbeit nicht gäbe, danke ich für das Erdenken des schönen DFG-Projekts, das mich den Rätseln der Antarktis näher gebracht hat, für die gute Betreuung, das freie Arbeiten und das "Liest sich gut, aber…".

Ganz besonders möchte ich Dr. Daniela Storch und Fredy Veliz Moraleda danken, ohne die ich den Löwenanteil meiner Doktorarbeit gar nicht hätte machen können (oder nicht in dieser Form), vor allem für die Hilfe bei der Tierbeschaffung aus Chile und die Mitbetreuung während meiner Larvenexperimente auf Helgoland.

Dr. Klaus Anger und Uwe Nettelmann danke ich für die Gastfreundschaft auf Helgoland, Klaus für die Diskussionsbereitschaft und die gute Zusammenarbeit beim Paperschreiben, und das tolle Larvenbuch.

Dr. Christian Bock und Rolf Wittig danke ich dafür, dass ich "ihren" Auswerteraum besetzen durfte und dafür, dass sie einfach nette Kollegen sind.

Allen übrigen Mitgliedern der AG Integrative Ökophysiologie danke ich für das positive Arbeitsklima und die schöne Zeit am AWI und im besonderen Timo Hirse und Nils Koschnick für alle größeren und kleineren Problemlösungen im Labor.

Der Salatgruppe danke ich für die leckeren Schlemmersalate und die nette Gesellschaft beim Mittagessen, vor allem Katrin, Heidi und Mareike.

Mareike danke ich außerdem für die Gesellschaft am "Katzentisch" und die Kurzurlaube am Deich beim Inline-skaten.

Dana danke ich für ihre verlässlichen Dienste als "Heinzelmännchen", sowie Jana und Christin, ohne die die Zeit auf Helgoland lange nicht so lustig gewesen wäre.

Meiner Familie möchte ich ganz herzlich dafür danken, dass sie mich bei allem bedingungslos unterstützt.

Volker danke ich dafür, dass er mir die Welt der Technik näher bringt, mich in der Politik auf dem Laufenden hält und einfach dafür dass er da ist.

Astrid Wittmann
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Bremerhaven, den 20. September 2010

Erklärung gem. § 5 (1) Nr. 3 PromO

(vom 14. März 2007)

Ich erkläre hiermit,

1. dass ich mich vor dem jetzigen Promotionsverfahren keinem anderen Promotionsverfahren unterzogen habe

und

2. dass ich außer dem jetzt laufenden Promotionsverfahren auch kein anderes beantragt habe.

_____

Astrid Wittmann