Temperature adaptation in the early ontogenesis of decapod crustaceans in the Humboldt Current under ENSO impact

Temperaturadaptation in der frühen Ontogenese dekapoder Krebse des Humboldtstroms unter ENSO-Einfluß

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List of abbreviations

AFLP	**A**mplified **F**ragment **L**ength **P**olymorphism
AMR	**A**ctive **M**etabolic **R**ate
C	**C**arbon
CS	**C**itrate **S**ynthase
CW	**C**arapace **W**idth
EN	**E**l **N**iño
ENSO	**E**l **N**iño **S**outhern **O**scillation
H	**H**ydrogen
HCS	**H**umboldt **C**urrent **S**ystem
HSPs	**H**eat **S**hock **P**roteins
LN	**L**a **N**iña
N	**N**itrogen
PK	**P**yruvate **K**inase
PO_2	**O**xygen **P**artial **P**ressure
SMR	**S**tandard **M**etabolic **R**ate
SO	**S**outhern **O**scillation
SST	**S**ea **S**urface **T**emperature
Tc	**c**ritical **T**emperature
Tp	**p**ejus **T**emperature

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Summary

The Humboldt Current System is a highly productive ecosystem supporting one of the world's largest fisheries. The oceanographic characteristics with year-round upwelling, low seasonality, and comparably low latitudinal changes allow wide distributional ranges of species. One of these species is the hairy crab *Cancer setosus* (Molina 1782) with a distributional range from 2°13'S to 46°00'S, which corresponds to a temperature range from ~10 to ~20 °C. The relatively temperature stable habitat is subjected to the El Niño Southern Oscillation, which might lead to drastic temperature changes of up to 10 °C. Temperature is one of the most important determining factors of species distribution. The early ontogeny of brachyurans was detected to be the bottleneck for survival, as the early life stages are especially sensitive to temperature changes. The pelagic phase presents the dispersal stage of the life cycle and influences biology, ecology, evolution and recruitment of a species. This study analyzed the influence of temperature changes on different levels of biological organisation at different simulated habitat temperatures ranging from 12 °C (LN temperature) to 24 °C (EN temperature). Results of this thesis show that *C. setosus* Zoeal instars react highly sensitive to temperature changes which is reflected in the whole organism function as well as in lower levels of organism complexity.

Survival rates of larvae reared at different temperatures define a very limited window of tolerated temperatures and the duration of larval instars varies dependent on the temperature. Those features seem to vary dependent on the latitude, showing thermal adaptation of larvae. The elemental composition of larvae revealed that the larval metabolism is very sensitive to temperature changes and the composition of larvae varies even within the small range of tolerated temperatures. Elemental analyses furthermore revealed that the temperature with maximum growth rates of *C. setosus* larvae varies with larval instar, probably being acclimated especially to the path of larval dispersal of larvae from the Antofagasta bay. Additionally thermal sensitivity seems to increase with instar. High temperature sensitivity was also found in larval morphometric traits. The temperature encountered during embryonic and larval development as well as the latitudinal origin of larvae influenced the morphometric parameters. Generally larger larvae are produced at cold temperatures. This phenotypic plasticity might be a key to the

species success to cover a wide distributional range and to survive highly variable habitat conditions.

The oxygen consumption rates show, that cold acclimated larvae display thermal plasticity (high Q10 values) being able to compensate for elevated temperatures. Routine metabolic rates of warm acclimated animals were found to function at its upper limits and had only very limited capacities to adjust the metabolism to further increased temperatures. The properties of the metabolic key enzyme citrate synthase (CS) revealed a certain level of acclimation in cold and warm acclimated animals, but CS was found to be a possible key for thermal limitation of *C. setosus* larvae at elevated temperatures.

Due to the highly temperature sensitive metabolism, larval survival during EN/LN events does not seem likely, although acclimation mechanisms could be identified within this study. Further studies on phenotypic and physiological plasticity and acclimation processes of different populations within the distributional range would provide further knowledge about mechanisms that allow such a wide geographical species distribution. It would be crucial to reveal if temperature conditioning of larvae might take place during embryonic development or if it is genetically defined, which would also have implications for larval recruitment. The maintenance of the northern stock that frequently is encountered by the fluctuations of ENSO seems to be dependent on the survival of adult specimen or a recolonization after strong catastrophic EN events. Further investigation of larval dispersal modes, recruitment and recolonization would be of great use when determining optimal spacing and size of potential protected areas

Zusammenfassung

Der Humboldtstrom ist eines der produktivsten Auftriebsgebiete und somit Grundlage für eine der größten Fischereiindustrien weltweit. Die ozeanographischen Gegebenheiten unter „normalen“ klimatischen Verhältnissen (keine El Niño (EN) / La Niña (LN) Verhältnisse) mit ganzjährigem Auftrieb, geringer Saisonalität und vergleichbar geringen latitudinalen Veränderungen erlauben eine weite Verbreitung von Arten. Eine dieser Arten ist der Taschenkrebs *Cancer setosus* (Molina 1782), der von 2°13’S bis 46°00’S vorkommt, was einem Temperaturbereich von ~10 bis ~20 °C entspricht. Im Vergleich mit anderen Küstenregionen entsprechender Breiten weist die lokale Meeresoberflächentemperatur unter „normalen“ klimatischen Bedingungen nur geringe jahreszeitliche Schwankungen auf. Im Gegensatz dazu führen El Niño und die südliche Oszillation (ENSO) zu drastischen Veränderungen der Temperatur. Während starker EN Ereignisse können die Wassertemperaturen um mehr als 10 °C ansteigen. Temperatur ist einer der wichtigsten Faktoren, die die Verbreitungsgrenzen von Arten bestimmen. Die frühe Ontogenese brachyurer Krebse kann als Engpass ihres Entwicklungszyklusses angesehen werden, da frühe Lebensstadien oft besonders empfindlich auf Veränderungen der Umwelt reagieren. Allerdings repräsentieren die Larven das Stadium des Entwicklungszyklusses, welches durch die pelagische Lebensweise für die Verbreitung der Art verantwortlich ist.

Die vorliegende Arbeit stellt den Einfluss von Temperatur und Veränderungen der Temperatur auf die Funktion des Metabolismus auf verschiedenen Ebenen der biologischen Organisation von *C. setosus* Larven dar. Für die Untersuchungen wurden die Larven bei simulierten Habitattemperaturen von 12 °C (LN Temperatur) bis 24 °C (EN Temperatur) gehältert und anschließend analysiert. Die Resultate dieser Arbeit zeigen, dass die Zoea Larven von *C. setosus* hochgradig sensibel auf Temperaturveränderungen reagieren. Dieses spiegelt sich sowohl auf der Ganztier-Ebene, als auch auf darunter gelegenen Organisationsebenen wider.

Erhöhte Mortalitätsraten bei Temperaturen außerhalb des Bereiches der „normalen“ Habitattemperatur (16 – 20 °C) zeigen, dass die Larven ein sehr eingeschränktes Temperaturtoleranzfenster besitzen. Generell nimmt die Stadiendauer der Larven mit steigender Temperatur ab. Das Temperatur-

toleranzfenster und die Larvaldauer bei einer bestimmten Temperatur variieren offensichtlich abhängig vom Breitengrad. Dies lässt auf eine Anpassung der Larven an die lokalen Temperaturen schließen.

Innerhalb des Fensters tolerierter Temperaturen zeigt sich durch die Analyse der elementaren Zusammensetzung der Larven, dass 20 °C die Optimumstemperatur von Larven des Untersuchungsgebietes während der südlichen Sommer- und Herbstmonate ist. Bei dieser Temperatur zeigen die Larven sowohl einen Zuwachs von Gewebe, als auch eine Speicherung von Energiereserven. Im Gegensatz dazu kommt es bei 16 und 20 °C zu einem Ungleichgewicht von Energieangebot und –nachfrage.

Auch die morphometrischen Merkmale der Larven wie Körpergröße und Länge der Appendices spiegeln die hohe Sensibilität der Larven gegenüber Temperaturveränderungen wieder. Die Hälterungstemperatur während der Embryonal- und Larvalentwicklung als auch die latitudinale Herkunft der Larven, haben einen Einfluss auf die morphometrischen Parameter. Im Allgemeinen führen kältere Temperaturen zu größeren Larven. Phänotypische Plastizität könnte der Schlüssel zum Erfolg einer Art sein, sich über ein so weites Gebiet auszubreiten und auch starke Schwankungen im Habitat zu überleben.

Die Sauerstoffverbrauchsraten, die bei Larven der Crustacea den Routine-metabolismus widerspiegeln, zeigen bei kalt akklimierten (angepassten) Larven (12 °C) eine thermale Plastizität (hohe Q10 Werte), was heißt, dass diese Larven in der Lage sind, die Auswirkungen von erhöhten Temperaturen zu kompensieren. Bei warm akklimierten Larven (20 und 22 °C) allerdings arbeitet der Metabolismus bereits an seiner oberen Grenze (niedrige Q10 Werte), was bedeutet, dass die Larven keine weiteren Kapazitäten mehr besitzen den Metabolismus an noch höhere Temperaturen anzupassen. Die Eigenschaften des metabolischen Schlüsselenzyms Citrat Synthase (CS) weisen auf einen gewissen Grad von Akklimierung bei ungünstigen Temperaturen hin. Kalt akklimierte Larven zeigen eine signifikante Erhöhung in der CS Aktivität, während bei warm akklimierten Larven die Temperatur der thermischen Inaktivierung des Enzyms nach oben verschoben zu sein scheint. Trotz dieser Anpassungen könnte CS ein möglicher Schlüssel für die Einschränkung der Temperaturtoleranz von *C. setosus* Larven bei erhöhten Temperaturen sein.

Die Temperaturen während starker EN/LN Ereignisse liegen außerhalb des Temperaturtoleranzfensters von *C. setosus* Larven (welches den saisonalen

Schwankungen in der Region entspricht: 16 – 20 °C). Dies macht ein Überleben der Larven währen dieser katastrophalen Ereignisse unwahrscheinlich, obwohl in der vorliegenden Studie Anpassungsmechanismen festgestellt werden konnten. Zukünftige Studien über die phänotypische und physiologische Plastizität und etwaige Prozesse der Anpassung der Larven innerhalb des Verbreitungsgebietes der Art würde weiteren Aufschluss über die Mechanismen geben, die die weite geographische Verbreitung von *C. setosus* erlauben.

Die Aufrechterhaltung der nördlichen Population, die am stärksten den Veränderungen von ENSO ausgesetzt ist, ist nach katastrophalen EN Ereignissen abhängig von einer Wiederbesiedlung. Zukünftige Studien über die Verbreitung der Larven, Rekrutierung und Wiederbesiedlungsprozesse sind ausschlaggebend für die Bestimmung von Größe und Ort potentieller mariner Schutzgebiete.

1 Overview

The Humboldt Current System (HCS) is characterized by nutrient rich, cold waters with weak seasonal temperature fluctuations compared to other coastal ecosystems at similar latitudes (Camus 2001, Thiel et al. 2007). The year round upwelling reduces the north – south temperature gradient and extends the cold temperate habitat further north (Camus 2001). The El Niño Southern Oscillation (ENSO) is known to evoke frequently occurring drastic changes in abiotic and biotic environmental factors e.g. in temperature, salinity and food availability that might result in a shift of species distribution. Temperature is one of the determining factors for species distribution. This study was conducted to reveal the consequences of ENSO on larval *Cancer setosus* and the underlying biochemical and physiological mechanisms in order to predict larval survival and assess the chance for recruitment. Assessed survival chances of larvae under changing temperature regimes also allow us to speculate about the implications for species distribution in the future ocean.

1.1 Concepts of thermal tolerance and functional entities

During the last fifteen years, the physiological mechanisms that define thermal sensitivity and limit thermal tolerance of marine ectothermal organisms, as well as its implications for marine fauna and ecosystems has been of particular interest in the context of climatic changes. The main focus of this thesis is the investigation of mechanisms setting thermal tolerance with respect to energetic limitations of crustacean larvae from the HCS under the influence of ENSO, as larval recruitment is crucial for species distribution and survival.

1.1.1. Temperature effects

Temperature is one of the main factors setting limits for life. It determines animal's geographical distribution (Perry et al. 2005), might lead to population collapses or local extinctions (Arntz et al. 1988) and is responsible for the timing of biological events (Wiltshire & Manly 2004). Especially sensitive to temperature are ectotherms as they cannot actively regulate their body temperature which thus depends on environmental temperature. Temperature influences and limits all levels of biological organisation, from biochemical processes to the whole organism function (Hochachka & Somero 2002). The underlying simple mechanism is that the speed of chemical reactions is dependent on temperature. This is described by

the temperature coefficient (Q10) which is a measure of the effect of temperature on reaction speed of a biological or chemical system, saying that chemical reactions at an elevated temperature of 10 °C are 2-4 folds faster.

A rise in the degree of organization in metazoan organisms induced a gain in physiological performance, leading to an increase in metabolic rate and oxygen demand (Pörtner 2002). The metabolic performance of an organism is governed by the thermal limits which are determined by hierarchical trade-offs at various structural and functional levels (Pörtner & Knust 2007). The effects of those thermal boundaries cascading from molecular to cellular to systemic levels are recognised first due to significant changes in the whole organism's aerobic performance (Pörtner 2002).

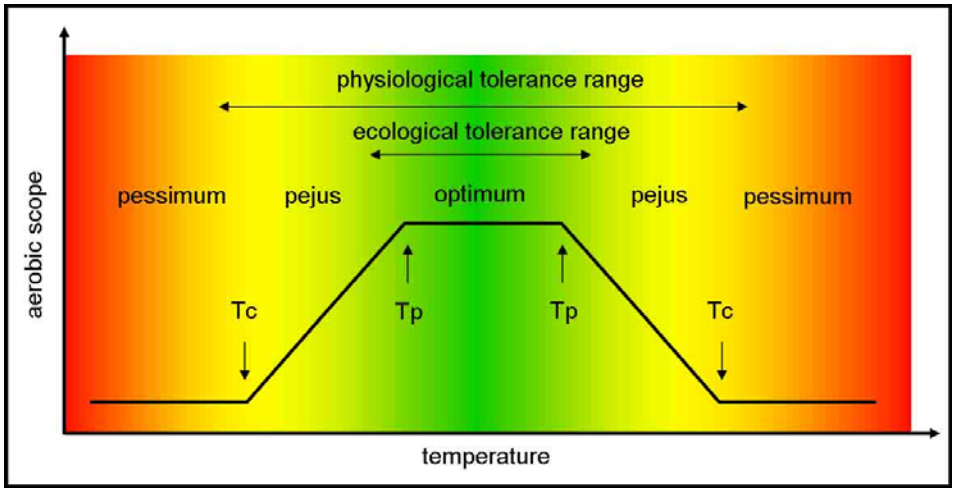

Figure 1. Temperature tolerance model

Oxygen limited thermal tolerance. Modified from Frederich & Pörtner (2000). See text for explanations.

Over the last decades the general model of Shelford (1931) that describes successive stages of tolerance of ectothermal organisms towards abiotic factors was developed further by several authors (Southward 1958; Weatherley 1970; Jones 1971). Eventually the quintessence of respiration experiments conducted mainly on fishes was the finding that oxygen supply and the decline of an animal's capacity to perform aerobically (aerobic scope) (Figure 1) are the key factors in thermal tolerance (Pörtner 2001). These findings have been verified by several studies (e.g. Frederich & Pörtner, Lannig et al. 2004). Environmental temperature influences the total metabolic rate in aquatic ectotherms and can also significantly affect metabolic regulation, eliciting transition to anaerobiosis even in fully

oxygenated waters (Figure 1) (reviewed by Pörtner 2001). Oxygen limitation sets in prior to functional failure and it appears that organism thermal tolerance is defined by the capacity limitations of the most complex organisational level, namely the oxygen supply mediated by the circulatory (i.e. cardio-vascular) system (Pörtner 2002; Lannig et al. 2004). Within pejus temperatures – defined as optimum temperature range - an increase in heart and ventilatory rate can maintain maximal PO_2 (Figure 1) (Frederich and Pörtner 2000). Beyond Pejus temperatures (Tp), heart and ventilation rates become limited, the PO_2 of the heamolymph/blood starts to decline leading to a mismatch between oxygen supply and demand (Pörtner 2001). Trespassing the critical temperature limits (Tc) the aerobic scope, which describes the capacity of an organism to perform aerobically (Pörtner & Knust 2007), becomes zero, energy provisioning depends on anaerobic metabolism and survival is restricted. Critical temperatures differ between species and populations depending on latitudinal or seasonal temperature acclimation and therefore they are related to geographical distribution (Pörtner 2001).

Especially in regions with wide temperature fluctuations (tides, seasons, oscillating climate phenomena), or in species with an extended geographic distribution (polar versus boreal), the significance of thermal tolerance becomes evident. The intertidal snail *Littorina obtusata* for example shows a latitudinal thermal adaptation in the way that key metabolic enzymes of animals adapted to a lower habitat temperature show higher activities than in animals from warmer regions (Sokolova & Pörtner 2001). In addition comparative investigation of *Littorina saxatilis* from the intertidal, where snails regularly are exposed to air, versus the subtidal zone revealed an enzymatic adaptation for an enhanced potential for anaerobic metabolism as an adaptation to high shore life (Sokolova & Pörtner 2001). In the lugworm *Arenicola marina* (Sommer & Pörtner 2002) and in the crabs *Cancer pagurus* and *Carcinus maenas* (Cuculescu et al. 1998) a shift in the temperature tolerance window and in the optimum temperature depending on seasonal and latitudinal variations in temperature regimes can be found. Those cases are only few examples for a whole branch of investigation during the last years, but knowledge is restricted concerning acclimation processes to irregular oscillating changes like ENSO.

- Complexity of organisms governs their sensitivity.
- Thermal tolerance is limited by restrictions in oxygen supply.
- Eurytherm species have the capacity to adjust metabolically to “normal” temperature fluctuations of their environment.
- We know little about the ecophysiological consequences of sporadic but drastic temperature changes such as during ENSO.

1.1.2. Ecological, physiological and biochemical aspects

Temperature induced changes can be detected at different organizational levels. At the intra-population level different temperatures might affect the characteristic of distinct morphometric features. At the population level habitat temperature determines the ecological tolerance range by developmental time and survival. At the whole organism level temperature affects the metabolic rate of an animal. Changes in the metabolic performance are also reflected in the enzymatic characteristics and the nutritional status of an organism. The following paragraphs describe the measured parameters to address the problem of thermal tolerance and thermal adaptation in crustacean larvae.

Oxygen consumption

Oxygen consumption is a reasonable approximation of metabolic activity. Thus, the metabolic response of organisms to a changing environment can be inferred from changes in oxygen consumption (Aarset & Aunaas 1990; Clarke & Johnston).

In crustaceans, oxygen consumption has been studied related to latitudinal gradients and temperature gradients. Salomon and Buchholz (2000) showed that increasing temperatures from 5 to 15 °C lead to 3 - 4 times higher respiration rates in two isopod species from different habitats. Saborowski et al. (2000) studied a population of Northern Krill (*Meganyctiphanes norvegica*, Malacostraca) in the Kattegat, where a pronounced thermal stratification leads to an intense thermal gradient. During daily vertical migrations the krill encounters acute temperature changes of more than 10 °C. Oxygen consumption rates increased exponentially with temperature within the temperature range from 4 to 16 °C. This stresses that

M. norvegica is a pronounced eurytherm species that does not show any thermal limitations within the encountered temperature range.

A study on energy metabolism of early life stages of the shrimp *Farfantepenaeus paulensis* (Decapoda: Caridea) revealed that cold acclimation temperatures result in lower respiration rates in the Protozoea II and the Mysis I stage whereas temperature had no effect on respiration rates of animals in subsequent stages (Lemos et al. 2003). That might be due to a thermal metabolic compensation in terms of an increase in the activities of the enzymes citrate synthase (CS) and pyruvate kinase (PK) in the higher stages. Generally, increasing temperatures lead to higher respiration rates in crustaceans (Saborowski et al. 2000; Salomon & Buchholz 2000; Lemos et al. 2003). At low temperatures low respiration rates can be compensated by increased activities of metabolic key enzymes (Lemos et al. 2003).

Few studies deal with respiration of *C. setosus* (Fenández et al. 2000, 2002, 2003; Brante et al. 2003), covering the topic of the oxygen provisioning of embryos. Those studies showed a positive relationship between oxygen consumption and progressive embryo stage. During the last years knowledge about respiration of crustacean larvae has improved (e.g. Hillyard & Vinegar 1972; Anger 1986; Lemos et al. 2001, 2003; Thatje et al. 2003; Storch et al. 2009), but the relation of temperature and oxygen consumption of different larval instars of *C. setosus* has not been investigated so far.

Enzymatic activities

The analysis of temperature-dependent changes in enzyme structure and function is traditionally used as a powerful tool in studies of temperature acclimation and adaptation of animals (e.g.: Buchholz & Saborowski 2000; Lange et al. 1998; Lannig et al. 2003; Lemos et al. 2003; Salomon & Buchholz 2000; Somero 2004). Different strategies for thermal enzyme adaptation are reviewed by Somero (2004): (i) changes in the amino acid sequence, (ii) shifts in concentrations of proteins, and (iii) changes in the milieu in which proteins function.

Citrate synthase (CS) is a key-enzyme of the aerobic metabolism (Childress & Somero 1979) and pyruvate kinase (PK) activity represents the capacity for anaerobic work in the glycolytic pathway (Johnston et al. 1977; Childress & Somero 1979).

Vetter et al. (1997) revealed for krill (*Meganyctiphanes norvegica* and *Euphausia superba*) that CS of cold adapted specimen has a reduced activation energy (the energy demand for onset of a chemical reaction), resulting in higher activity in spite of low temperatures, compensating for a lower available thermal energy in cold environments. Lemos et al. (2003) describes that cold acclimation resulted in increased enzyme activities of CS and PK in early life stages of shrimp *Farfantepenaeus paulensis* to offset the negative effect of low temperature due to reduced respiration rates.

A positive correlation between enzyme activity and metabolic rate was found (Childress & Somero 1979; Torres & Somero 1988), which might indicate that metabolic key enzymes could be used to predict metabolic rates in larvae of marine animals (Torres & Somero 1988; Lemos et al. (2003)).

Elemental composition - CHN

Carbon (C), nitrogen (N) and hydrogen (H) present the dominant elements of the organic fraction of biomass (C > 35%, N ~8 - 11%, H ~5 – 6%) (Anger 2001 and publications cited therein). Further elements like oxygen, phosphorus and sulphur are also omnipresent, but quantitatively less important (Anger 2001). The elements C, H and N can be measured with high precision with a CHN analyzer and have become standard measures in planktonic organisms (e.g. Anger & Dawirs 1982; Anger 1988; Ikeda & Skjoldal 1989; Clarke et al. 1992; Lovrich et al. 2003).

Absolute values of carbon and nitrogen are a precise reflection of growth, while relative values (% of dry weight (W)) display the detailed elemental composition. The C/N ratio reflects changes in the relative proportions of lipids and proteins (Anger 2001) and therefore allows conclusions about the nutritional status of an organism.

According to Anger (1988) changes in the elemental composition observed during single larval moult cycles show a typical pattern. During and immediately after ecdysis larvae take up water and minerals, followed by a period of tissue growth. The C/N ratio indicates a higher lipid than protein accumulation in the beginning of an instar (postmoult phase), which is balanced by protein accumulation during the intermoult and early premoult phase. Proteins normally contribute two to three times more to total biomass compared to lipids, while carbohydrates play only a minor role in crustacean larvae (Anger 1988).

Changes in the metabolic efficiency and fitness of an organism are well reflected in its dry weight (W) and its elemental composition (C, H, N) (Dahlhoff 2004) and correlate with changes in extrinsic factors like food availability (Anger 1988), salinity (Torres et al. 2002) or temperature (Dawirs & Dietrich 1986).

Morphometric traits

Phenotypic plasticity in invertebrate larval offspring has often been discussed as an important mechanism to respond to sudden changes in their habitat like temperature and/or food conditions (e.g. Criales & Anger 1986; Shirley et al. 1987).

In decapod crustaceans there is evidence that phenotypic patterns of larvae may be indicative of their energetic status or changes in the physical habitat conditions like salinity and pH (Silva et al. 2009) as well as temperature (Wehrtmann & Albornoz 1998; Wehrtmann & Kattner 1998; Thatje & Bacardit 2000; Giménez 2002). Most of the knowledge about phenotypic plasticity of decapods is limited to studies on caridean shrimp larvae, which are known to express high variability in larval developmental pathways, number of instars, as well as size and energy contents in response to food and temperature condition (e.g. Criales & Anger 1986; Wehrtmann 1991).

In comparison, and despite known energetic trade-offs in crab eggs, phenotypic plasticity in crab larvae is generally assumed to be more conservative; a view that might be largely driven by the lack of sufficient information available to date (Anger 2001). For example for *Neohelice* (formerly *Chasmagnathus*) *granulata* a conservative phenotypic plasticity has already been disproved. A supplementary larval instar has been found frequently in rearing experiments (Pestana & Ostrensky 1995), and field-collected larvae revealed varying morphological traits (Cuesta et al. 2002).

Regarding the effect of a latitudinal (temperature) gradient, it has been shown that crab larvae display larger sizes in the colder regions of the distributional area (Shirley et al. 1987; Storch et al. 2009). In cancrid larvae, the character of larval appendages is prominent and subject of modification. Changes in the characteristics of antennae, spines and body length might have ecological implications like protection against predators or modified swimming behaviour.

- Temperature induced metabolic changes are visible. on the whole organism level, but are manifested on lower levels of organismic organisation as well. Thus, we looked at various parameters from these different levels:
  - survival rates and larval duration
  - oxygen consumption
  - morphometric traits
  - elemental composition
  - enzymatic activity of key metabolic enzymes

1.2 The Humboldt Current System

The Humboldt Current is one of the strong Eastern Boundary Currents which, to a certain degree, have similar characteristics. Cold, nutrient rich deep sea water is transported to the euphotic zone. The nutrients elicit high primary production which is the source of the highly productive ecosystems that sustain the world's largest fishing grounds. In the following chapter the oceanographic characteristics of the Humboldt current including the oscillating atmospheric – ocean coupled climate phenomenon ENSO and a view on future scenarios of the east Pacific is presented.

1.2.1. Upwelling region

Due to the pressure difference between the Indonesian Low and the South Pacific High, steady southeast trade winds blow along the South American Pacific coast. The co-action of Coriolis and Ekman forces produce a netto transport at right angle to the wind force. Due to the co-action of the described forces, warm surface water is transported away from the South American coast and is piled up in the West Pacific, whereby a tilted thermocline is produced (Figure 2). A pressure gradient emerges, which results in a compensatory current – upwelling occurs. The resulting gradient in SST between the warm water in the west and the cold water in the east drives an east-west overturning circulation in the atmosphere known as Walker Cell. Is the pressure gradient permanent that drives the upwelling, the Coriolis force results in a current normal to the pressure gradient, namely along the coast. This drives the Humboldt Current (Arntz & Fahrbach 1991; Bearman 2002). The same genesis is based for all eastern boundary currents (Benguela Current,

Canary Current, California Current) (Bearman 2002). The upwelling deep sea water is nutrient rich, providing permanent phytoplankton blooms in the euphotic zone. This phytoplankton represents the base of one of the world's largest fisheries (Bertrand et al. 2004). Additionally seasonal temperature variability in the Humboldt Current System (HCS) is limited compared to that in other coastal ecosystems at similar latitudes (e.g. Arntz et al. 1987, Camus 2001, Thiel et al. 2007).

- The HCS is characterised by cold nutrient rich water.
- High productivity based on high nutrients supports one of the worlds' largest fisheries.
- Within the HCS limited seasonal variations in temperature can be found.

1.2.2. ENSO

Differences in the strength of the Walker Cell are inducing the Southern Oscillation (SO). A strong South Pacific High intensifies the South East Trade Winds and upwelling is more pronounced. An increased upwelling results in cold temperatures on the coast line. This phase is called La Niña (LN). Cold temperatures in the eastern Pacific strengthen the Walker Circulation, resulting in higher precipitation and lower air pressure in Indonesia. At the same time the Hadley Circulation (atmospheric circulation that shows a rising motion near the equator, pole ward flow 10-15 kilometres above the surface, descending motion in the subtropics, and equator-ward flow near the surface) is weakened due to a reduction of the air pressure of the South Pacific High. South East Trade Winds slow down, the thermocline becomes horizontal and warm tropical water masses spread over the Pacific Ocean (Figure 2). The already reduced upwelling only transports warm, nutrient poor water from the upper water masses to the surface. These factors lead to warmer water temperatures on the South American Pacific coast, initiating the onset of an El Niño (EN). Due to the weakened Trade Winds, waves occur in the western Pacific that produce upwelling of cold water. The signal of the cooled water is transported to the east by Kelvin waves, presenting the onset of a LN phase. The Hadley Circulation intensifies and results in a strengthening of the South Pacific High.

The El Niño Southern Oscillation (ENSO) has a frequency of 2 – 10 years with strong events every 13-70 years (Figure 3) (e.g. 1925-1926, 1982-1983 and 1997-1998) (Thatje et al. 2008). The events may last between half a year and several years.

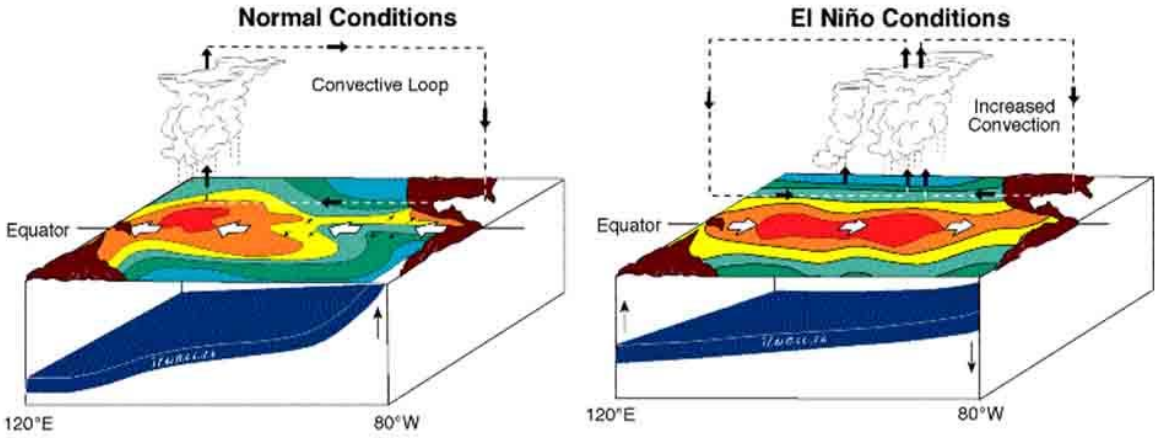

Figure 2. Schematic diagram of normal and El Niño conditions in the Pacific Ocean
See text for explanations. Source: NOAA (2009).

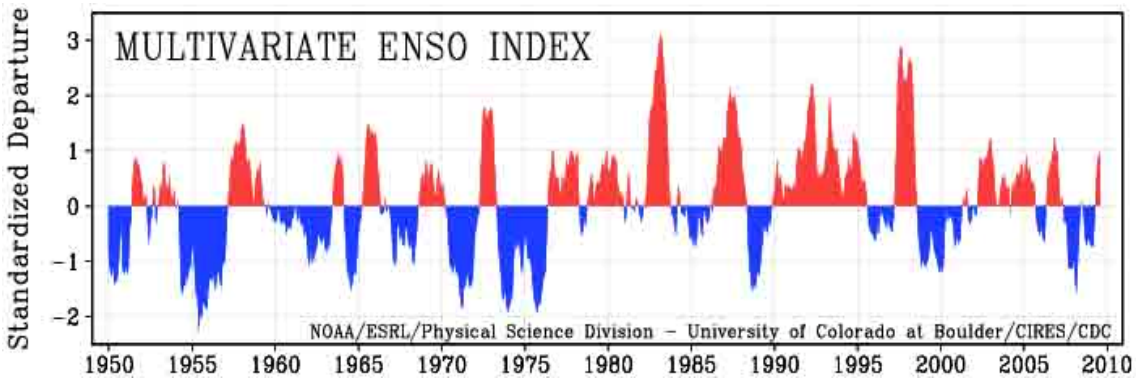

Figure 3. Multivariate ENSO Index (MEI)
Weighted average of main ENSO features. Source: NOAA (2009).

The coastal region underlies severe changes during EN events. E.g. an intense precipitation in arid regions of Peru and Chile causes a significant increase of freshwater input, leading to reduced salinities in coastal waters, especially close to river estuaries (Arntz & Fahrbach 1991; Thatje et al. 2008). This increase of freshwater input is accompanied by an increased sediment load. Additionally changes in the oxygen concentration, agitated sea and changes in UV radiation occur. One of the most striking effects for the ectotherm fauna of the ecosystem of the Humboldt Current is the severe change in temperature that can increase up to > 10 °C within several weeks (Arntz & Fahrbach 1991). The intensity of temperature changes during EN decreases towards higher latitudes. EN thermal

anomalies are propagated southward by coastal Kelvin waves, whose magnitude generally declines towards higher latitudes, producing a latitudinal impact gradient (Camus 2008).On the one hand, the changes during EN events have some positive effects like an outbreak of local commercially exploited species, e.g. the scallop *Argopecten purpuratus* and the immigration of valuable tropical species (e.g. the shrimp *Xiphopenaeus riveti*) (Arntz et al. 1988). On the other hand, the devastating negative effect on the endemic flora and fauna prevails by far and particularly damages artisanal fisheries (Arntz et al. 1988).

- ENSO is an ocean – atmosphere coupled climate phenomenon with a frequency of 2 – 10 years.
- EN/LN impose severe changes in physical properties on the ecosystem of the HCS.
- During EN events tropic species invade.
- Strong EN events can cause mass mortalities in endemic species.

1.2.3. Evolutionary timescale and future scenarios of ENSO

To reveal the evolutionary driving force of ENSO, the question of its age, frequency and regularity has to be answered. The last five centuries since the conquest of America are quite well recorded. Since the early 20th century, modern oceanographic techniques allow a precise routine monitoring of ENSO events. For earlier centuries, historical records of mass mortalities of marine species, mud floods or heavy rainfalls serve as a quite precise record of ENSO. From the past archaeology, sedimentology, trace elements, ice cores and growth rings of trees allowed conclusions about frequency and strength of EN events.

Discoveries of cold water mollusc shells in 5000 year old kitchen middens from northern Peru, accompanied by EN specific sediments is interpreted as the birth of EN by some authors (Arntz & Fahrbach 1991). Andrus et al. (2002) concluded from otolith $\delta^{18}O$ data that upwelling intensified and the “modern” EN as it exists today developed during that time. Other findings like sediment discharges further inland point at an EN existence of at least 40 000 years (Pleistocene). With the utmost probability ENSO always was a changing phenomenon, vanishing and

reappearing during several epochs. As the history of the phenomenon shows, it underlies changes of an unpredictable irregularity, although - to some extent - stability may be displayed on a large temporal scale.

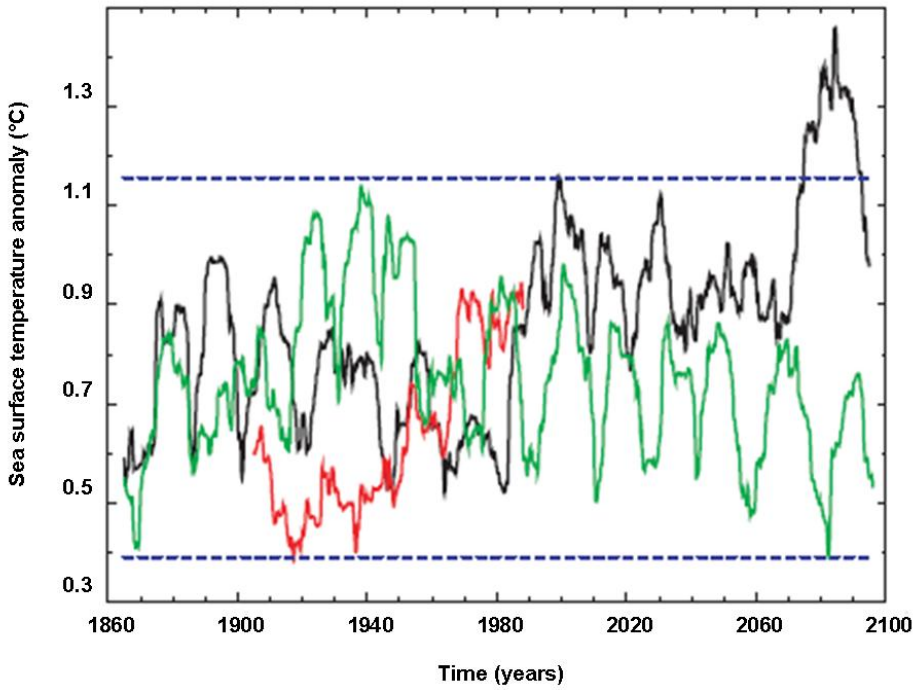

Figure 4. Future ENSO

Inter-annual variability of observed and simulated sea surface temperature (SST) anomalies. Greenhouse warming simulation (black) and observed (red) SST anomalies exhibit trends towards stronger inter-annual variability, with pronounced inter-decadal variability superimposed. The minimum and maximum standard deviations derived from the control run (green) with present-day concentrations of greenhouse gases are denoted by the dashed lines. With friendly allowance from Macmillan Publishers Ltd (http://www.macmillan.com/). Reprint from Timmermann et al. 1999.

ENSO occurrences and consequences remain hard to predict. The more frequent occurrence of EN during the last decades with the strong EN events 1982-1983 and 1997-1998 raise the question, to which extent the progressing global change affects ENSO (Timmermann et al. 1999).

Different models of future climate scenarios have been developed. The opinion that global warming will lead to a shutdown of the ENSO variability, enforcing a permanent El Niño like condition (e.g. Cane 1998) has been controversially discussed during the last decade (for review see Huber & Caballero

2003). Latest publications underline the continuity of ENSO (Timmermann et al. 1999; Cole 2001; Huber & Caballero 2003; Vecchi & Wittenberg 2009).

Nevertheless results of the developed models vary. Some of those future scenarios are predicting more frequent EN events and stronger cold events in the tropical Pacific Ocean (Figure 4) (Timmermann et al. 1999), or only little change in the frequency but slightly greater amplitudes (Huber & Caballero 2003). Others reveal that the variations in ENSO do not rely on radiative forcing, meaning that the changes in ENSO are not obligatory coupled to global warming (Vecchi & Wittenberg 2009). Recently developed models showed that even the probability exists that no change in ENSO will appear (reviewed in Vecchi & Wittenberg 2009).

- It remains yet undetermined during which geological epoch ENSO like patterns formed the first time, but there is evidence that the “modern” ENSO has an age of ~5000 years.
- Over the last millennia, both frequency and amplitude of EN/LN events varied widely.
- Future scenarios of ENSO range from a permanent EN like state to a more frequent, higher amplitude EN/LN pattern.

1.2.4. Study area in the HCS

The main study area of Antofagasta is located in the II. region of Chile, within the centre of the model species distributional range (Figure 5). Large scale oceanographic patterns on the northern Chilean coast show a distinct pattern (Figure 6) which, despite some changes, seems to be persistent during “normal conditions”.

The surface currents are dominated by the coastal branch of the northward Humboldt Current in the region of 73°W, the southward directed Peru-Chile Undercurrent around 71.5°W that results in a southward transport, and the northward directed Chile Current north of 26°S that is assumed to be a part of an anticyclone, which seems to be a permanent feature of the Iquique – Antofagasta region (Silva 1983).

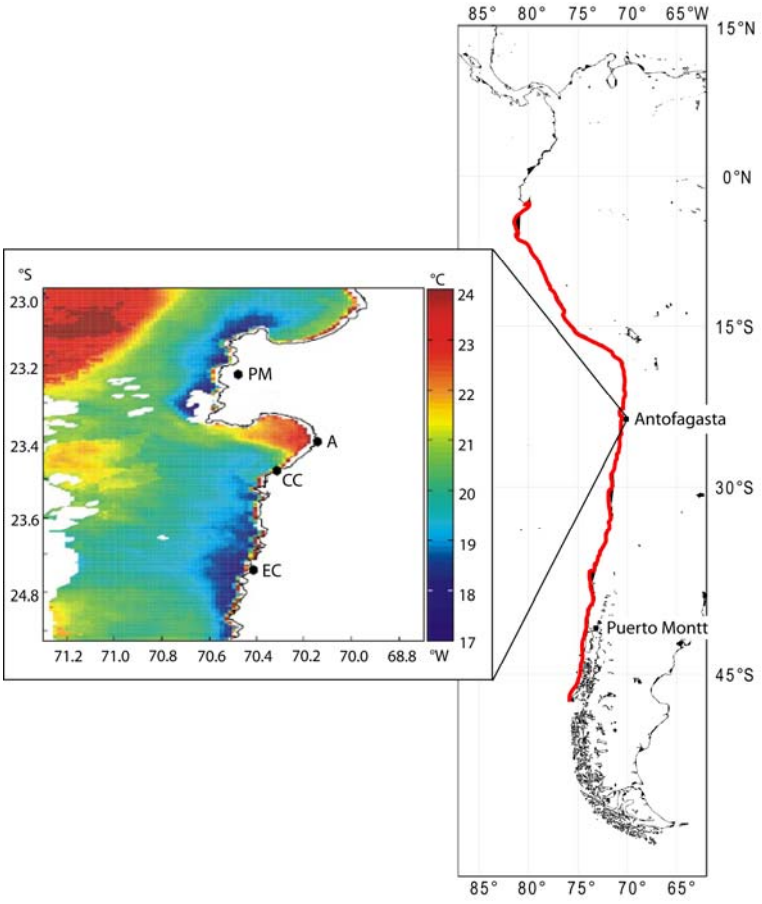

Figure 5. Experimental site and latitudinal distribution of *C. setosus*

Right side: distributional range of *C. setosus* along the South American Pacific coast in red. Experimental sites Antofagasta and Puerto Montt. Left side: Satellite image of the main experimental site Antofagasta with mean SST in January 2000 (°C). PM – Peninsula Mejillones, A – Antofagasta, CC – Caleta Colosso, EC – El Cobre. Picture after Piñones et al. 2007, with friendly allowance of Ciencias Marinas.

In general it is assumed, that strong upwelling is correlated with higher advection and that low upwelling favours retention of water masses. However, oceanographic studies have shown that advection can be influenced by more than upwelling strength in local coastal regions (Giraldo et al. 2009). The advection of coastal waters strongly depends on the interaction between local (e.g. winds) and remote (e.g. coastally trapped waves) forcing, coastline geometry, bottom topography, and physical characteristics (e.g. density) of the water column (Giraldo et al. 2009). In particular, strong upwelling events might result in local retention

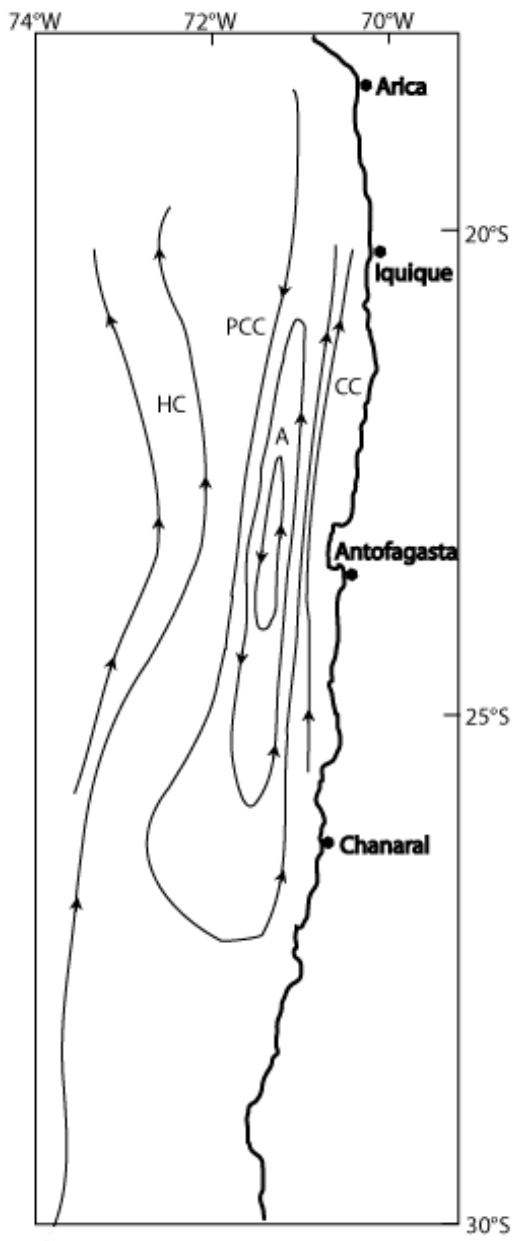

Figure 6. Surface currents off northern Chile
HC – Humboldt Current, PCC – Peru Chile Counter Current, A – Anticyclon, CC – Chile Current. Schematic drawing after Silva 1983.

areas, while low upwelling might result in advection of water masses (Giraldo et al. 2009). The core study area is located in the region of Antofagasta (23°45'S, 70°27'W), which is characterised by a semi enclosed, southward closed bay with the Mejillones Peninsula on the downwind side. Strong upwelling plumes exist on the seaside of Mejillones and further south in El Cobre (Figure 5) (Piñones et al. 2007). Prevailing northward directed winds result in water transport from the latter upwelling plume into the bay. Radiation is high in this region and surface water masses retain in the Antofagasta bay for several days, leading to extraordinary high SSTs in the bay. Additionally, a persistent but spatially variable thermal gradient structure can be found between the upwelling plumes and the warm surface water in the bay. These permanent features present key factors for the dynamics and structure of pelagic and benthic communities. Mean monthly SST in Antofagasta range between ~16 and ~20 °C at the measurement station of the Chilean armada (Figure 7). The Caleta Colosso, where the egg bearing females used in this study were caught, is situated outside the region of extended upwelling or warming (Figure 5).

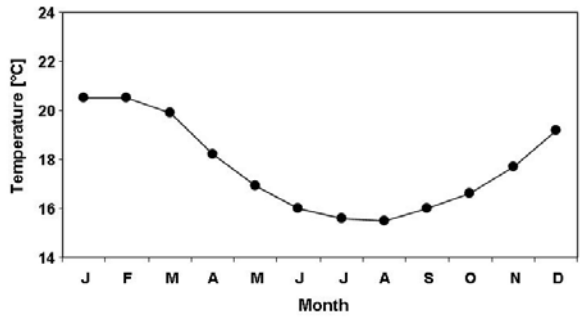

Figure 7. Mean monthly SST in Antofagasta.
Mean monthly SST in Antofagasta during 20 years (1980 – 2000).Source:SHOA (2009).

The larval dispersal of *Cancer* larvae that hatch in this region is unknown. However, since surface waters are moved to the north and remain within the bay for several days up to two weeks, it is very likely that the larvae, as "inhabitants" of the euphotic zone, are transported into the bay and reside there for a restricted period of time.

The temperatures in the study area correspond to model species northern limits, but also lower temperatures in the region of the plumes occur. Therefore the study area of Antofagasta presents a habitat with heterogenic temperature patterns, and also unique oceanographic conditions which makes it an ideal site for investigations of thermal tolerance of the marine fauna.

- Different surface currents characterise the ocean off the experimental area.
- The waters off Antofagasta are characterised by high small scale variability, e.g. upwelling plumes, cold and warm retention areas and local current variations.
- SST in the experimental area ranges from ~16 °C in austral winter to ~20 °C in austral summer.

1.3 The model species

"Rock crabs" of the genus *Cancer* (Brachyura, Decapoda) are of relatively large size and have strong claws which often make them a favoured target of high economic value in their area of distribution, especially for artisanal fisheries. *Cancer* crabs are represented by 24 contemporary species that are distributed in the Pacific and the North Atlantic Ocean between 4 and 24 °C mean annual SST (Figure 8) (MacKay 1943; Nations 1975, 1979). The few species found in the

tropics, e.g. *C. borealis* (Florida) and *C. johngarthi* (Panama), are restricted to low temperature water masses on the deeper shelf.

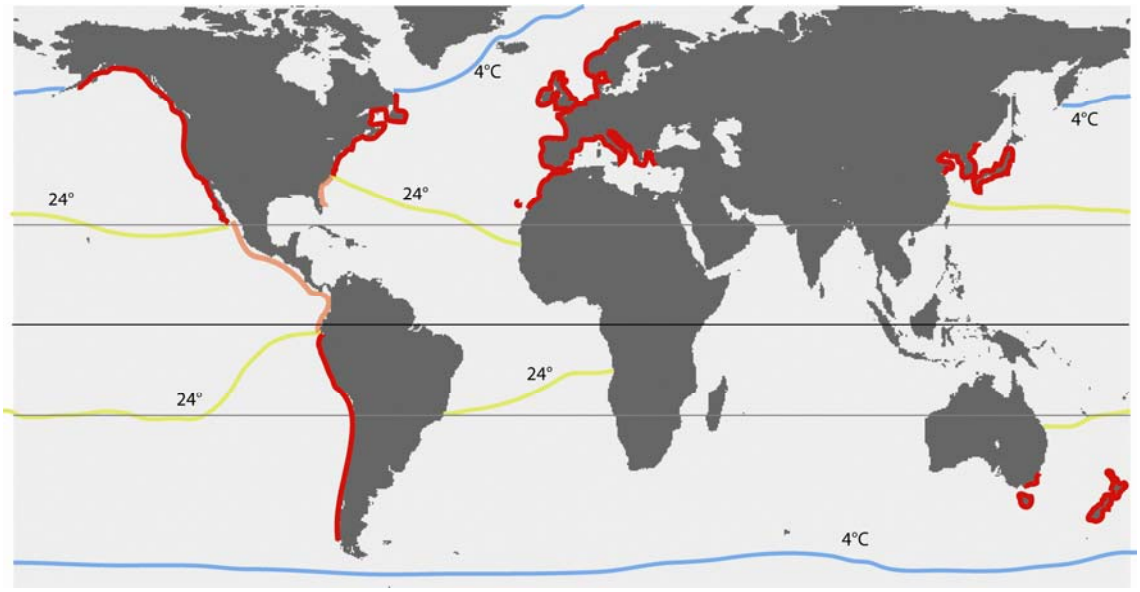

Figure 8. World distribution of the genus *Cancer* (red areas)
4°C and 24 °C SST isotherms indicate the principle borders of genus distribution.

Within their habitats different species encounter varying environmental conditions. *C. setosus* occurs along the South American Pacific coast where year-round upwelling leads to low seasonal changes, resulting in relatively stable "normal" conditions that are disturbed by the oscillating changes of ENSO. In contrast *C. pagurus* underlies strong regular seasonal changes in its habitat of the North Atlantic shoreline. *Cancer* species attracted the interest of evolutionary biologists, palaeontologists, taxonomists, behavioural ecologists, fisheries researchers and physiologists during the last century. Hence, a broad range of information is available but information concerning the early ontogeny of the named species is scarce. Within this thesis *C. setosus* serves as a model species from a relatively stable habitat that undergoes strong and irregular changes. *C. pagurus* shall serve as a reference species from a cold temperate region with regular seasonal changes. The most important characteristics of distribution and ecology of the two investigated species are summarized in Table 1.

1.3.1. Model species profile: *Cancer setosus*

Phylogeny

The genus *Cancer* most likely originated in the North East Pacific coast in the early Miocene where the highest cancrid diversity of nine species is found

nowadays. A rapid species diversification occurred about 5 million years ago (mya) (Harrison & Crespi 1999a). Cytochrome oxydase I analyses, which in phylogenetics serves as a "molecular clock", complementary to fossil records of a cancrid species suggest that *Cancer* species immigrated into the Atlantic 6 to 12 mya (Harrison & Crespi 1999a). Specification processes most likely occurred independently in the different areas of distribution. Specification was obviously habitat-dependent resulting in smaller species in heterogeneous, structurally complex environments such as rocky coasts, and the largest species in homogeneous habitats like sand beds (Harrison & Crespi 1999b).

At the South American Pacific coast four recent *Cancer* species have been identified up to now, namely *C. coronatus* (syn. *C. plebejus* (Poeppig 1836), *C. edwardsii*, *C. porteri* and *C. setosus (syn. C. polyodon,* (Poeppig 1836)). The colonization from the North Pacific via submergence to deeper, colder water masses, to avoid high SSTs in the tropics was supposed (Garth 1957). This theory is supported by fossil records of *C. setosus* in the region of California (Nations 1975).

Ecology and distribution

Cancer setosus (local names in Chile "Jaiba" or "Jaiba peluda") is one of the key predators in the heterogeneous benthic habitat on the shores along the South American Pacific coast line and frequently occurs in high abundances in the highly productive Humboldt Current ecosystem (e.g. Wolff & Cerda 1992, Wolff & Soto 1992; Cerda & Wolff 1993). The hairy crab mainly preys upon small crustaceans, molluscs, echinoderms and polychaetes, and shows a high rate of cannibalism (Cerda & Wolff 1993). The mollusc prey includes clams and scallops (Wolff and Soto 1992) and *C. setosus* is supposed to play an important role in the Humboldt Current ecosystem by regulating/controlling recruitment of the scallop *Argopecten purpuratus* (Wolff 1987). Though the species is occasionally found on unstructured sand grounds down to 25 m water depth, *C. setosus* is most abundant between 4 and 8 m water depth on heterogeneous sand or muddy sand grounds with refuges of rocks, shells and macroalgae (Wolff & Soto 1992). Age at first maturity is about 2 years at a carapace width (CW) (width of the carapace at its broadest location) of 98 mm in females and 124 mm in males (Wolff & Soto 1992), maximum sizes found in this species (CW) are 164 mm and 199 mm, respectively (Pool et al. 1998; Fischer 2009).

Along the coastline of Chile and Peru, *C. setosus* is one of the target crab species of artisanal diving and trapping fisheries. Together with *C. edwardsii* and *Homolaspis plana*, *C. setosus* is the most important commercially exploited brachyuran species in Chile (Pool et al. 1998). Due to the oceanographic characteristics of the Humboldt Current (see Chapter 1.2.1), all three species have a broad geographic range, however, *C. setosus* is the only species that is commercially exploited in almost its entire area of distribution. *C. setosus* fishery is managed in Chile and most recently (April 2009) as well in Peru. The Chilean law regulates minimum size at catch of 120 mm CW and prohibits landing of egg-bearing females of all Brachyuran species (DCTO. N°9/90). In Peru the "RESOLUCIÓN MINISTERIAL N° 159-2009-PRODUCE" regulates a minimum size at catch of 110 mm CW and prohibits landing of egg-bearing females of the brachyuran species *C. setosus*, *C. porteri* and *Platyxanthus orbignyi*.

The geographic distribution of *C. setosus* ranges from Guayaquil in Ecuador (2°13'S, 79°53'W) to the Peninsula of Taitao in southern Chile (46°00'S, 75°00W) (Garth & Stephenson 1966) (Figure 5), and is most likely limited by water temperature (Nations 1975). It therefore covers an extent of about 4500 km and the encountered SST spans from ~10 °C in the South to ~20 °C in the North (SHOA 2009, IMARPE 2009). Maintaining such a broad geographic range requires adaptation, acclimation and plasticity in life history and physiology. Genetic studies using allozyme and AFLP (Amplified Fragment Length Polymorphism) analyses revealed that there seems to be no separation of the stocks of *C. setosus* along a wide range of 2500 km coast line (Gomez-Uchida et al. 2003). The lack of stock separation is most likely the result of long-distance larval dispersal along the coast within the HCS. Therefore, *C. setosus* represents an optimum model organism for the comparison of metabolic adaptation across a latitudinal temperature gradient (Sokolova & Pörtner 2001). Northward dispersal of the crabs is obviously constrained by El Niño (EN) events, as temperature fluctuation during strong EN events might result in mass mortalities of adult specimen (Arntz & Fahrbach 1991). Though investigation of the effect of temperature fluctuations on the metabolism of adults and embryonic development was subject of many studies during the last years (see Baeza & Fernandez 2002; Fernández et al. 2002; Brante et al. 2003; Fischer & Thatje 2008; Fischer et al. 2009a; Fischer et al. 2009b), the impact on the larval phase remains largely unknown. Since the early ontogeny is known to be the most delicate part in the life cycle of brachyuran crabs (Anger 2001), it is of

particular importance to study the impact of temperature on larval physiology and ecology to predict recruitment success under different temperature regimes.

Life cycle

The life cycle of *C. setosus* includes a prezoeal stage (lasting only a few minutes), 5 pelagic zoeal stages (instars (ZI-ZV), 1 megalopa stage, several juvenile stages without any further morphological changes, and the adult life stage (Figure 9). *C. setosus* is a very fecund species with ≥ 2.5 million eggs per egg clutch in large females (~ 140 – 150 mm CW) (Fischer 2009). Females show active brood care by carrying the eggs under the abdomen (Figure 10) and providing sufficient oxygen supply by abdominal flapping. Reproductive output as well as egg size are inversely related to the ambient water temperature, and are thus changing with latitude, resulting in higher numbers and larger eggs in higher latitudes (Brante et al. 2003, 2004). This temperature-dependency is attributed to increasing energetic costs due to a higher oxygen demand of eggs at higher temperatures, which is compensated by an increased abdominal flapping rate of the female (Fernández et al. 2000; 2002; 2003). After hatching, the pelagic planktotrophic phase is initiated. Pelagic larvae are of particular importance for the dispersal of relatively slow moving benthic species like *C. setosus* (Cowen & Sponaugle 2009). In particular in a habitat such as the HCS, pelagic life stages are dispersed over long distances (Scheltema 1986). Pelagic larval phases are most likely crucial for the recolonization after mass mortalities due to catastrophic events like strong El Niño phases. After the dispersal and a completion of zoeal development, which might last about 1 - 2 months (Quintana 1986; Weiss et al. 2009a), the development into the megalopa stage involves transition to a benthic life style. The benthic environment provides shelter and food for the megalopa. The transition is apparently coupled with the occurrence

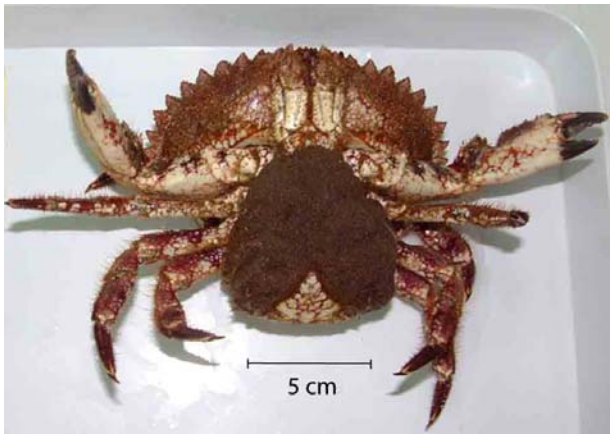

Figure 9. Ovigerous *C. setosus* with eggs.
Eggs in a late stage of development (eye-placode stage), CW – 13.5 cm.

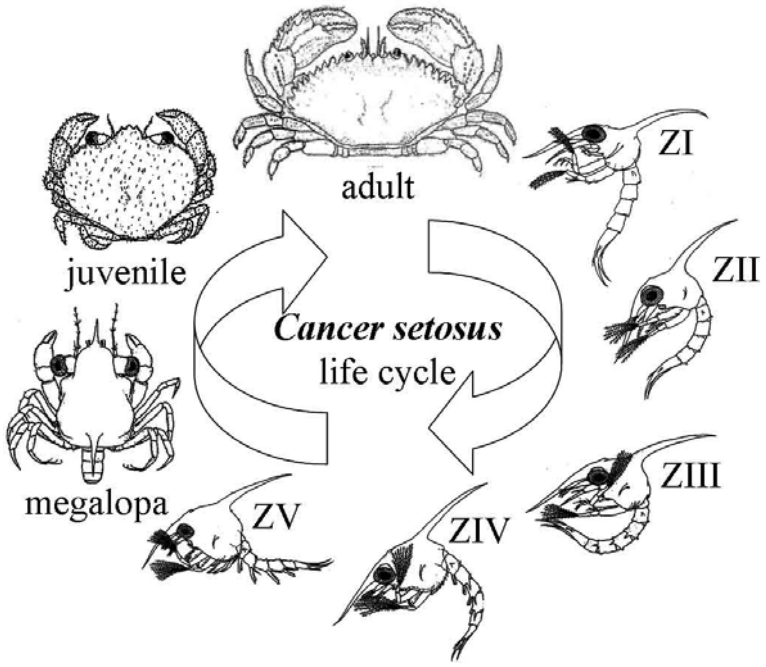

Figure 10. Life cycle of *C. setosus*
"ZI – ZV" – zoeal instars. Drawings by Quintana 1986.

of particular physical or chemical cues that induce the metamorphosis, for example chemical cues and odours from adult substrate, aquatic vegetation, bio films, conspecifics, estuarine water, humic acids, related crab species, and potential prey (summarized in Forward et al. 2001). This assumption is supported by the fact that the megalopa stage is mainly found between rocks in the intertidal zone (Wolff & Soto 1992) and obviously needs a cue to find the fitting substrate for settlement.

- The genus *Cancer* most likely had its origin in the eastern North Pacific.
- Cancer crabs occur within a temperature range of 4 to 24 °C mean annual SST.
- Many *Cancer* species are commercially important.
- *C. setosus* has a broad geographical distribution (>40° of latitude).
- Mass mortalities of adult *C. setosus* can occur during strong EN events.
- Effects of temperature variation on larval ecophysiology are widely unknown.

1.3.2. Reference species: *Cancer pagurus*

The so-called “edible crab”, *Cancer pagurus*, has a broad geographical distribution from northern Norway to West Africa and has been reported to be also abundant in the Mediterranean Sea (Hayward & Ryland 1995). The southern border of distribution, however, remains questionable, as the water temperatures in some areas exceeds the limiting habitat temperature range reported for *Cancer* crabs (see Chapter 1.1.1). According to Pinho et al. (2001) *C. bellianus,* a species that is abundant in deeper waters with lower temperatures was misidentified as *C. pagurus* in waters around the Azores. Moreover, in his book “Mediterranean seafood: a comprehensive guide with recipes” Davidson (2002) reported that large crabs identified as *C. pagurus* in the Mediterranean, actually were blue crabs, *Callinectes sapidus*.

However, according to verified capture data from artisanal fisheries, the southerly distribution of *C. pagurus* extents to at least 28°N (Fischer et al. 1981), suggesting a wide range of temperature tolerance in adult populations of the species. Studies of occurrence, abundance and catch rates show that *C. pagurus* is expanding its biogeographical range further northwards (Woll et al. 2006) due to rising water temperatures (Weiss et al. 2009b). The reproductive cycle of *C. pagurus* seems to be adapted to the seasonal cycle of the ecosystem: Eggs are incubated during winter and larval hatching seems to correlate with plankton blooms in spring/summer, as it has been reported for other brachyuran crab species from temperate regions, as well (Stevens et al. 2008).

A study conducted on the island of Helgoland with *C. pagurus* larvae (Publication I) serves as a baseline for a comparison with *C. setosus* larvae from an ecosystem where temperature fluctuations are sporadic.

- The species range of *C. pagurus* covers a wide latitudinal range of ~40°.
- The habitat of *C. pagurus* is subject to high seasonal temperature fluctuations.
- The life cycle is adapted to the habitats strong seasonal changes.

Table 1. Comparison of habitat and breeding ecology of two *Cancer* species: *C. setosus* and *C. pagurus*

	C. setosus	*C. pagurus*	Source
Distribution	South American Pacific coast 2°13'S - 46°00'S	North Atlantic east coast, northern Norway to West Africa + Mediterranean 28° - 70°N	Garth & Stephenson 1966; Fischer et al. 1981; Woll et al. 2006
Habitat	rocky substrata + on muddy sand - 25 m depths	rocky substrata + on muddy sand - 100 m depths	Wilson 1999
Experimental area	23°45'S	54°11'N	
Size at maturity	♀ 9.8 cm CW ♂ 12.4 cm CW	♀~12.7 cm CW ♂ ~11 cm CW	Wolff & Soto 1992; Fish & Fish 1989
Age at maturity	2 years	3 years	Wolff and Soto 1992
Max size	♀ 16.4 cm CW ♂ 19.9 cm CW	28.5 cm	Pool et al. 1998; Fischer 2009
Species temperature range	~10 - ~20 °C	~ 6 - ~23°C	SHOA (2009) NOAA (2009)
Populations temperature range at experimental site	16 – 20 °C (non EN)	2 – 18°C	SHOA (2009) Wiltshire & Manly 2004
breeding	Mainly ovigorous year round, ≥2.5 million eggs	Breeding in winter for 6-9 months, larvae hatch in spring / summer, up to 2-3 million eggs	Woll 2003 Tallack 2007
No of egg masses per year	1 - multiple	1	Fischer & Thatje 2008; Shields et al. 1991
Duration of larval phase	35 – 85 days	30 - 40 days	Weiss 2009a,b

1.4 Concept of the thesis

The objective of this thesis is to apply an integrative approach to reveal the influence of temperature changes due to ENSO on the development of *C. setosus* larvae by means of the above-described mechanisms of thermal tolerance. Herein we linked ecological and physiological approaches to draw a precise picture of larval fate. The thesis will centre around four questions, which focus on the existence of thermally induced capacity limitations.

- **Are *C. setosus* larvae able to survive the temperature fluctuations induced by ENSO?**
  Larval survival and development are strongly dependent on the temperature experienced during development. A study was conducted to determine the temperature tolerance window of *C. setosus* larvae. Threshold temperatures were determined by means of the survival capabilities and the instar duration reflects the speed of development.

- **How is the larval metabolism reacting to temperature fluctuations?**
  Temperature affects all levels of biological organization ranging from cellular to organism level and cause changes in the metabolic efficiency or fitness of an organism. This is reflected in the animal's elemental and biochemical composition as well as in its oxygen limited thermal tolerance and in the capacity to display aerobic and anaerobic metabolism. Therefore measurements of the elemental composition (carbon, hydrogen, nitrogen) were conducted, the oxygen consumption was determined and the activity of the two metabolic key enzymes citrate synthase (CS) and pyruvate kinase (PK) was analysed.

- **To which extent are larvae able to display phenotypic plasticity dependent on temperature and latitudinal distribution?**
  Phenotypic plasticity is an important ability that enables organisms, within species-specific physiological limits, to respond to gradual or sudden extrinsic changes in their environment and it may be a key to species' success to occupy a wide distribution range and/or to thrive under highly variable habitat conditions. In this part of the thesis the influence of

morphometric traits of larvae of the hairy crab *Cancer setosus* was studied, whose embryonic development took place at different temperatures at two different sites (Antofagasta, 23°45' S; Puerto Montt, 41°44' S) along the Chilean Coast.

- **How do salinity changes during EN affect larval development?**
  Salinity change influences the survival, metabolism and cumulative larval growth of crustacean larvae. The influence of changes in salinity has been the subject of several estuarine and rocky shore species, but only little is known about the physiological responses of larvae of a stenohaline species. The survival rate and the influence on elemental composition of Zoea I larvae was examined under hyposaline conditions of *C. setosus* from a salinity and temperature stable region.

2 Publications

List of publications and my contribution towards them

Publication I

Monika Weiss, Sven Thatje, Olaf Heilmayer, Klaus Anger, Thomas Brey, Martina Keller (2009)

Influence of temperature on the larval development of the edible crab, *Cancer pagurus*

Journal of the Marine Biological Association, UK 89(4), 753-759

The concept and the experimental design was established by the sixth and fourth author. Data analysis and interpretation was done by me in cooperation with the first, second, third and fourth author. The preparation of the manuscript was within my responsibility in cooperation with the second and third author, the final version was improved by discussions with all co-authors.

Publication II

Monika Weiss, Olaf Heilmayer, Thomas Brey, Sven Thatje (2009)

Influence of temperature on the zoeal development and elemental composition of the cancrid crab, *Cancer setosus* (Molina 1782) from Pacific South America

Journal of Experimental Marine Biology and Ecology, 376(1): 48-54.

I developed the idea the concept and the experimental design and conducted experiments and analyses. Data analysis and interpretation was done by me, the second and the third author. Manuscript preparation was done by me in cooperation with the other authors.

Publication III

Monika Weiss, Sven Thatje, Olaf Heilmayer (2009)

Temperature effects on zoeal morphometric traits and intraspecific variability in the hairy crab *Cancer setosus* across latitude

Helgoland Marine Research, in press. DOI10.1007/s10152-009-0173-8

Idea and concept originated from me. Experiments were conducted by S. Fischer and me and analyses were done by me. Data analysis and interpretation was done by me and the second author. Manuscript preparation was done by me in cooperation with the second and third author.

Publication IV

Monika Weiss, Olaf Heilmayer, Thomas Brey, Magnus Lucassen, Hans-Otto Pörtner (2009)

Physiological capacity of *Cancer setosus* larvae – adaptation to El Niño conditions

In preparation

I developed the idea, the conceptual approach and the experimental design. I conducted the experiments and the sample analyses. Data analysis and interpretation was done by me, the third and the fourth author. Manuscript preparation was done by me in cooperation with the second and fourth author and was discussed with the fifth author.

Publication V

Monika Weiss & Olaf Heilmayer

Effect of salinity changes during El Niño on the development of *Cancer setosus* Zoea I larvae

In preparation

I developed the idea, the conceptual approach and the experimental design. I conducted the experiments and the data analyses. Data interpretation and the preparation of the manuscript were done by me in cooperation with the second author.

2.1 Publication I

Influence of temperature on the larval development of the edible crab, *Cancer pagurus*

Weiss M; Thatje S; Heilmayer O; Anger K; Brey T; Keller M

(2009)

Journal of the Marine Biological Association
UK 89(4): 753-759

Journal of the Marine Biological Association of the United Kingdom, 2009, 89(4), 753–759.
doi:10.1017/S0025315409003269 Printed in the United Kingdom

Influence of temperature on the larval development of the edible crab, *Cancer pagurus*

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The influence of temperature on larval survival and development was studied in the edible crab, Cancer pagurus, *from a population off the island of Helgoland, North Sea. In rearing experiments conducted at six different temperatures (6°, 10°, 14°, 15°, 18° and 24°C), zoeal development was only completed at 14° and 15°C. Instar duration of the Zoea I was negatively correlated with temperature. A model relating larval body mass to temperature and developmental time suggests that successful larval development is possible within a narrow temperature range (14° ± 3°C) only. This temperature optimum coincides with the highest citrate synthase activity found at 14°C. A comparison for intraspecific variability among freshly hatched zoeae from different females (CW 13–17 cm, N = 8) revealed that both body mass and elemental composition varied significantly. Initial larval dry weight ranged from 12.1 to 17.9 µg/individual, the carbon content from 4.6 to 5.8 µg/individual, nitrogen from 1.1 to 1.3 µg/individual, and the C:N ratio from 4.1 to 4.4. A narrow larval temperature tolerance range of* C. pagurus *as well as the indication of intraspecific variability in female energy allocation into eggs may indicate a potential vulnerability of this species to climate change. Large-scale studies on the ecological and physiological resilience potential of this commercially fished predator are needed.*

Keywords: Brachyura, early ontogeny, edible crab, elemental composition

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INTRODUCTION

The embryonic and larval development of marine invertebrates is affected by extrinsic and intrinsic factors, such as temperature, maternal energy provisioning (Ouellet & Plante, 2004) and both pre- and post-hatching environmental conditions (Torres & Escribano, 2003; Giménez *et al.*, 2004; Fischer & Thatje, 2008). Variation in environmental key factors such as food availability (Anger & Dawirs, 1982), quality (Harms *et al.*, 1991), salinity (Giménez & Torres, 2002; Giménez & Anger, 2003) or temperature (Dawirs, 1979, 1985) can have unfavourable effects on growth in the early ontogeny of decapod crustaceans (for review, see Anger, 2001).

The early life cycle of the edible crab, *Cancer pagurus*, consists of five planktotrophic zoeal stages and a megalopa before reaching the first crab stage (Ingle, 1981). This species has a broad geographical distribution from northern Norway to West Africa and is also abundant in the Mediterranean Sea, which implies a wide range of temperature tolerance in adult populations of the species. Studies of abundance and catch rates show that *C. pagurus* is expanding its biogeographical range further northwards (Woll *et al.*, 2006).

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Since 1962 mean annual sea-surface temperature in the North Sea around the island of Helgoland rose 1.1°C, with milder winters and rising summer maxima (Wiltshire & Manly, 2004). Such a shift in ecological conditions may cause changes in the metabolic efficiency or fitness of an organism (Pörtner, 2001; Heilmayer *et al.*, 2004), which presumably is reflected in its elemental and biochemical composition (Dahlhoff, 2004). Studies on the complete temperature tolerance window of invertebrate larvae are extremely scarce, but may be a clue in future assessments of the potential of species to cope with climate change (e.g. Anger, 2001; Pörtner *et al.*, 2001, 2005; Thatje *et al.*, 2005).

In the present study, we provide evidence for temperature-induced changes in the chemical composition and aerobic capacities of *C. pagurus* larvae. Based on the hypothesis that the early ontogeny is the most vulnerable part of a life cycle (Anger, 2001), we discuss the physiological capability of the species to cope with elevated temperatures.

MATERIALS AND METHODS

Sampling and maintenance of adults and larvae

Ovigerous *Cancer pagurus* (carapace width (CW) 125 to 171 mm) were caught in May 2005 near the island of Helgoland in the North Sea (54° 11′N 7° 53′E) using a bottom

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Influence of temperature on the larval development of the edible crab, *Cancer pagurus* L.

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Abstract

The influence of temperature on larval survival and development was studied in the edible crab, *Cancer pagurus*, from a population off the Island of Helgoland, North Sea. In rearing experiments conducted at six different temperatures (6°, 10°, 14°, 15°, 18°, 24° C), zoeal development was only completed at 14° and 15° C. Instar duration of the Zoea I was negatively correlated with temperature. A model relating larval body mass to temperature and developmental time suggests that successful larval development is possible within a narrow temperature range (14° ± 3° C) only. This temperature optimum coincides with the highest citrate synthase (CS) activity found at 14° C. A comparison for intraspecific variability among freshly hatched zoeae from different females (CW 13 - 17cm, N = 8) revealed that both body mass and elemental composition varied significantly. Initial larval dry weight ranged from 12.1 to 17.9 µg/individual, the carbon content from 4.6 to 5.8 µg/individual, nitrogen from 1.1 to 1.3 µg/individual, and the C:N ratio from 4.1 to 4.4. A narrow larval temperature tolerance range of *C. pagurus* as well as the indication of intraspecific variability in female energy allocation into eggs may indicate a potential vulnerability of this species to climate change. Large-scale studies on the ecological and physiological resilience potential of this commercially fished predator are needed.

Keywords: Brachyura, early ontogeny, edible crab, elemental composition

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The early life cycle of the edible crab, *Cancer pagurus*, consists of five planktotrophic zoeal stages and a megalopa before reaching the first crab stage (Ingle, 1981). This species has a broad geographical distribution from northern Norway to West Africa and is also abundant in the Mediterranean Sea, which implies a wide range of temperature tolerance in adult populations of the species. Studies of abundance and catch rates show that *C. pagurus* is expanding its biogeographic range further northwards (Woll *et al.*, 2006).

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In the present study, we provide evidence for temperature-induced changes in the chemical composition and aerobic capacities of *C. pagurus* larvae. Based on the hypothesis that the early ontogeny is the most vulnerable part of a life cycle (Anger, 2001), we discuss the physiological capability of the species to cope with elevated temperatures.

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Ovigerous *Cancer pagurus* (carapace width, CW 125 to 171 mm) were caught in May 2005 near the island of Helgoland in the North Sea (54° 11' N, 7° 53' E) using a bottom trawl. Animals were immediately transported to the laboratory of the Marine Biological Station Helgoland (Biologische Anstalt Helgoland, BAH) where they were maintained individually in flow-through seawater aquaria (15 -20 l) at sea surface temperature (15.2 – 17.2 °C) and salinity (ca. 32 psu) in a 12:12-h light/dark cycle. Adults were fed twice a week either with isopods (*Idotea* sp.) or pieces of mussel (*Mytilus edulis*) meat. One day after feeding, remains were removed from the aquaria to maintain good water quality.

Freshly hatched larvae were collected in filters receiving water from the overflow of the aquaria. Since most larvae hatched at night, samples were taken every morning. Filters were cleaned every evening to ensure daily larval age did not vary by more than 12 hours (Lovrich *et al*., 2003). Solely actively moving larvae were used for experiments.

Influence of temperature on larval development

Randomly selected larvae from one randomly selected female (A) were kept in 500 ml glass bowls with a density of 20 to 30 individuals per bowl. In daily intervals, water was changed; larvae were checked for moults or mortality and subsequently were fed with freshly hatched *Artemia* spp. nauplii. One hatch (female A, Table 1) was divided on the day of hatching and subsequently reared at five constant temperatures (6, 10, 14, 18, 24 °C). Larvae reared at 15 °C resulted from a female caught in 1985 and were maintained and reared under the same condition as outlined above.

Minimum time of development for each instar was recorded assuming optimal developmental conditions in larvae (see Figure 1). Samples for determinations of larval dry weight (W) and elemental composition were taken immediately after hatching and later in intervals of one to ten days (see Table 1). Five replicates were collected, or less, when too few larvae were available. Each replicate consisted of 20-25 individuals in the Zoea I (Z I), but less (see Table 1) in the following (larger) instars.

Elemental analyses (CHN)

Carbon (C), hydrogen (H), nitrogen (N) contents were determined following Anger and Dawirs (1982), in brief: Larvae were gently rinsed in distilled water, blotted on filter paper, placed into tin cartridges, vacuum-dried for 48 h at < 0.01 mbar in a Lyovac GT 2E (Leybold-Heraeus) apparatus, weighed to the nearest 0.1 µg on a Mettler UM3 microbalance, and stored frozen at -20 °C. CHN content was measured with a Fisons (Carlo Erba Science) Model 1108 Elemental Analyzer.

In order to study intraspecific variability in the maternal energy provisioning of offspring, we sampled freshly hatched larvae from eight ovigerous females (always after the first night of the hatching period; female CW = 13 - 17cm; Table 2) and compared their initial body mass (measured as dry weight, W) and elemental composition (carbon, hydrogen, nitrogen; collectively CHN).

Enzyme assay

Citrate synthase (CS) (E.C. 4.1.3.7) is a key regulatory enzyme in the tricarbonic acid (TCA) cycle and was chosen as an indicator of aerobic capacity. Investigations on metabolic enzymes in larval stages are scarce and studies on shrimp larvae show that CS activity is dependent on growth during ontogeny (Lemos *et al*., 2003). Samples for determinations of enzyme activity of CS were taken in the premoult period of the Zoea I instar of larvae reared at three temperatures (10, 14, 18 °C) and analyzed following a modified method of Sidell *et al.* (1987). Three replicates were collected, each consisting of 10 larvae. No samples are taken for 6° and 24 °C as an insufficient amount of material was available.

Frozen samples were homogenized in ~0.3 µl extraction buffer (75 mM Tris-HCl, 1 mM EDTA; pH 7.6) per 1µg larval W (dry weight) to get a 1:10 (w/v) ratio with a Branson Sonifier 450 (0° C, output control 8, duty cycle 50%, 15 min). Homogenates were centrifuged for 5 minutes at 7400 g and 0° C with an Eppendorf Centrifuge 5810R. The concentration of soluble protein in the extracts was measured after Bradford. The samples were first diluted 1:5 with 0.9 % NaCL and were then applied in duplicate (5 µl) on microplates. Subsequently 250 µl dye reagent (Biorad protein assay 500 0006, diluted 1:5 with aqua dest) were added and the optical density was measured at 620 nm in a microplate reader (FLUOstar Galaxy). Bovine serum albuminum (BSA, 0 - 3.5 µg per well) was run parallel as standard.

For enzyme assays the absorption of the supernatant was measured at four temperatures (10, 14, 18, 24 °C) in three aliquots with a microplate reader at 405 nm. Homogenates (2 µl / well) were assayed in 150 µl of 100 mM Tris-HCl buffer (pH 8.0), 10 µl 5 mM DTNB (5.5'-Dithio-bis-(2-nitrobenzoic acid)) and 4 µl Acetyl-CoA (20 mM). 4 µl Oxalacetat (20 mM) was added to start the reaction (omitted for the blanks). Standards of 0.5 mM Dithiothreitol DTT (5 – 40 µl per well) were run in parallel. The activity was expressed as the change of absorption per time and protein weight units ($\Delta A\ min^{-1}{}_{gprt}{}^{-1}$).

Statistical analyses

All data were tested with the Nalimov test to exclude outliers from analysis (Kaiser & Gottschalk, 1972). A general additive model (Hastie & Tibshirani 1990) was used to describe larval mass (µg C) as a function of time (t, days) and temperature (T, Kelvin):

$$C_{BC} = a + b_1 \times t + b_2 \times f(T) + b_3 \times t \times f(T) \quad [\mu g, d, K]$$

where C_{BC} is the Box-Cox transformed larval mass (Sokal & Rholf 1981) and f(T) a function that models the temperature effect according to a normal distribution with mean M_T, standard deviation SD_T and skewing factor SK_T. The latter was introduced to allow for asymmetric effects of temperatures above and below the optimum, as observed in many temperature tolerance studies (see e.g. Pörtner *et al.*, 2001; Pörtner *et al.*, 2005) and implied by the original data:

$$f(t) = \left(1/\left(SD_T \times \sqrt{2\pi}\right)\right) \times e^{-0.5\times(((T-M_T)+SK_T\times(T-M_T))/SD_T)^2} \qquad \text{for T >= } M_T$$

$$f(t) = \left(1/\left(SD_T \times \sqrt{2\pi}\right)\right) \times e^{-0.5\times(((T-M_T)-SK_T\times(T-M_T))/SD_T)^2} \qquad \text{for T < } M_T$$

For the comparison of elemental composition of larvae of different females and temperature dependence of CS activity a one-way ANOVA was used. Post hoc tests were conducted with the Student-Newman Keuls method. C/N ratio data were transformed logarithmically prior to analysis in order to achieve a normally distributed data set.

Results

Influence of temperature on larval development

Complete zoeal development occurred only at 14 and 15 °C (Table 1, Figure 1). At 6 °C, the Zoea I survived for up to 25 days without moulting to the Zoea II stage. At 10 °C, first larvae moulted after 12 days to the Zoea II and survived only for another three days. Larvae reared at 14 °C reached the Zoea V 33 days after hatching. At 18 °C, larvae died already after 6 days in the Zoea II stage, and at 24 °C they reached the Zoea III, dying ten days later.

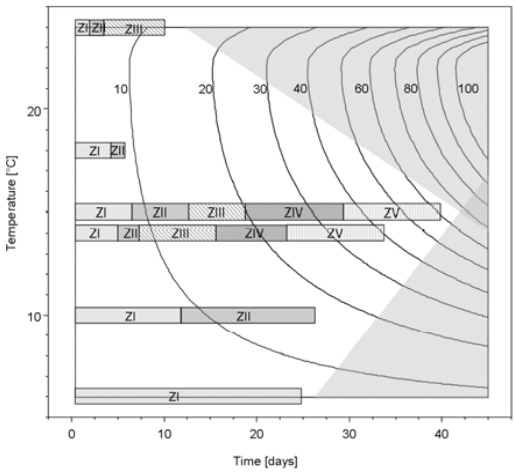

Figure 1. Model of body mass increase (C) throughout the larvae development of the edible crab *Cancer pagurus*. Equation for the model is: C_{BC} = -7.97 – 10.755 x t + 919.52 x f(T) + 476.634 x t x f(T); N = 463, F = 1599.51, R^2 = 0.954; T represents the temperature in Kelvin and t the time in days. Isolines represent carbon content in µg. ZI-ZV are the larval instars. White areas show the realistic range of the model.

The minimum duration of development through the ZI stage decreased with increasing temperature. This pattern can be described as a linear relationship between ln temperature and ln instar duration with the equation:

$$\ln D = \ln 6.5482 - 1.8096 \times \ln T \; ; \; R^2 = 0.991, \; P < 0.001$$

Where D = time of development (days) and T = temperature (°C). Changes in W and CHN during the course of larval development are shown in Table 1.

Table 1. *Cancer pagurus*. Changes in dry weight (W), carbon (C), nitrogen (N) and C:N ratio during time days after hatch (± SD) at five temperatures. Larvae reared at 15°C are from different female.

Temp	**instar**	**ind/sample**	**time**	**DM** [µg]		**C** [µg]		**N** [µg]		**C/N**	
[°C]			[days]	mean	± SD	mean	± SD	mean	± SD	mean	± SD
6	ZI	25	0	15.6	0.5	5.00	0.09	1.19	0.02	4.22	0.01
	ZI	24	2	16.0	0.8	5.27	0.21	1.21	0.05	4.35	0.02
	ZI	22	4	18.0	0.6	5.51	0.11	1.28	0.02	4.31	0.03
	ZI	21	6	17.7	0.5	5.60	0.18	1.30	0.04	4.30	0.02
	ZI	21	8	19.2	0.3	5.56	0.16	1.31	0.04	4.26	0.02
	ZI	21	10	19.0	0.5	5.68	0.21	1.35	0.05	4.22	0.02
	ZI	21	12	19.0	1.0	5.36	0.20	1.28	0.05	4.19	0.02
	ZI	21	15	18.3	0.5	5.45	0.04	1.33	0.04	4.15	0.02
	ZI	21	17	19.4	0.3	6.29	0.07	1.48	0.01	4.26	0.03
	ZI	21	19	19.8	0.8	5.97	0.15	1.40	0.04	4.27	0.03
	ZI	21	21	22.9	0.5	7.34	0.03	1.74	0.01	4.22	0.01
	ZI	21	25	20.8	0.1	6.97	0.14	1.69	0.04	4.12	0.01
10	ZI	25	0	15.6	0.5	5.00	0.09	1.19	0.02	4.22	0.01
	ZI	24	2	18.4	0.5	5.93	0.19	1.32	0.04	4.47	0.01
	ZI	22	4	20.3	0.2	6.34	0.15	1.43	0.03	4.44	0.03
	ZI	21	6	20.2	0.2	6.49	0.16	1.48	0.03	4.38	0.04
	ZI	21	8	21.3	0.3	6.26	0.12	1.47	0.04	4.32	0.01
	ZI	21	10	20.6	0.6	5.97	0.25	1.41	0.07	4.23	0.02
	ZI	21	12	20.7	0.7	5.94	0.26	1.42	0.06	4.19	0.01
	ZII	20	15	26.1	0.8	9.36	0.30	2.26	0.03	4.21	0.01
14	ZI	25	0	15.6	0.5	5.00	0.09	1.19	0.02	4.22	0.01
	ZI	24	2	18.3	0.7	5.69	0.17	1.28	0.04	4.45	0.02
	ZI	22	4	18.5	1.4	5.98	0.43	1.35	0.10	4.45	0.01
	ZI	21	6	21.5	0.3	6.95	0.33	1.58	0.08	4.39	0.03
	ZII	20	7	21.6	0.2	6.80	0.21	1.55	0.04	4.39	0.02
	ZIII	7	16	41.0	1.3	14.21	0.43	3.36	0.10	4.22	0.03
	ZIV	5	23	63.4	4.7	23.32	1.96	5.42	0.45	4.30	0.05
	ZV	2	33	105.6	3.9	40.86	0.99	9.23	0.30	4.43	0.05

Temp [°C]	**instar**	**ind/sample**	**time** [days]	**DM** [µg] mean	± SD	**C** [µg] mean	± SD	**N** [µg] mean	± SD	**C/N** mean	± SD
15	ZI	20	0	14.7	0.8	5.0	0.2	1.2	0.1	4.15	0.18
	ZI	15	1	19.1	0.5	6.2	0.1	1.5	0.0	4.1	0.08
	ZI	15	2	21.0	0.9	7.2	0.4	1.7	0.1	4.29	0.1
	ZI	15	3	21.8	0.9	7.6	0.4	1.7	0.1	4.54	0.12
	ZI	15	4	22.2	1.4	8.4	0.5	1.9	0.1	4.52	0.09
	ZI	15	5	23.9	1.5	8.8	0.6	2.0	0.1	4.4	0.11
	ZI	15	6	23.9	1.4	8.7	0.6	2.0	0.2	4.28	0.1
	ZI	15	7	22.4	0.6	8.6	0.2	2.1	0.1	4.09	0.11
	ZII	15	8	25.3	1.1	8.8	0.3	2.1	0.1	4.15	0.08
	ZII	10	10	34.8	1.9	12.6	0.6	2.8	0.1	4.54	0.09
	ZII	10	12	39.9	1.6	14.7	0.6	3.4	0.2	4.37	0.09
	ZII	10	14	37.4	3.5	14.0	1.4	3.4	0.4	4.17	0.06
	ZIII	7	15	43.7	3.0	14.2	0.7	3.4	0.2	4.17	0.17
	ZIII	7	17	52.1	6.5	17.0	2.4	3.7	0.5	4.57	0.23
	ZIII	7	19	63.9	1.9	22.3	0.7	5.0	0.3	4.5	0.15
	ZIII	7	21	62.2	7.5	23.9	3.4	5.5	0.8	4.35	0.16
	ZIV	7	22	63.0		19.9		4.8		4.12	
	ZIV	5	23	83.9	4.3	27.5	2.1	6.0	0.5	4.6	0.1
	ZIV	5	24	93.9	18.0	32.2	6.9	7.0	1.4	4.57	0.11
	ZIV	4	26	120.4		43.0		9.6		4.49	
	ZV	3	38	129.0	8.5	45.7	3.7	11.0	0.6	4.15	0.23
18	ZI	25	0	15.6	0.5	5.00	0.09	1.19	0.02	4.22	0.01
	ZI	24	2	18.8	0.7	5.55	0.17	1.25	0.03	4.42	0.01
	ZI	20	3	19.3	0.8	6.03	0.25	1.36	0.05	4.41	0.02
	ZI	22	4	20.8	0.3	6.46	0.26	1.51	0.06	4.28	0.01
	ZII	20	6	24.3	1.9	8.23	0.38	1.87	0.16	4.41	0.18
24	ZI	25	0	15.6	0.5	5.00	0.09	1.19	0.02	4.22	0.01
	ZI	24	2	19.6	2.9	5.86	0.83	1.31	0.22	4.50	0.11
	ZII	22	4	22.8	0.3	8.15	0.05	1.90	0.01	4.30	0.03
	ZIII	17	10	33.9	3.8	11.55	1.52	2.73	0.38	4.24	0.03

Larval body mass (C in µg/ind) is predicted from day (t) and temperature (T in K) by the model

$$C_{BC} = -7.97 - 10.755 \times t + 919.52 \times f(T) + 476.634 \times t \times f(T)$$

N = 463, F = 1599.51, R^2 = 0.954, P < 0.001 for the whole model and each term with

$$f(T) = 0.02452 \times e^{-0.5 \times \left(\left(\left(\left(T - 295.243\right) + \left(T - 295.243\right) + 0.620 \times \left(T - 295.243\right)\right) \div 16.273\right)^2\right)} \quad \text{for T >= 295.243}$$

$$f(T) = 0.02452 \times e^{-0.5 \times \left(\left(\left(\left(T - 295.243\right) + \left(T - 295.243\right) - 0.620 \times \left(T - 295.243\right)\right) \div 16.273\right)^2\right)} \quad \text{for T < 295.243}$$

using

$$C_{BC} = \left(C^{0.2} - 1\right) / 0.0283318325559$$

this solves to

$$C = \left(0.779 - 0.305 \times t + 26.0517 \times f(T) + 13.504 \times t \times f(T)\right)^5$$

Note that this model predicts a larval mass for just any combination of time and temperature, whereas our experiments indicate that the time x temperature space where larvae do exist is limited (see discussion). Figure 1 provides a plot of larval mass in the time x temperature space, where the "unrealistic" range has been shadowed. The residual plot (Figure 2) indicates that the model fit the data quite well up to about 40 µg C, but severely underestimates larval mass >50 µg C. At the upper threshold temperatures in higher instars, changes in carbon values were generally lower than predicted by the model (see Figure 2). Further increase or decrease in temperature cause death of the larvae. To give an example: at 6 °C larvae did not moult and die after 25 days; at 24 °C, carbon values in the Zoea III are much lower than they were predicted by the model (also compare Table 1) for e.g. 20 °C, and the larvae died before reaching the Zoea IV. According to the model, the final Zoea V body mass of 60 µg C observed at 16

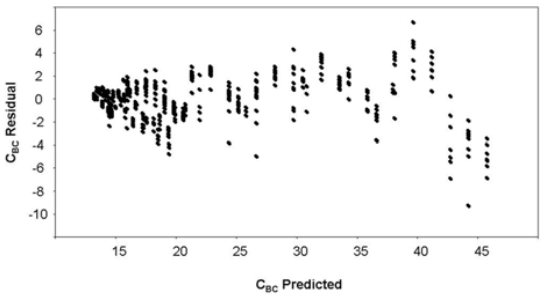

Figure 2. Residual by predicted plot from the model predicting larval growth: plot of regression residuals versus predicted values. The plot does not indicate a problem with the model.

°C would be obtained after 36 days at 18 °C, which already lies outside the realistic assumption, and would be unattainable at temperatures < = 10 °C.

Table 2. *Cancer pagurus*. Size of eight different females and elemental composition (dry weight (W), carbon (C), nitrogen (N), hydrogen (H), C:N and C:H ratio of freshly hatched larvae (hatch 1).

Female	CW [cm]	W [µg]		C [µg]		N [µg]		H [µg]		C/N [µg]	
		mean	±SD	mean	±SD	mean	±SD	mean	±SD	mean	±SD
A	16.8	15.6	0.5	5.00	0.09	1.19	0.02	0.71	0.01	4.22	0.01
B	14.2	18.0	0.4	5.83	0.01	1.33	0.00	0.84	0.01	4.40	0.02
C	14.3	17.1	0.1	5.54	0.07	1.29	0.01	0.78	0.00	4.28	0.01
D	14.2	16.1	0.2	5.01	0.05	1.14	0.01	0.69	0.02	4.38	0.01
E	12.5	14.4	0.3	4.79	0.06	1.13	0.01	0.66	0.02	4.22	0.01
F	13.2	12.5	0.3	4.87	0.08	1.16	0.01	0.68	0.01	4.17	0.01
G	17.1	15.7	0.2	5.56	0.08	1.28	0.01	0.79	0.01	4.34	0.02
H	13.6	12.1	0.3	4.65	0.15	1.13	0.04	0.67	0.03	4.13	0.01

Citrate synthase activity (CSA)

Activities of citrate synthase (CS) in *C. pagurus* larvae measured at four different temperatures are shown in Figure 3. Larvae of all acclimation temperatures show lowest activity levels at 10 °C assay temperature. 14 °C acclimated larvae show the highest activity at all assay temperatures. 18° acclimated larvae show no significant differences over the whole measured temperature range. Significantly higher CSA rates compared to larvae at hatching are only observed at 14 °C assay temperature.

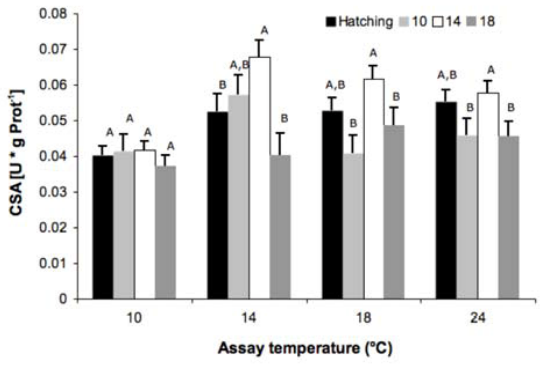

Figure 3. Citrate synthase activity [U * g Prot^{-1}] of *Cancer pagurus* larvae acclimated to three different temperatures (light grey bars – 10° C acclimated; white bars – 14° C acclimated; dark grey bars – 18° C acclimated) compared with larvae immediately after hatching (black bars). Bars within on assay temperature not connected with the same letter are significantly different (P > 0.05).

Variability in initial larval body mass among broods

Female carapace width (CW), larval body weight (W) and elemental composition (CHN) are compared in Table 2. Initial body mass and elemental composition of freshly hatched Zoea I larvae varied significantly among the eight females (Table 3) without showing a clear pattern between larval energy provisioning and female size. For example, females B and D had the same CW, but larval W and C, H, and N contents differed significantly.

Table 3. *Cancer pagurus*. One-way ANOVA to evaluate the maternal influence on body mass [as dry weight (W), carbon (C), and nitrogen (N)] and C:N ratio of freshly hatched larvae (first day of the hatching period 1). *: ANOVA on ranks because equal variance test failed.

	df	MS	F	p
W	7	16.873	177.957	< 0.001
C	7	0.765	99.901	< 0.001
N*	7			< 0.001
C/N	7	0.0023	244.893	< 0.001

Discussion

Larval development of *C. pagurus* of the Helgoland population up to Zoea V is achieved within a narrow temperature window (15 – 16 °C) only. Below and above this window we see initial development that completes stages Zoea I to Zoea III, depending on temperature. Zoea I development times indicate a positive temperature effect on larval growth up to 24 °C, too. Our predictive model captures this feature quite well, indicating that optimum temperature should be around 22 °C. Hence, what causes the failure of larval development outside the 15 – 16 °C window? Obviously, this is not a straight forward temperature effect on metabolism, e.g. through cellular oxygen deficiency beyond Pejus temperatures as postulated by Pörtner (2001). Additionally, reduced feeding activity at cold temperatures can result in insufficient ingestion rates, while at high temperatures higher maintenance costs cannot be compensated (Dawirs & Dietrich, 1986, Anger *et al.*, 2004; Heilmayer *et al*., 2008). We hypothesize that the transition from one stage to the next is the phase of failure. Either, energy investment and thus oxygen demand is distinctly enhance during transition from one stage to the next, and/or the complex metamorphostic process by itself is more temperature sensitive, as it has long been known that ecdysis is the critical point in the development of decapod larvae (Anger 2001).

The residual plot (Figure 2) shows a general good fit of the model (i.e. randomly distribution of residuals) and slightly lower carbon values in higher instars (Z V) than predicted by the model (Figure 2), which might be due to problems during metamorphosis to the megalopa stage. It is known that larvae are able to postpone their metamorphosis to the megalopa stage if the cue for the suitable habitat can not be detected (Krimsky & Epifanio 2008), but the enduring lack of those cues cause stress and the depletion of resources.

Changes in the kinetic characteristics of enzymes reflect differences in metabolic regulation and are inevitably involved in adaptation and acclimation to ambient temperature (Wells *et al.*, 2001; Somero, 2005). A decrease in CS activity is a strong indication for metabolic reduction. In *C. pagurus* CSA decreases with increasing enzyme assay temperature, indicating an optimum temperature at 14 °C. The generally lower citrate synthase activity, i.e. lower aerobic capacity, of 18 °C larvae over the whole measured temperature range indicate that acclimation to above-optimum temperatures cannot be compensated. The compensation over certain temperature ranges has been reported for several fish and crustaceans (e.g. Salomon & Buchholz, 2000; Lannig *et al.*, 2003; Lemos *et al.*, 2003).

No complete larval development was observed in our laboratory experiments at 18 °C, although this is a temperature which larvae may encounter in Helgoland waters during summer. Only little information is available about the southern distribution boundaries, where larvae should encounter much warmer water temperatures. There is evidence for a northern expansion of this species in Norway (Woll *et al.*, 2006), probably indicating that warming of the ocean drives an expansion if not shift of the geographic range of *C. pagurus* towards the northern North Sea (see also Wiltshire & Manly, 2004). It is thus necessary to determine the significance of the temperature tolerance window of this species for its distribution boundaries more accurately.

In this context it might be important to recognize that initial larval biomass at hatching varied significantly within our small sample size (N = 8) but without statistically significant correlation between female size and the body mass of freshly hatched Zoea I. However, it must be taken into consideration that our data (12.5 – 17.1 cm CW) do not cover the whole size range of mature females and thus further study investigating the full range of female maturity (11.5 to 19 cm; Neal & Wilson, 2004) may come to different conclusion. Energy allocation of females into offspring

may be controlled by genetic disposition (Reznick, 1981), size (DeMartini *et al.* 2003), temperature (Fischer & Thatje, 2008), or the nutritional status of the female (Bernardo, 1996). A positive relationship between maternal size and offspring size has been observed in both invertebrates (Marshall & Keough, 2004) and vertebrates (Birkeland & Dayton, 2005), but not universally. Among crustaceans, there are species that show such a relationship, e.g. the xanthoid crab *Pseudocarcinus gigas* (Gardner, 1997), but also other species not following this pattern, e.g. the American lobster (*Homarus americanus*) (Ouellet & Plante, 2004).

Future studies covering the full size range of mature *C. pagurus* need to reveal whether intraspecific variability in energy offspring is a matter of individual variability in female fitness and/or a female size related trade off. Intraspecific variability may also affect larval fitness and survival and thus a future large-scale study is needed to reveal whether the herein reported temperature tolerance pattern for larvae from single female can be easily translated into populations and consequently management approaches of this species.

Acknowlegements

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Erratum for Publication I

Table 1. There has been a confusion of the column headers. The correct table is:

Table 1. *Cancer pagurus*. Changes in dry weight (W), carbon (C), nitrogen (N) and C:N ratio during time (days) after hatch (± SD) at five temperatures. Larvae reared at 15 °C are from different female.

Temp	instar	ind/sample	time	DM [µg]		C [µg]		N [µg]		C/N	
[°C]			[days]	mean	± SD	mean	± SD	mean	± SD	mean	± SD
6	ZI	25	0	15.6	0.5	5.00	0.09	1.19	0.02	4.22	0.01
	ZI	24	2	16.0	0.8	5.27	0.21	1.21	0.05	4.35	0.02
	ZI	22	4	18.0	0.6	5.51	0.11	1.28	0.02	4.31	0.03
	ZI	21	6	17.7	0.5	5.60	0.18	1.30	0.04	4.30	0.02
	ZI	21	8	19.2	0.3	5.56	0.16	1.31	0.04	4.26	0.02
	ZI	21	10	19.0	0.5	5.68	0.21	1.35	0.05	4.22	0.02
	ZI	21	12	19.0	1.0	5.36	0.20	1.28	0.05	4.19	0.02
	ZI	21	15	18.3	0.5	5.45	0.04	1.33	0.04	4.15	0.02
	ZI	21	17	19.4	0.3	6.29	0.07	1.48	0.01	4.26	0.03
	ZI	21	19	19.8	0.8	5.97	0.15	1.40	0.04	4.27	0.03
	ZI	21	21	22.9	0.5	7.34	0.03	1.74	0.01	4.22	0.01
	ZI	21	25	20.8	0.1	6.97	0.14	1.69	0.04	4.12	0.01
10	ZI	25	0	15.6	0.5	5.00	0.09	1.19	0.02	4.22	0.01
	ZI	24	2	18.4	0.5	5.93	0.19	1.32	0.04	4.47	0.01
	ZI	22	4	20.3	0.2	6.34	0.15	1.43	0.03	4.44	0.03
	ZI	21	6	20.2	0.2	6.49	0.16	1.48	0.03	4.38	0.04
	ZI	21	8	21.3	0.3	6.26	0.12	1.47	0.04	4.32	0.01
	ZI	21	10	20.6	0.6	5.97	0.25	1.41	0.07	4.23	0.02
	ZI	21	12	20.7	0.7	5.94	0.26	1.42	0.06	4.19	0.01
	ZII	20	15	26.1	0.8	9.36	0.30	2.26	0.03	4.21	0.01
14	ZI	25	0	15.6	0.5	5.00	0.09	1.19	0.02	4.22	0.01
	ZI	24	2	18.3	0.7	5.69	0.17	1.28	0.04	4.45	0.02
	ZI	22	4	18.5	1.4	5.98	0.43	1.35	0.10	4.45	0.01
	ZI	21	6	21.5	0.3	6.95	0.33	1.58	0.08	4.39	0.03
	ZII	20	7	21.6	0.2	6.80	0.21	1.55	0.04	4.39	0.02
	ZIII	7	16	41.0	1.3	14.21	0.43	3.36	0.10	4.22	0.03
	ZIV	5	23	63.4	4.7	23.32	1.96	5.42	0.45	4.30	0.05
	ZV	2	33	105.6	3.9	40.86	0.99	9.23	0.30	4.43	0.05
15	ZI	20	0	14.7	0.8	5.0	0.2	1.2	0.1	4.15	0.18
	ZI	15	1	19.1	0.5	6.2	0.1	1.5	0.0	4.1	0.08
	ZI	15	2	21.0	0.9	7.2	0.4	1.7	0.1	4.29	0.1
	ZI	15	3	21.8	0.9	7.6	0.4	1.7	0.1	4.54	0.12
	ZI	15	4	22.2	1.4	8.4	0.5	1.9	0.1	4.52	0.09
	ZI	15	5	23.9	1.5	8.8	0.6	2.0	0.1	4.4	0.11
	ZI	15	6	23.9	1.4	8.7	0.6	2.0	0.2	4.28	0.1
	ZI	15	7	22.4	0.6	8.6	0.2	2.1	0.1	4.09	0.11
	ZII	15	8	25.3	1.1	8.8	0.3	2.1	0.1	4.15	0.08
	ZII	10	10	34.8	1.9	12.6	0.6	2.8	0.1	4.54	0.09
	ZII	10	12	39.9	1.6	14.7	0.6	3.4	0.2	4.37	0.09
	ZII	10	14	37.4	3.5	14.0	1.4	3.4	0.4	4.17	0.06
	ZIII	7	15	43.7	3.0	14.2	0.7	3.4	0.2	4.17	0.17
	ZIII	7	17	52.1	6.5	17.0	2.4	3.7	0.5	4.57	0.23
	ZIII	7	19	63.9	1.9	22.3	0.7	5.0	0.3	4.5	0.15
	ZIII	7	21	62.2	7.5	23.9	3.4	5.5	0.8	4.35	0.16
	ZIV	7	22	63.0		19.9		4.8		4.12	
	ZIV	5	23	83.9	4.3	27.5	2.1	6.0	0.5	4.6	0.1
	ZIV	5	24	93.9	18.0	32.2	6.9	7.0	1.4	4.57	0.11
	ZIV	4	26	120.4		43.0		9.6		4.49	
	ZV	3	38	129.0	8.5	45.7	3.7	11.0	0.6	4.15	0.23
18	ZI	25	0	15.6	0.5	5.00	0.09	1.19	0.02	4.22	0.01
	ZI	24	2	18.8	0.7	5.55	0.17	1.25	0.03	4.42	0.01
	ZI	20	3	19.3	0.8	6.03	0.25	1.36	0.05	4.41	0.02
	ZI	22	4	20.8	0.3	6.46	0.26	1.51	0.06	4.28	0.01
	ZII	20	6	24.3	1.9	8.23	0.38	1.87	0.16	4.41	0.18
24	ZI	25	0	15.6	0.5	5.00	0.09	1.19	0.02	4.22	0.01
	ZI	24	2	19.6	2.9	5.86	0.83	1.31	0.22	4.50	0.11
	ZII	22	4	22.8	0.3	8.15	0.05	1.90	0.01	4.30	0.03
	ZIII	17	10	33.9	3.8	11.55	1.52	2.73	0.38	4.24	0.03

Figure 3. There has been a mistake in the protein content calculation. Correct values are twice as high as they have been calculated. Since CS activity is expressed per protein, the CS values in the Figure should be half as high as they are presented.

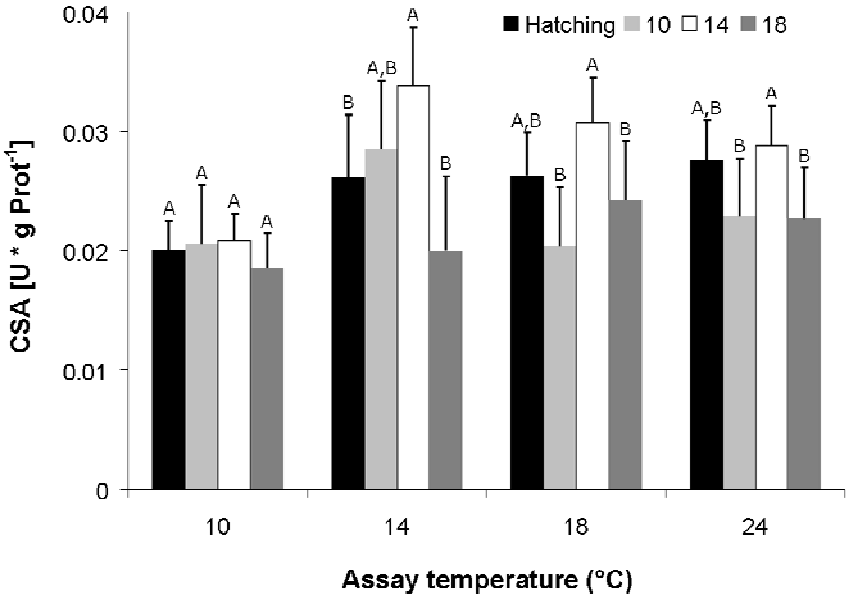

Figure 3. Citrate synthase activity (U * g $Prot^{-1}$) of *Cancer pagurus* larvae acclimated to three different temperatures (light grey bars -10 °C acclimated; white bars -14 °C acclimated; dark grey bars -18 °C acclimated) compared with larvae immediately after hatching (black bars). Bars within an assay temperature not connected with the same letter are significantly different ($P > 0.05$).

2.2 Publication II

Influence of temperature on the zoeal development and elemental composition of the cancrid crab, *Cancer setosus* (Molina 1782) from Pacific South America

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Influence of temperature on the zoeal development and elemental composition of the cancrid crab, *Cancer setosus* Molina, 1782 from Pacific South America

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ABSTRACT

Temperature changes during ENSO cause mass mortalities of adult *Cancer setosus*, but the effects on early life stages are unknown. The influence of temperature on survival, development and biochemical composition was studied in larvae of the hairy crab, *C. setosus*, from a population off the northern Chilean coast. In rearing experiments conducted at four different temperatures (12, 16, 20, 22 °C), zoeal development was only completed at 16 and 20 °C, after 78 and 36 days, respectively. Instar duration was negatively correlated with temperature. A multiple linear model relating larval body mass (in carbon) to temperature and developmental time suggests that successful larval development is possible within a narrow temperature range only. The biochemical composition, measured as carbon, hydrogen, and nitrogen (*C, H, N*) content, show in general the typical oscillating changes during the moult cycle of brachyuran crab larvae. However, at high (22 °C) and low (16 °C) temperatures, CHN values show deviations from the typical pattern, indicating threshold temperatures for larval activity and survival. These findings indicate that the larval development of *C. setosus* is compromised under conditions of El Niño, with temperatures exceeding the upper thermal temperature tolerance threshold of larvae. Effects of El Niño on early life history stages and recruitment rates should be increasingly taken into account in fisheries management strategies.

1. Introduction

Most cancrid crabs are found in cold temperate and boreal waters (MacKay, 1943) and provide a huge proportion of crustacean fisheries in those regions (Bennett, 1995; Johnson and Shanks, 2002). Due to their commercial importance population structure and recruitment of cancrid crabs have been the subject of several studies (e. g. Hankin et al., 1997; Eaton et al., 2003; Taggart et al., 2004; Fischer and Thatje, 2008; Fischer et al., 2009). Successful recruitment is based on larval development. Larval hatching normally occurs during spring when food availability (plankton bloom) (Park et al., 2007) and temperatures are favourable for larval growth. This seasonal dependency is not valid for larvae in the highly productive upwelling region of the Humboldt Current, which provides a huge amount of plankton year-round during upwelling periods. The persistent stable temperate conditions in a broad range of the Humboldt Current allow larvae to develop all year round (for review see Fischer and Thatje, 2008). As one of the world's most productive ecosystems, the Humboldt Current supports one of the world's largest fisheries (Bertrand et al., 2004) which is of high economical importance for the adjacent countries (Ryther, 1969; Urban and Tarazona, 1996; Food and Agricultural Organization, 2006). The availability of resources is highly dependent on the global coupled ocean-atmosphere phenomenon El Niño Southern Oscillation (ENSO) (Lehodey et al., 2006). During El Niño various abiotic and biotic conditions change: i.e., temperature rises, salinity is reduced, sedimentation and turbidity increases in some regions, radiation increases due to clear oceanic water in others, predation and competition increase by invaders and food shortage occurs (Arntz et al., 1988). Those changing conditions during El Niño have some positive effects like immigration of commercially valuable tropical species (e.g. the shrimp *Xiphopenaeus riveti*) and an outbreak of some commercial exploited local species, such as the scallop *Argopecten purpuratus* (Arntz et al., 1988). However, the negative effects and damage caused by collapsing local populations of other important species, like the hairy crab, *Cancer setosus*, likely caused by the sudden and drastic temperature rise prevails by far in local artisanal fisheries (Arntz et al., 1988). Only few artisanal fishermen are able to take advantage of the immigration of tropical species during El Niño since they do not have the right fishing gears (O'Riordan, 1998).

Cancer setosus (Molina 1782; synonymous *C. polyodon* Poeppig 1836) ranges in its distribution from Guayaquil in Ecuador (2°13′ S, 79°53′ W) to the Peninsula of Taitao in Southern Chile (46°00′ S, 75°00′ W) (Garth and Stephenson, 1966; Fischer and Thatje, 2008) and its commercial value for the Chilean and Peruvian artisanal fishery increased during the last decades (Wolff and Soto, 1992; SERNAPESCA, 2006; Thatje et al., 2008). The early ontogeny, which in *C. setosus*

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Influence of temperature on the larval development and elemental composition of the cancrid crab, *Cancer setosus* Molina, 1782 from Pacific South America

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Abstract

Temperature changes during ENSO cause mass mortalities of adult *Cancer setosus*, but the effects on early life stages are unknown. The influence of temperature on survival, development and biochemical composition was studied in larvae of the hairy crab, *Cancer setosus*, from a population off the northern Chilean coast. In rearing experiments conducted at four different temperatures (12, 16, 20, 22 °C), zoeal development was only completed at 16 and 20 °C, after 78 and 36 days, respectively. Instar duration was negatively correlated with temperature. A multiple linear model relating larval body mass (in carbon) to temperature and developmental time suggests that successful larval development is possible within a narrow temperature range only. The biochemical composition, measured as carbon, hydrogen, and nitrogen (C, H, N) content, show in general the typical oscillating changes during the moult cycle of brachyuran crab larvae. However, at high (22 °C) and low (16 °C) temperatures, CHN values show deviations from the typical pattern, indicating threshold temperatures for larval activity and survival. These findings indicate that the larval development of *C. setosus* is compromised under conditions of El Niño, with temperatures exceeding the upper thermal temperature tolerance threshold of larvae. Effects of El Niño on early life history stages and recruitment rates should be increasingly taken into account in fisheries management strategies.

KEY WORDS: Brachyura, early ontogeny, survival rate, hairy crab, elemental composition, El Niño

Introduction

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Cancer setosus (Molina 1782; synonymous *C. polyodon* Poeppig 1836) ranges in its distribution from Guayaquil in Ecuador (2°13' S, 79°53' W) to the Peninsula of Taitao in Southern Chile (46°00' S, 75°00' W) (Garth and Stephenson 1966, Fischer and Thatje 2008) and its commercial value for the Chilean and Peruvian artisanal fishery increased during the last decades (Wolff and Soto 1992, SERNAPESCA 2006, Thatje et al. 2008). The early ontogeny, which in *C. setosus* consists of five planktotrophic zoeal stages (zoea I – V = Z I – V) and one megalopa before reaching the first crab stage (Quintana and Saelzer 1986), is considered as the most delicate part within the life cycle of brachyuran and in particular cancrid crabs (Anger 2001, Weiss et al. 2009). A unique physiological plasticity to respond to latitudinal and seasonal changes in temperature, has been observed in early egg traits of *C. setosus*. Among the most conspicuous characteristics is a synchronization of a single egg batch release with seasonality at the species southernmost distribution boundary in central southern Chile, contrasted by multiple annual ovipositions in northern Chile (Antofagasta). In addition, a correlation of changes in egg energy contents and temperature was observed along latitude as well as in subsequent ovipositions in the same female (Fischer et al. 2009).

Temperature affects all levels of biological organization ranging from cellular to organismal level (Guderley and St-Pierre 2002) and cause changes in the metabolic efficiency or fitness of an organism (Pörtner 2001), which presumably is reflected in its elemental and biochemical composition (Dahlhoff 2004). In this study we draw a picture of larval destiny during ENSO temperature oscillations to improve our knowledge of mechanisms of temperature adaptations in *C. setosus* larvae. We define the temperature window of *C. setosus* larvae for the Antofagasta region (northern Chile), and reveal the influences of temperature changes throughout ENSO on larval elemental composition and survival.

Materials and Methods

Sampling and maintenance of adults

Ovigerous *Cancer setosus* (carapace width, CW 101 to 201 mm) were caught between February and May 2007 by fishermen of the "Caleta Colosso" (54°11' N, 7° 53' E) by scuba diving and were immediately transferred to the

laboratory of the Instituto de Investigaciones Oceanológicas of the Universidad de Antofagasta, Chile. Animals were maintained individually in flow-through seawater aquaria (12 l) at ambient temperature ~16.0 °C and salinity 34 psu in a 12:12-h light/dark cycle and fed *ad libitum* with living *Perumytilus purpuratus*.

Experimental set-up

Freshly hatched larvae were collected in filters receiving water from the overflow of the aquaria. Since most larvae hatched at night, samples were taken every morning. Filters were cleaned every evening to ensure daily larval age did not vary by more than 12 hours (after Lovrich et al. 2003). Solely actively moving larvae were used for experiments.

A) Influence of temperature on larval survival and development

Randomly selected larvae from one randomly selected female (A) were kept individually in glass bowls containing 100 ml filtered seawater. For each experimental temperature (12, 16, 20, 22 °C) an initial number of 100 larvae was cultured in order to describe larval development and mortality. Water was changed daily; larvae were daily checked for moults or mortality and fed *ad libitum* with freshly hatched *Artemia* spp. nauplii. Survival rates and time of development for each instar was recorded. The mean duration of instars was calculated from all larvae that successfully passed through the moult into the subsequent stage.

B) Influence of temperature on elemental composition

Larvae from a randomly selected second female (B) were reared at 16, 20 and 22 °C. 12 °C as rearing temperature was excluded in this experiment, as the instar duration experiment showed extremely extended developmental times and exceptionally high mortality. Larvae were kept in 500 ml glass bowls (20 to 30 individuals/bowl) and provided with water and alimentation as described above. At each temperature an initial amount of 1000 larvae were randomly distributed into 35 glass bowls. At 20 °C, due to unforeseen technical problems, all larvae died, and a second set of experiments was conducted at 20 °C with larvae of a third female (C).

For the analyses of dry weight (W) and carbon (C), hydrogen (H) and nitrogen (N) content throughout larval development, it was inevitable to pool

specimens from the same hatch and developmental day in order to reach minimum required sample W. Each sample consisted of 30 individuals in the zoea I (Z I) on the day of hatching, but less (see Table 1) in the following days and (larger) instars, in order to obtain minimum sample W necessary for elemental analysis. Samples for elemental analyses were immediately taken after hatching and later every second day at 20 and 22 °C. At 16°C samples were taken every fourth day due to the extended developmental time at lower temperatures. Three replicates of pooled larvae from the same hatch and developmental day were collected. Number of replicates was reduced, when too few larvae were available.

Elemental analyses (CHN)

Carbon (C), hydrogen (H), nitrogen (N) contents were determined following Anger and Dawirs (1982). In brief: Larvae were gently rinsed in distilled water, blotted on filter paper, placed into pre-weighed tin cartridges and stored at -80 °C. Afterwards samples were vacuum-dried for 48 hours in a Virtis Benchtop SLC freeze dryer at -70º C and a pressure below 0.01 mbar before being stored in airtight boxes with silica gel. At the home institute samples were dried again, at 50 °C in a dry oven for 24 hours and weighed to the nearest 0.1 µg on a Sartorius M2P microbalance. CHN content was measured with a HEKAtech EURO EA 3000 CHNS-Analyzer using Acetanilid as a standard.

Statistical analyses

All data were tested with the Mahalanobis distances test (Mahalanobis 1936) to exclude outliers from analysis. The effects of temperature and instar on instar duration and carbon gain per instar were analysed with a full interaction analysis of covariance (ANCOVA). Data were Box-Cox transformed (Sokal and Rohlf 1981) to reach the best transformation to approach the normal distribution of residuals. A t-test was conducted to test if there are significant differences in the biochemical composition (C, H, N) between freshly hatched larvae of female B and C. To test whether slopes and intercepts of C % and C:N with time are significantly different from zero, we conducted a slope analyses. A general additive model (Hastie and Tibshirani 1990) was used to describe larval mass (µg C) as a function of time (t, days) and temperature (T, Kelvin):

$$C_{BC} = a + b_1 \times t + b_2 \times f(T) + b_3 \times t \times f(T) \quad [\mu g, d, K]$$

where C_{BC} is the Box-Cox transformed larval mass (Sokal and Rohlf 1981) and *f*(T) a function that models the temperature effect according to a skewed normal distribution with mean M_T, standard deviation SD_T and skewing factor SK_T following the method described in Weiss et al. (2009):

$$f(t) = 1/\left(SD_T \times \sqrt{2\pi}\right) \times e^{-0.5 \times (((T - M_T) + SK_T \times (T - M_T))/SD_T)^2} \quad \text{for T >= } M_T$$

$$f(t) = 1/\left(SD_T \times \sqrt{2\pi}\right) \times e^{-0.5 \times (((T - M_T) - SK_T \times (T - M_T))/SD_T)^2} \quad \text{for T < } M_T$$

Results

Influence of temperature on larval survival and development

Complete zoeal development occurred at 16° and 20 °C only (Figure 1). At 12° and 22 °C development proceeded until zoea IV. At all temperatures mortality was high especially during ecdysis and only few larvae reached the higher instars (zoea IV/V). At 12 °C mortality was exceptionally high in the zoea I instar. 85 % of the larvae died within the first five days after hatching. Development up to zoea IV took 84 days and another 27 days until all remaining larvae died. At 16 °C mortality in the zoea I stage was lower (33 % within 5 days) and the whole zoeal development took 84 days until the last zoea V died. At 20 °C mortality was comparatively low within zoea I (22 % within 5 days) and total development took 36 days. Zoeal development at 22 °C was fast; the last larvae died after 22 days in a zoea IV instar. Mortality in zoea I was on an intermediate level.

The mean instar duration was significantly shorter at higher temperatures ($p < 0.0001$, $F = 1230.814$, $DF = 3$,), this temperature effect is instar specific ($p < 0.0001$, $F = 377.351$, $DF = 2$,) and the interaction term between temperature and instar also had a significant influence, indicating that slopes of instar duration depending on temperature are not parallel.

Table 1. *Cancer setosus*. Changes in dry weight (W), carbon(C), nitrogen (N) and C:N ratio during time days after hatch (± SD) at four temperatures.

T [°C]	instar	ind/sample	day	W [µg]		N [µg]		C [µg]		H [µg]		C/N	
				mean	± SD	mean	± SD	mean	± SD	mean	± SD	mean	± SD
16	ZI	30	0	10.624	0.390	0.805	0.010	3.364	0.045	0.436	0.009	4.177	0.035
	ZI	25	4	20.753	0.906	1.310	0.087	6.248	0.367	0.820	0.051	4.771	0.040
	ZI	20	8	27.307	0.698	2.027	0.024	9.679	0.118	1.291	0.023	4.775	0.002
	ZI	15	12	24.136	3.675	1.679	0.093	7.212	0.195	0.899	0.027	4.308	0.344
	ZII	15	0	36.658	1.496	2.360	0.071	11.258	0.434	1.479	0.055	4.769	0.042
	ZII	15	4	54.902	0.938	3.751	0.091	18.400	0.493	2.474	0.084	4.906	0.029
	ZII	10	8	61.850	2.203	4.703	0.242	21.588	1.084	2.863	0.159	4.590	0.013
	ZII	12	12	56.700	4.268	4.251	0.396	17.553	1.730	2.322	0.245	4.128	0.023
	ZIII	10	0	63.537	3.703	4.838	0.135	21.257	1.052	2.862	0.139	4.392	0.097
	ZIII	10	4	91.073	4.707	6.312	0.416	28.434	2.325	3.913	0.386	4.502	0.109
	ZIII	10	8	94.787	3.689	6.903	0.308	29.535	1.488	4.082	0.224	4.278	0.034
	ZIII	5	12	83.780	5.082	6.044	0.448	23.967	2.139	3.139	0.320	3.963	0.062
	ZIII	5	16	73.940	1.782	5.595	0.011	22.021	0.089	2.819	0.006	3.936	0.008
	ZIV	1	0	96.000		4.908		21.645		2.210		4.410	

T [°C]	instar	ind/sample	day	W [µg]		N [µg]		C [µg]		H [µg]		C/N	
				mean	± SD	mean	± SD	mean	± SD	mean	± SD	mean	± SD
20	ZI	30	0	13.618	0.341	0.806	0.019	3.688	0.079	0.445	0.009	4.576	0.011
	ZI	25	2	24.693	0.340	1.279	0.003	6.912	0.015	0.897	0.019	5.404	0.023
	ZI	20	4	26.100	0.786	1.475	0.058	7.542	0.297	0.971	0.038	5.112	0.043
	ZI	15	6	30.689	0.847	1.941	0.111	9.970	0.476	1.284	0.070	5.139	0.053
	ZI	15	8	29.200	0.406	1.784	0.044	9.050	0.217	1.132	0.031	5.074	0.025
	ZII	15	0	37.733	1.453	2.223	0.067	11.755	0.401	1.552	0.045	5.293	0.279
	ZII	10	2	58.022	2.076	3.460	0.168	18.475	1.052	2.511	0.156	5.339	0.082
	ZII	10	4	100.400	1.323	6.624	0.142	34.702	0.965	4.842	0.159	5.239	0.041
	ZII	10	6	63.000	2.600	4.330	0.214	21.119	1.322	2.810	0.201	4.875	0.066
	ZIII	5	0	75.067	2.914	4.993	0.161	23.819	0.611	3.288	0.126	4.771	0.064
	ZIII	5	2	111.333	2.532	6.869	0.203	34.663	1.116	4.555	0.156	5.046	0.080
	ZIII	5	4	121.733	3.478	8.156	0.254	39.838	1.379	5.271	0.184	4.885	0.061
	ZIII	5	6	124.800	2.771	8.795	0.203	41.397	1.054	5.516	0.183	4.707	0.012
	ZIV	5	0	147.133	7.100	9.969	0.325	44.733	1.391	6.045	0.222	4.488	0.052
	ZIV	5	2	197.933	17.900	11.831	1.408	55.763	6.902	7.656	1.054	4.712	0.046
	ZIV	5	4	206.733	14.607	13.737	1.559	63.686	7.673	8.842	1.166	4.634	0.039
	ZIV	3	6	226.556	17.513	15.753	1.466	70.853	7.335	9.526	1.078	4.495	0.057
	ZIV	3	8	263.667	20.809	20.758	2.239	93.527	10.309	13.166	1.417	4.507	0.146
	ZV	3	0	307.222	28.578	21.302	1.467	94.240	6.722	13.390	1.038	4.423	0.026
	ZV	3	2	384.667	27.966	26.668	2.059	124.221	9.925	17.886	1.489	4.657	0.013
	ZV	3	4	400.444	50.536	29.906	4.006	132.893	21.163	19.382	2.978	4.434	0.124
	ZV	3	6	360.222	174.243	27.299	13.165	120.631	57.603	17.670	8.892	4.431	0.045
	ZV	3	8	168.667		13.793		61.377		8.284		4.450	

10

T [°C]	instar	ind/sample	day	W [µg]		N [µg]		C [µg]		H [µg]		C/N	
				mean	± SD	mean	± SD	mean	± SD	mean	± SD	mean	± SD
22	ZI	30	0	10.624	0.390	0.805	0.010	3.364	0.045	0.436	0.009	4.177	0.035
	ZI	25	2	20.693	1.054	1.331	0.049	6.075	0.299	0.786	0.044	4.565	0.073
	ZI	20	4	25.083	1.382	1.896	0.144	8.296	0.598	1.068	0.080	4.377	0.045
	ZI	15	6	23.711	0.948	1.830	0.096	7.506	0.469	0.925	0.059	4.100	0.067
	ZII	15	0	27.178	0.454	2.124	0.053	8.512	0.284	1.081	0.043	4.008	0.109
	ZII	10	2	51.511	1.699	3.522	0.190	16.304	0.683	2.178	0.097	4.631	0.062
	ZII	10	4	58.633	4.801	4.816	0.628	19.827	2.337	2.582	0.339	4.124	0.141
	ZII	10	6	52.500	2.458	3.520	0.267	17.160	1.433	2.391	0.194	4.873	0.038
	ZIII	10	0	63.833	2.230	4.066	0.126	19.503	0.568	2.730	0.092	4.797	0.066
	ZIII	5	2	93.867	2.120	5.698	0.145	29.590	0.689	4.044	0.104	5.194	0.026
	ZIII	5	4	107.333	3.911	7.186	0.354	35.599	2.011	4.931	0.269	4.953	0.070
	ZIII	5	6	117.933	3.754	8.324	0.377	40.611	1.735	5.624	0.277	4.879	0.017
	ZIV	5	0	134.867	8.011	8.415	0.230	39.200	1.147	5.458	0.124	4.661	0.201
	ZIV	5	2	156.000	6.409	9.894	0.750	46.890	4.064	6.650	0.650	4.256	0.241
	ZIV	5	4	196.400	15.565	13.565	1.456	64.325	6.670	9.226	1.010	4.743	0.080
	ZIV	3/3/2	6	161.667	26.359	10.859	2.316	51.237	12.776	6.921	1.623	4.697	0.181
	ZIV	5/1	8	121.600	31.961	6.865	4.233	32.661	16.939	4.230	2.930	4.936	0.576

Influence of temperature on elemental composition

All changes in W and CHN during the course of larval development are shown in Table 1. As an example the initial larval W for zoea I at 20 °C was 13.62 ± 0.34 µg, while a zoea V at 20 °C had a maximal dry weight of 400.44 ± 50.54 µg, which means a growth rate of 2840 %. The t-test did not show significant differences in the elemental composition between freshly hatched larvae of female B and female C.

Larval body mass (C in µg/ind) is predicted from day (t) and temperature (T °C) by the model

$$\log_e(C) = -9.575 - 10.927 \times t + 926.034 \times f(T) + 899{,}829 \times t \times f(T)$$

N = 151, F = 637.9388, R^2 = 0.93, P < 0.001 for the whole model and each term with

$$f(T) = 0.012234462 \times e^{-0.5 \times \left(\left(\left((T-21.58)+(T-21.58)+0.5\times(T-21{,}58)\right)\div 32{,}608\right)^2\right)} \quad \text{for T >= 21.58 °C}$$

$$f(T) = 0.012234462 \times e^{-0.5 \times \left(\left(\left((T-21.58)+(T-21.58)-0.5\times(T-21{,}58)\right)\div 32{,}608\right)^2\right)} \quad \text{for T < 21.58 °C}$$

The Box-Cox test found $\log_e$ to be the most appropriate transformation.

$$C_{BC} = \log_e(C)$$

Note that this model (Figure 4) predicts a larval mass for just any combination of time and temperature; whereas our experiments indicate that the "time x temperature" space where larvae do exist is limited (see discussion). Outside these margins larval development beyond zoea I is not possible (24 °C, preliminary experiments), or mortality is exceptionally high and zoeal development theoretically took several months and could not be completed (12 °C). The residual plot (Figure 5) indicates that the model fit the data well, but slightly overestimates body mass of freshly hatched larvae. In late premoult stages of higher instars, changes in carbon values were generally lower than predicted by the model (compare Figures 4 and 5).

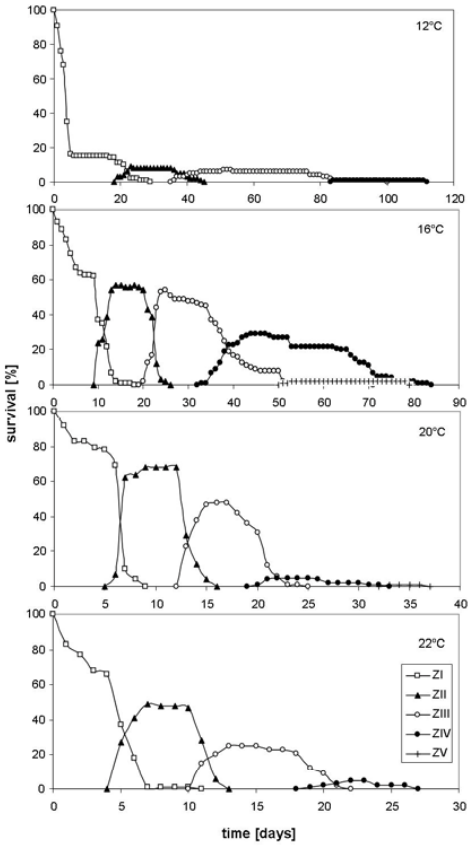

Figure 1. *Cancer setosus*. Instar duration and larval survival at four temperatures (12, 16, 20 and 22 °C). ZI – ZV are zoeal instars I – V.

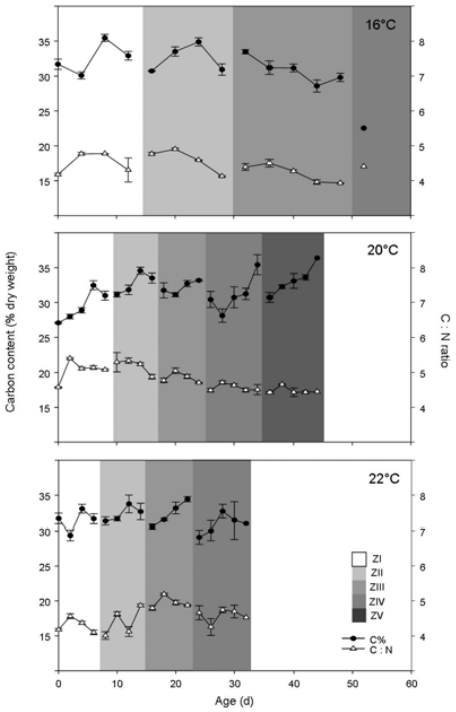

Figure 2. *Cancer setosus*. Changes during larval development in the carbon content (% of body mass, W) and in the carbon: nitrogen (C:N) weight ratio of larvae reared at three temperatures.

At 20 °C the slope of the relative carbon content (% W) (Figure 2) shows a significant positive tendency, while the slopes of 16° and 22 °C do not show significant trends. At 22 °C the slope increases within the first three zoeal instars and decreases in zoea IV. The C:N ratio generally increases during the postmoult, reaches a maximum in the intermoult and has a decreasing tendency in the premoult phase (Figure 2). The slopes of the C:N ratio over the complete recorded larval development where significantly different from zero ($p < 0.0001$). Slopes show a decrease at 16° and 20 °C, and increases at 22 °C.

The variation in growth of the single instars is shown in Figure 3, here the carbon gain in % of initial carbon per instar is shown separately (given as percentage of the initial value measured for each instar)

$$\%\ gain = \frac{\left(C\mu g \times ind^{-1}{}_{initial} - C\mu g \times ind^{-1}{}_{final}\right)}{\left(C\mu g \times ind^{-1}{}_{final} \times 100\right)}.$$

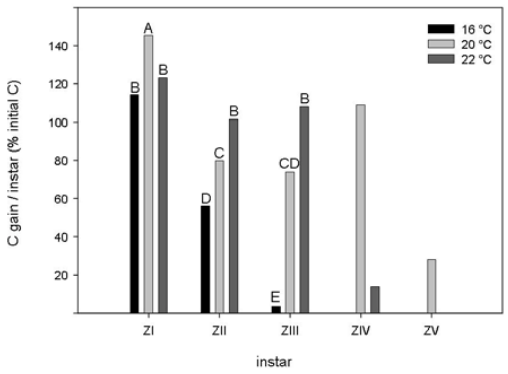

Figure 3. *Cancer setosus*. Increment in carbon (carbon gain in % of initial carbon per instar) in each instar of larvae reared at three temperatures.

The results of the ANCOVA showed that temperature ($P < 0.0001$, $F = 16883.666$, $DF = 2$) and instar ($P < 0.0001$, $F = 71.571$, $DF = 2$), as well as the interaction term ($P < 0.0001$, $F = 10634.729$, $DF = 4$) have a significant influence on the instar growth rates. The significantly highest growth rates occur in the zoea I at all three tested temperatures with the highest increment at 20 °C, followed by an intermediate growth at 22 °C and a slightly lower rate at 16 °C. In the zoea II and zoea III instar the highest growth rates occur at 22 °C, followed by an intermediate increment at 20 °C and the smallest increment at 16 °C. Data for zoea IV and V were not included in the statistical analyzes due to missing values (zoea IV, 16 °C; zoea V 16 and 22 °C), caused by death of all larvae. Nonetheless data are presented in Figure 3 to show the consecutive pattern. In the zoea IV instar larvae reared at 20 °C show a clearly higher increment than at 22 °C. The growth rate of larvae reared at 20 °C was lowest in zoea V. It has to be mentioned that we did not include the last sampling day in the graph, as at day 8 of the zoea V only few larvae were alive for sampling and we could not take any replicates.

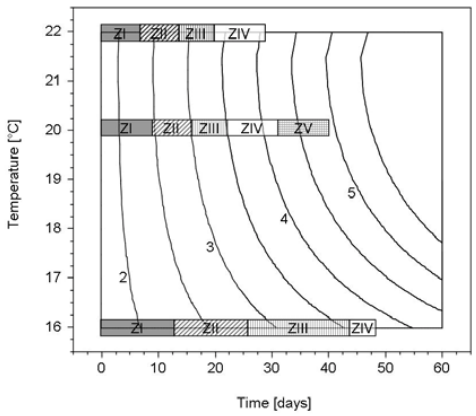

Figure 4. Model of body mass increase (C) throughout the larval development of the edible crab *Cancer setosus*. Equation for the model is: $\log_e(C)$ = -9.575 -10.927 x t +926.034 x f(T) +899.829 x t x f(T) ; N = 151, F = 637.9388, R^2 = 0.93, P < 0.001; T represents the temperature in °C and t the time in days. Isolines represent carbon content in ln µg. ZI-ZV are the larval instars.

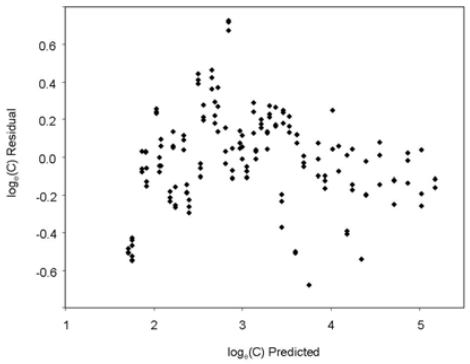

Figure 5. Residual by predicted plot from the model predicting larval body mass in *Cancer setosus*: plot of regression residuals versus predicted values.

Discussion

In the present study, complete zoeal development from hatching to zoea V was only successful within a narrow temperature range of approximately 16° and 20 °C (Figure 1). The general increase of the relative carbon content (Figure 2) combined with an decrease of the C:N ratio at 20 °C, points at lipid storage and likely construction of muscle proteins (Torres and Childress 1985). The growth of larvae measured as the increment in carbon per instar (Figure 3) is relatively high at 20 °C during each instar, but significantly the highest in the zoea I. Those findings indicate 20 °C as the optimal temperature for larvae of the Antofagasta *C. setosus* population during summer and early fall months. The temperature range of 16° to 20 °C corresponds with the normal annual temperature fluctuation in the Antofagasta region (SHOA 2008). At temperatures below or above the optimal range, development could not be completed, suggesting that lower and upper Pejus temperatures for successful zoeal development (*sensu* Pörtner 2001) are > = 12 °C and < = 22 °C, respectively. Beyond Pejus temperatures survival is

likely restricted due to a mismatch between oxygen supply and demand (Pörtner 2001).

The growth problems of larvae that occur at 16° and 22 °C are also reflected in their elemental composition. At 16 °C the overall relative carbon content decreases slightly and the C:N increases (Figure 2), because the absolute nitrogen content stays constant (Table 1). This indicates a metabolism that is mainly based on carbon, which refers to a higher overall lipid than protein metabolism. The general decrease in C (% W, Figure 2) indicates that 16 °C is below the temperature optimum for *Cancer setosus* larvae. Similar patterns were found in *Carcinus maenas* larvae suffering from starvation (Dawirs 1986), temperature (Dawirs et al. 1986) and osmotic stress (Torres et al. 2002). Here, carbon is metabolized at higher rates than nitrogen, which indicates that unfavourable conditions have comparable effects on the metabolism of larvae (Anger 2001). At 22 °C, the generally constant overall relative carbon content and the increasing C:N ratio points to protein degradation due to high metabolism, which is also described for larvae after long periods of food deprivation (Anger 2001) and likely the result of a mismatch of energy supply and energy demand.

Short term temporal changes in the relative carbon content (% W) show the typical cyclic pattern (Figure 2) (Anger 1988, Anger 2001) within individual moult cycles with decreased carbon values after ecdysis due to rapid water and mineral uptake during the initial phase of a moult cycle. One exception occurs after ecdysis from zoea II to zoea III where the carbon content is higher after moulting. The C:N (carbon : nitrogen) ratio, is an index of the lipid : protein ratio (e.g. Anger and Harms 1990, Minagawa et al. 1993). Both, lipids and proteins serve as the most important energy reserves in decapod crustaceans (Cockcroft 1997). The C:N values also shows a typical short term pattern described for several brachyurans, with low values after moulting and an increase on the following days (Dawirs et al. 1986, Anger 1988, Minagawa et al. 1993) and a slight decrease towards moulting. This indicates a higher lipid metabolism shortly after ecdysis, followed by an increasing protein accumulation.

Growth of larvae, measured as carbon increment per instar (Figure 3) at 16 °C clearly declines with instar, indicating a temperature limitation of growth. At 20 °C increment is high in each instar, but decreases in the zoea V. This instar is characterized by growth in the first four days and a following decrease in absolute

carbon values (Table 1), giving evidence that the cue for metamorphosis to the megalopa stage is missing. It is known that larvae are able to postpone their metamorphosis to the megalopa stage if the cue for the suitable habitat can not be detected (Krimsky and Epifanio 2008), but the enduring lack of those cues cause stress and the depletion of resources. We conducted an additional preliminary experiment following Forward et al. (2001) to detect potential chemical or physical cues for metamorphosis with exudates of adults, rubble, macroalgae, shells and mesh. None of the larvae moulted successfully to the megalopa stage. Another reason that moulting to the megalopa fails might be that *Artemia* spp. nauplii are an insufficient food source for higher zoeal instars, although this is unlikely as growth was successful during the first days and larvae are known to stop food uptake shortly before ecdysis (Al-Mohanna and Nott 1989). Successful metamorphosis has never been achieved in any cancrid larva to date and requires much closer investigation (Quintana and Saelzer 1986, Anger 2001, Weiss et al. 2009).

Instar dependent increment is highest at 20 °C in the zoeal I instar, but in zoea II and III maximal growth can be detected at 22 °C. In contrast in the zoea IV instar the increment is clearly higher at 20 °C than at 22 °C. According to these changes in the maximum growth in successive instars, *C. setosus* larvae show an ontogenetic shift in the instar specific temperature optimum (Anger 2001). While in the zoea I and II instar growth is visible at all tested temperatures (Figure 3), a steep drop in growth occurs in the zoea III at 16 °C and in the zoea IV at 22 °C. These findings indicate that lower instars are more temperature tolerant. Shifts in the temperature preference of larvae can be due to seasonal increase in water temperature during larval development as described for *Hyas araneus* larvae by Anger (2001). Since *C. setosus* in northern Chile is egg-bearing all year round (Fischer and Thatje 2008), it is unlikely that this shift in temperature optimum is due to seasonal acclimation. It is known that larval crabs migrate vertically and that these movements change during ontogeny (Shanks 1986, Hobbs and Botsford 1992), but no consistent pattern within a genus could be found. Therefore, different temperature optima during ontogeny might reflect the preference of water masses with different temperatures dependent on the instar.

The positive temperature effect described for the time of development of larvae is also reflected in the growth model (Figure 4). Beyond Pejus temperatures

a reduced feeding activity in the cold and an unbalanced equilibrium between ingestion rates and maintenance costs at warm temperatures lead to reduced growth rates and cause the death of larvae. Herein, the most vulnerable part of the larval moult cycle is the transition from one instar to the next (also obvious in survival rates, see Figure 1) (Anger 2001). The residual plot (Figure 5) shows a general good fit of the model (i.e. randomly distribution of residuals) and slightly lower carbon values in higher instars (zoea V) than predicted by the model (Figure 4), which might be due to problems during metamorphosis to the megalopa stage.

In comparison with findings of Quintana (1986), who reared *C. setosus* larvae of a population from Coliumo Bay (Concepción, central Chile) under natural temperature conditions (13.5 - 14.6 °C), larvae from the Antofagasta region have a 1.5 times extended zoeal development at 16 °C (Figure 1) and show higher survival rates, while larvae from Concepción are capable to develop through all zoeal instars at lower temperatures. These findings indicate a better cold adaptation of larvae originating from a southern population (see also Fischer and Thatje 2008, Fischer et al. 2009). This adaptive variation in growth has been described for several ectotherms like fishes (Yamahira and Conover 2002), Polychaeta (Levinton 1983) and Crustacea (Lonsdale and Levinton 1985). Additionally those findings show that not only egg-size, reproductive output per egg batch (Brante et al. 2003) and oviposition (Fischer and Thatje 2008) but also larval growth follows a latitudinal cline. This indicates that the species temperature tolerance window is much wider than the window of one single population, and this knowledge is important for a successful adaptive fisheries managing during and post-El Niño.

Conclusion

The optimum temperature for larval development of *C. setosus* from the Antofagasta population lies between 16° and 20 °C. Higher and lower temperatures cause changes in the elemental composition, which reflects a lower metabolic efficiency. A narrow temperature tolerance window throughout early ontogeny is surprising in a species that faces strong temperature oscillations beyond its upper thermal tolerance limit during conditions of El Niño. Future experiments should reveal if the temperature optimum is genetically defined or if

the temperature experienced at embryonic development is crucial for temperature preference in later life.

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2.3 Publication III

Temperature effects on zoeal morphometric traits and intraspecific variability in the hairy crab *Cancer setosus* across latitude

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ORIGINAL ARTICLE

Temperature effects on zoeal morphometric traits and intraspecific variability in the hairy crab *Cancer setosus* across latitude

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Abstract Phenotypic plasticity is an important but often ignored ability that enables organisms, within species-specific physiological limits, to respond to gradual or sudden extrinsic changes in their environment. In the marine realm, the early ontogeny of decapod crustaceans is among the best known examples to demonstrate a temperature-dependent phenotypic response. Here, we present morphometric results of larvae of the hairy crab *Cancer setosus*, the embryonic development of which took place at different temperatures at two different sites (Antofagasta, 23°45′ S; Puerto Montt, 41°44′ S) along the Chilean Coast. Zoea I larvae from Puerto Montt were significantly larger than those from Antofagasta, when considering embryonic development at the same temperature. Larvae from Puerto Montt reared at 12 and 16°C did not differ morphometrically, but sizes of larvae from Antofagasta kept at 16 and 20°C did, being larger at the colder temperature. Zoea II larvae reared in Antofagasta at three temperatures (16, 20, and 24°C) showed the same pattern, with larger larvae at colder temperatures. Furthermore, larvae reared at 24°C, showed deformations, suggesting that 24°C, which coincides with temperatures found during strong EL Niño events, is indicative of the upper larval thermal tolerance limit. *C. setosus* is exposed to a wide temperature range across its distribution range of about 40° of latitude. Phenotypic plasticity in larval offspring does furthermore enable this species to locally respond to the inter-decadal warming induced by El Niño. Morphological plasticity in this species does support previously reported energetic trade-offs with temperature throughout early ontogeny of this species, indicating that plasticity may be a key to a species' success to occupy a wide distribution range and/or to thrive under highly variable habitat conditions.

Keywords Brachyura · Early ontogeny · Morphological variability · El Niño · Humboldt Current · Chile

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Introduction

Phenotypic plasticity in larval offspring of invertebrates has often been discussed as an important mechanism to respond to sudden changes in their habitat, namely temperature and food conditions (e.g. Criales and Anger 1986; Shirley et al. 1987). However, quantitative experimental studies on this subject are scarce and most insight is probably available from experimental laboratory studies of decapod crustaceans. There is evidence that phenotypic conditions of larvae may be indicative of their energetic status or conditions experienced during oogenesis like contrasting habitats (Silva et al. 2009) and including across geographic temperature gradients (Wehrtmann and Albornoz 1998; Wehrtmann and Kattner 1998; Thatje and Bacardit 2000; Giménez 2002). Most of this knowledge is limited to studies of caridean shrimp larvae (i.e. Criales and Anger 1986; Wehrtmann 1991), which may

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Temperature effects on zoeal morphometric traits and intraspecific variability in the hairy crab *Cancer setosus* across latitude

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Abstract

Phenotypic plasticity is an important but often ignored ability that enables organisms, within species-specific physiological limits, to respond to gradual or sudden extrinsic changes in their environment. In the marine realm, the early ontogeny of decapod crustaceans is among the best known examples to demonstrate a temperature dependant phenotypic response. Here, we present morphometric results of larvae of the hairy crab *Cancer setosus*, whose embryonic development took place at different temperatures at two different sites (Antofagasta, 23°45' S; Puerto Montt, 41°44' S) along the Chilean Coast. Zoea I larvae from Puerto Montt were significantly larger than those from Antofagasta, when considering embryonic development at the same temperature. Larvae from Puerto Montt reared at 12 and 16 °C did not differ morphometrically, but sizes of larvae from Antofagasta kept at 16 and 20 °C did, being larger at the colder temperature. Zoea II larvae reared in Antofagasta at three temperatures (16, 20, and 24°C) showed the same pattern, with larger larvae at colder temperatures. Furthermore larvae reared at 24 °C, showed deformations, suggesting that 24 °C, which coincides with temperatures found during strong EL Niño events, is indicative of the upper larval thermal tolerance limit. *C. setosus* is exposed to a wide temperature range across its distribution range of about 40° of latitude. Phenotypic plasticity in larval offspring does furthermore enable this species to locally respond to the inter-decadal warming induced by El Niño. Morphological plasticity in this species does support previously reported energetic trade-offs with temperature throughout early ontogeny of this species, indicating that plasticity may be a key to a species' success to occupy a wide distribution range and/or to thrive under highly variable habitat conditions.

Keywords Brachyura, early ontogeny, morphological variability, El Niño, Humboldt Current, Chile

Introduction

Phenotypic plasticity in larval offspring of invertebrates has often been discussed as an important mechanism to respond to sudden changes in their habitat, namely temperature and food conditions (e.g. Criales and Anger 1986; Shirley et al. 1987). However, quantitative experimental studies on this subject are scarce and most insight is probably available from experimental laboratory studies of decapod crustaceans. There is evidence that phenotypic conditions of larvae may be indicative of their energetic status or conditions experienced during oogenesis like contrasting habitats (Silva et al. 2009) and including across geographic temperature gradients (Wehrtmann and Albornoz 1998; Wehrtmann and Kattner 1998; Thatje and Bacardit 2000; Giménez 2002). Most of this knowledge, is limited to studies of caridean shrimp larvae (i.e. Criales and Anger 1986; Wehrtmann 1991), which are known to may express high variability in for example larval developmental pathways, number of instars, and size and energy contents in response to food and temperature condition. In comparison, and despite known energetic trade-offs in crab eggs, phenotypic plasticity in crab larvae is generally assumed to be more conservative; a view that might be largely driven by the lack of sufficient information available to date (Anger 2001) and that has already been disproved for example for *Neohelice* (formerly *Chasmagnathus*) *granulata* (Pestana and Ostrensky 1995; Cuesta et al. 2002).

The hairy crab *Cancer setosus* (Molina 1782; synonymous *C. polyodon* Poeppig 1836) ranges in its distribution from Guayaquil in Ecuador (2°13' S, 79°53' W) to the Peninsula of Taitao in Southern Chile (46°00' S, 75°00' W) (Garth and Stephenson 1966) and is of commercial value to local artisanal fisheries (Wolff and Soto 1992; SERNAPESCA 2006; Thatje et al. 2008). The early ontogeny, which in *C. setosus* consists of five planktotrophic zoeal and one megalopa stage before reaching the first crab stage (Quintana and Saelzer 1986), is considered the most delicate part within the life cycle of brachyuran and in particular cancrid crabs (Anger 2001; Weiss et al. 2009a, b). A unique physiological plasticity to respond to latitudinal and seasonal changes in temperature has been observed in reproductive traits of *C. setosus*. Among the most conspicuous characteristics is a synchronization of a single egg batch release with seasonality at the species southernmost distribution boundary in central southern Chile (Puerto Montt, 41°44' S), contrasted by multiple annual

ovipositions in northern Chile (Antofagasta, 23°45' S) (Fischer and Thatje 2008). In addition, a correlation of changes in egg energy contents and temperature was observed along latitude as well as in subsequent ovipositions in the same female (Fischer and Thatje 2008; Fischer et al. 2009a). These findings are consistent with the concept of a latitudinal gradient in energy provisioning into offspring of a broad variety of marine invertebrates (for review see Thatje et al. 2005). The underlying pattern of larger eggs with better energy provisioning at colder temperatures (higher latitudes) is discussed to be an adaptive response to limited food supply and longer pelagic life stage phases (Thatje et al. 2005). The latitudinal cline in energy provisioning of eggs therewith corresponds with the energy demand of the embryo. Egg development is generally faster at higher temperatures, but causes an altered metabolic efficiency often resulting in a size reduction of later life stages. This has been shown for the Dungeness crab, *Cancer magister* (Shirley et al. 1987), the spider crab, *Hyas araneus* (Kunisch and Anger 1984) as well for caridean shrimps like *Nauticaris magellanica* (Wehrtmann and Kattner 1998), *Betaeus emarginatus* (Wehrtmann and Lopez 2003), and *Pandalus borealis* (Brillon et al. 2005) in laboratory cultures, and for *Nauticaris magellanica* from the field (Thatje and Bacardit 2000). In *C. setosus* eggs from a southern Chilean population were larger containing higher lipid content, resulting in bigger larvae than eggs from northern Chile (Fischer et al. 2009b). Egg development in the southern population is primarily based on the metabolism of energy rich lipids, which finally enhances the energetic fitness of the hatching larvae, and which has been discussed as an adaptive response to colder temperatures and prolonged egg and larval developmental time (Fischer et al. 2009b).

The size and elemental composition of eggs and hatchlings itself has been subject to some studies, but fewer studies have addressed the question of phenotypic effects on larvae (Shirley 1987; Thatje and Bacardit 2000; Wehrtmann and Albornoz 2003). Following the assumption that larval size will follow the known latitudinal cline in egg size, we hypothesize that larvae from Puerto Montt have larger sizes and tested whether the temperature experienced during embryonic development triggers phenotypic plasticity of the zoeal stages in this species.

Materials and methods

All samples are reference samples of parallel experiments. For further explanations of the experimental setup see Table 1.

Table 1. *Cancer setosus*. Origin of samples with reference to related works. (A=Antofagasta; PM=Puerto Montt)

female ID	experimental site	rearing temperature °C	date	experiment	reference samples for
A+B	A	16	02/2007	larval development	Weiss et al. 2009a
C+D	A	16	11/2005	embryonic development	Fischer et al. 2009a
E+F	A	20	01/2006	embryonic development	Fischer et al. 2009a
G+H	PM	12	09+11/2006	embryonic development	Fischer et al. 2009a
I+J	PM	16	09+11/2006	embryonic development	Fischer et al. 2009a

Sampling and maintenance of adults

Fishermen caught ovigerous *Cancer setosus* by scuba diving at different sites around Antofagasta, Northern Chile (23°45' S, 70°27'W; November 2005, January 2006, February 2007) and in Carelmapu, close to Puerto Montt in Central Southern Chile (41°44'S, 73°41'W, September and November 2006). Crabs were immediately transferred to the laboratory and only crabs with eggs in the early blastula stage were considered for subsequent experiments. Sampling sites were chosen for representing the upper and lower temperature conditions encountered by *C. setosus*, which roughly range from 10 - 20 °C mean annual sea surface temperature (SST) throughout its geographical range (Fischer 2009). Puerto Montt is located close to the southern limit of this species. In Antofagasta Bay, SST is significantly higher than in the surrounding Humboldt Current upwelling system (+ 2 – 3 °C) due to the bay's particular oceanographic conditions (Piñones et al. 2007), and thus SST is comparable to the temperature encountered by *C. setosus* at its northern distributional limit off Ecuador (Fischer et al. 2009b).

Two females (IDs A, B) (influence of temperature encountered during larval development) in Antofagasta were maintained individually in flow-through seawater aquaria (12 l) at ambient temperature ~16.0 °C, while four females (IDs C, D, E, F) (influence of temperature encountered during embryonic development on larval morphology) were held in 3200 l flow-through aquaria (≤ 12 ind. / basin) under natural

seasonal temperature conditions (up to 10 months; 16 – 23 °C) in Antofagasta. Larval samples of females C and D were taken immediately after hatching at 16 °C in November 2005 and larval samples E and F were taken immediately after hatching at 20 °C in January 2006. In Puerto Montt, four females (IDs G, H, I, J) where held in 500 l aquaria (≤ 9 ind. / basin). Here, temperatures were kept constant at 12 (IDs G, H) and 16 °C (IDs I, J) in order to simulate the temperature conditions at oviposition and embryonic development from Northern and Southern Chile. The whole embryonic development of the eggs from the early blastula stage on took place at the respective experimental temperature. All females were kept in seawater at a salinity of 34, in a 12:12-h light/dark cycle and were fed *ad libitum* with living *Perumytilus purpuratus*. The aquaria were cleaned and water temperature was recorded daily.

Rearing of larvae

Freshly hatched larvae were collected in filters receiving water from the overflow of the aquaria. Since most larvae hatched at night, samples were taken every morning. Filters were cleaned every evening to ensure daily larval age did not vary by more than 12 hours (after Lovrich et al. 2003). For the experiment on the influence of temperature encountered during embryonic development on larval morphology, samples of freshly hatched, actively moving larvae were taken in Antofagasta (females A-F) and Puerto Montt (females G-J) (Tab. 1). For the experiment of the influence of temperature on larval development, solely actively moving larvae of females A and B (Antofagasta) were transferred to bowls with 16 °C filtered seawater and afterwards allowed to acclimate to the experimental temperatures (16, 20, 24 °C). Samples were taken at the first day of each available instar. We were only able to get Zoea II and Zoea III larvae from each experimental temperature, because unfavorable temperatures lead to high mortality rates of *C. setosus* larvae (Weiss et al. 2009a, b). Zoea IV and V larvae were only available at the optimum temperature of 20 °C (Weiss et al. 2009a). Larvae were transferred into Eppendorf caps with 4% formalin buffered seawater. Samples were transferred to the Alfred Wegener Institute, Bremerhaven, for further analyses.

Larval morphometry

Digital pictures (Color view soft imaging system) of each larva were taken using a stereo-microscope (Leica $MZ12_5$). Images where processed with the programme Cell* imaging software for life sciences microscopy (Olympus Soft

Imaging Solutions GmbH). All measurements (Fig. 1) were taken along the dorsal surface of the morphological feature.

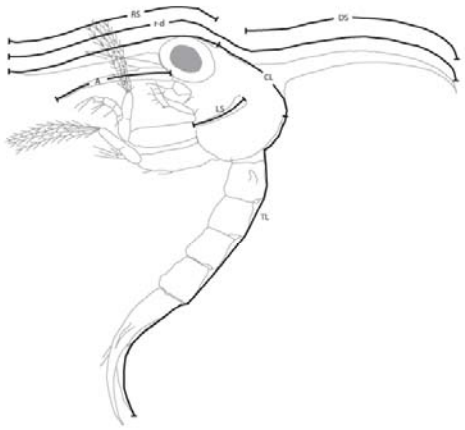

Figure 1. *Cancer setosus*. Zoea I larvae. Thick black lines indicate measured parameters. TL = total body length, r-d = rostral – dorsal distance, RS = rostral spine, DS = dorsal spine, LS = lateral spine, A = antenna

Zoeal total length (TL) was measured from the telson furca along the dorsal curvature to the tip of the rostral carapace spine. The antenna was measured from its tip to the point of attachment at the carapace.The rostral carapace spine was measured from its tip to its basal indentation of the spine between the eyes. The dorsal and lateral spines were measured from the point of attachment of the carapace to the distal tip. The carapace length (CL) was measured from the base of the rostrum to the posterior margin of the carapace. Additionally, the distance between the tip of the rostral and the tip of the dorsal spine was measured (r-d distance). The number of measured individuals per feature is given in tables 2 and 3.

Statistical analyses

All data were tested with the Mahalanobis distances test (Mahalanobis 1936) to exclude outliers from analysis. Ratios were transformed with an arcsin transformation prior analysis. The effects of temperature and location on instar characteristics were analysed with analysis of variance (ANOVA). Normal distribution of the data set was tested with a Saphiro-Wilk W test. Post-hoc tests were conducted with the student's t-test.

Results

Geographic differences

Freshly hatched Zoea I larvae of females reared at 16 °C in Puerto Montt (southern Chile) were significantly larger in all measured parameters than Zoea I larvae of females reared at 16 °C in Antofagasta (northern Chile) (Fig. 2).

In Puerto Montt, TL of freshly hatched Zoea I larvae was 13% longer ($p = 0.0031$, $f = 9.55$); the rostral spine was 5% longer ($p = 0.0093$, $f = 7.24$), the dorsal spine was 7 % longer ($p < 0.0001$, $f = 18.98$), the lateral spine was 4% longer ($p = 0.0088$, $f = 7.37$), the r-d was 17% longer ($p < 0.0001$, $f = 52.86$), the carapace was 10% longer ($p < 0.0001$, $f = 26.68$) and the antenna was 4% longer ($p = 0.0007$, $f = 12.76$) than in Antofagasta (Fig. 2). The ratio of the measured parameters versus the carapace length (CL) revealed significant differences between the two locations.

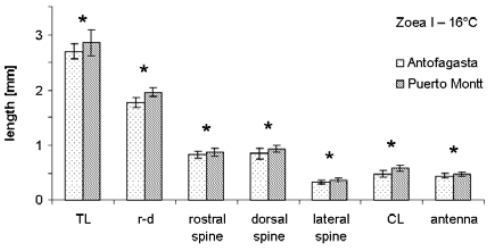

Figure 2. *Cancer setosus*. Morphometric measurements of Zoea I larvae hatched from eggs incubated at 16°C in Antofagasta (23°45' S) and Puerto Montt (41°44' S), Chile. Measures given are: Total Length (TL), the distance between the tip of the rostral and the tip of the dorsal spine (r-d), lengths of the rostral, dorsal, and lateral spine. For details on measurements see materials and methods section. All measured parameters were significantly different ($p < 0.01$), * = significant difference.

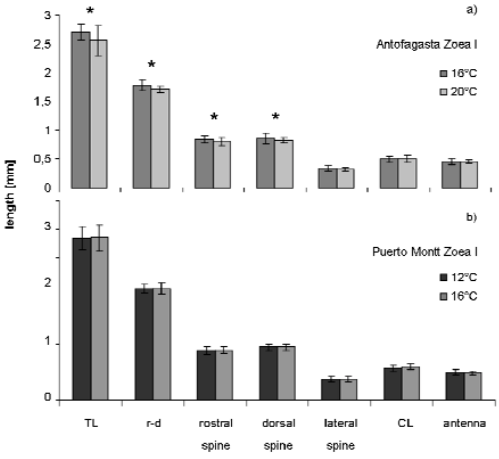

Figure 3. *Cancer setosus*. Morphometric measurements of Zoea I larvae hatched from eggs incubated at a) 16 and 20 °C in Antofagasta (23°45' S), and b) 12 and 16 °C in Puerto Montt (41°44' S), Chile. Measures given are: Total Length (TL), the distance between the tip of the rostral and the tip of the dorsal spine (r-d), lengths of the rostral, dorsal, and lateral spine. For details on measurements see materials and methods section ($p < 0.05$, * = significant difference).

The TL:CL ($p = 0.0001$, $f = 17.09$), the r-d:CL ($p < 0.0001$, $f = 2129.98$), the RS:CL ($p = 0.0003$, $f = 14.66$), the DS:CL ($p < 0.0345$, $f = 4.70$) and the Antenna:CL ($p < 0.0001$, $f = 1335.30$) were significantly longer in larvae from the Antofagasta region (Tab. 4).

Temperature effect during embryonic development

Freshly hatched Zoea I larvae which experienced embryogenesis at 16 and 20 °C in Antofagasta (Fig. 3a) revealed that larvae with embryonic development at colder temperatures have significantly (16%) longer total length ($p = 0.0082$, $f = 7.50$), a 7% longer rostral spine ($p = 0.0279$, $f = 5.09$), a 8% longer r-d distance ($p = 0.0049$, $f = 8.59$) and a

3% longer dorsal spine (p = 0.0226, f = 5.49). The ratio of appendages and CL showed that freshly hatched Zoea I reared at 20 °C had slightly shorter spines than larvae reared at 16 °C, this effect was significant (p = 0.0203, f = 5.71) in the TL:CL ratio (Tab. 4). Freshly hatched Zoea I larvae from Puerto Montt in contrast, which experienced embryogenesis at 12 and 16 °C did not show any significant differences in the measured parameters (total length, r-d distance, rostral spine, dorsal spine, lateral spine, antenna) (Fig. 3b) or in the calculated appendages versus carapace length ratios.

Temperature effect during larval development

In Antofagasta, Zoea II larvae reared at 16, 20, and 24 °C showed significant differences in larval TL (p = 0.0468, f = 3.40), the length of the rostral (p = 0.0009, f = 8.76) and dorsal spine (p = 0.0014, f = 8.13), the r-d distance (p = 0.0001, f = 12.07) and the CL (p = 0.0072, f = 5.64) (Fig. 4a). In those differing parameters larvae reared at 24 °C were significantly smaller than larvae at 16 and 20 °C, except for the larval TL at 16 °C, which showed an intermediate TL to that found at 16 and 24 °C (Fig. 4a). The appendage versus carapace length ratios showed that the DS:CL was significantly smaller (p = 0.023, f = 4.24) in Zoea II larvae reared at 24 °C (Tab. 4). Additionally those larvae showed deformations of the carapace and the dorsal spine; the dorsal spine is not broken, but reduced and the carapace shows lateral bulging (Fig. 5). Zoea III larvae were only available at 16 and 20 °C, because all larvae maintained at 24 °C died during ecdysis, as this is known to be the most critical point during the larval cycle (Anger 2001, Weiss et al. 2009a). Zoea III larvae reared at 20 °C were significantly smaller than those reared at 16 °C, as evident in total length

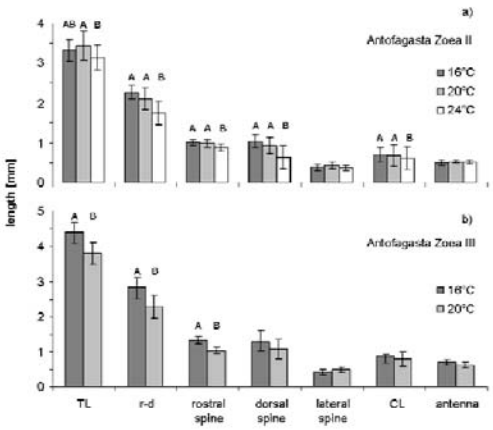

Figure 4. *Cancer setosus.* Morphometric measurements of zoeal stages for a) Zoea II larvae incubated at 16, 20, and 24 °C and b) Zoea III larvae incubated at 16 and 20 °C in Antofagasta (23°45' S), Chile. Measures given are: Total Length (TL), the distance between the tip of the rostral and the tip of the dorsal spine (r-d), lengths of the rostral, dorsal, and lateral spine. For details on measurements see materials and methods section. Significant differences are marked with different letters (ZII: p < 0.01, ZIII: p < 0.05).

Table 2. *Cancer setosus*. Female identifications, and morphometric measures of Zoea I larvae hatched at different incubation temperatures in Antofagasta (A) (23°45' S), and Puerto Montt (PM) (41°44' S), Chile. Measures given are: Total Length (TL), the distance between the tip of the rostral and the tip of the dorsal spine (r-d), lengths of the rostral, dorsal, lateral spine, carapace length (cl) and antenna length. For details on measurements see materials and methods section; N=10 in each treatment.

location	T [°C]	female	TL [mm]		r-d [mm]		rostral spine [mm]		dorsal spine [mm]		lateral spine [mm]		CL [mm]		antenna [mm]	
		ID	mean	± SD	mean	± SD	mean	± SD	mean	± SD	mean	± SD	mean	± SD	mean	± SD
A	16	A/B/C/D	2.56	0.26	1.71	0.05	0.80	0.07	0.83	0.05	0.32	0.03	0.49	0.01	0.45	0.03
	20	E/F	2.70	0.14	1.78	0.10	0.84	0.06	0.85	0.09	0.34	0.05	0.50	0.01	0.45	0.05
PM	12	G/H	2.85	0.20	1.96	0.07	0.87	0.06	0.94	0.06	0.39	0.05	0.57	0.01	0.50	0.06
	16	I/J	2.85	0.23	1.96	0.09	0.89	0.07	0.94	0.06	0.37	0.05	0.59	0.01	0.49	0.04

Table 3. *Cancer setosus*. Morphometric measures of Zoea II (ZII), Zoea III (ZIII), Zoea IV (ZIV) and Zoea V (ZV) larvae reared at different incubation temperatures in Antofagasta (A) (23°45' S), Chile. Measures given are: Total Length (TL), the distance between the tip of the rostral and the tip of the dorsal spine (r-d), lengths of the rostral, dorsal, lateral spine, carapace length (cl) and antenna length. For details on measurements see materials and methods section.

instar	T [°C]	n	incubation time [days]	TL [mm]		r-d [mm]		rostral spine [mm]		dorsal spine [mm]		lateral spine [mm]		CL [mm]		antenna [mm]	
				mean	± SD	mean	± SD	mean	± SD	mean	± SD	mean	± SD	mean	± SD	mean	± SD
ZII	16	16	10-23	3.43	0.26	2.27	0.17	1.01	0.08	1.03	0.16	0.38	0.07	0.70	0.02	0.50	0.06
	20	18	7-17	3.43	0.36	2.10	0.26	0.99	0.01	0.92	0.20	0.42	0.09	0.68	0.02	0.52	0.03
	24	21	6-18	3.15	0.32	1.74	0.29	0.88	0.01	0.63	0.30	0.36	0.07	0.61	0.02	0.52	0.04
ZIII	16	8	23-34	4.39	0.30	2.84	0.27	1.34	0.10	1.29	0.33	0.42	0.07	0.88	0.04	0.70	0.06
	20	3	16-28	3.80	0.31	2.29	0.34	1.04	0.10	1.08	0.28	0.48	0.07	0.80	0.06	0.62	0.08
ZIV	20	14	25-33	5.85	0.49	3.94	0.34	1.71	0.10	1.83	0.24	0.44	0.10	1.26	0.14	0.82	0.11
ZV	20	6	27-39	7.69	0.34	4.83	0.31	2.01	0.10	2.21	0.21	0.42	0.13	1.47	0.16	0.99	0.13

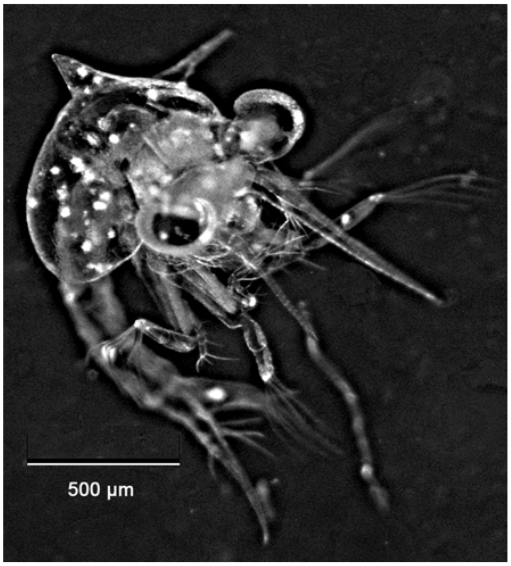

Figure 5. *Cancer setosus*. Zoea III larvae reared at 24 °C, showing a reduced dorsal spine and lateral bulging of the carapace.

(p = 0.0159, f = 8.39), r-d distance (p = 0.0162, f = 8.33) and rostral spine (p = 0.0011, f = 20.46) (Fig. 4b). The appendage versus carapace length ratios showed that larvae reared at 20 °C have shorter appendages, but this was only significant in the RS:CL (p = 0.0196, f = 6.64).

Discussion

In the present study, the intraspecific variability of morphological characteristics of *Cancer setosus* early zoeal stages in relation to rearing temperature and geographical origin is presented. Early zoeal stages from the southern region, where temperatures are colder (SST 10 - 16 °C) (mean values of the years 1982 - 2006, SHOA 2009) were significantly larger than larvae from the northern population (SST 16 - 20 °C). Latitudinal variation (accompanied by variation in temperature) is generally known to lead to higher growth efficiencies at lower temperatures (e.g.: Clarke 1983; Clarke (2003); Heilmayer et al. (2004)). Animals from colder regions have to invest less energy in the maintenance of the standard metabolism and therefore can direct more energy into growth. Fischer et al. (2009a) suggested that blastula eggs of *C. setosus* at Puerto Montt at 12 °C were provided by 30% more fatty acids as blastula eggs produced in Antofagasta at 20 °C, and larvae utilize a higher percentage of fatty acids in colder temperatures. Indeed, these findings may indicate that a metabolism based on fatty acids might produce larger larvae. Surprisingly, the appendages of freshly hatched Zoea I larvae from the Antofagasta region were relatively longer than those in Puerto Montt. This might be explained by a thermal acclimation of larvae. In the Antofagasta region 16 °C can already be regarded as a cold temperature, where the formation of longer spines can serve as a protection against predators during the elongated pelagic life phase. Another explanation would be that instead of an increase in size, warm temperatures might result in the rapid formation of

morphological structures due to a certain imbalance between development and growth (Wehrtmann and López 2003 and publications cited therein).

A comparison of the morphology of freshly hatched Zoea I larvae of females from Puerto Montt reared at 12 ° and 16 °C did not show any differences in total length, r-d distance, rostral spine, dorsal spine, lateral spine, carapace length, antenna or in the calculated ratios of appendages versus carapace length. The energy provisioning with higher lipid content of larvae from the south seems to act as a protection shield not only for unfavourable conditions like food shortage but also makes larvae more tolerant to temperature changes. Freshly hatched Zoea I larvae from the northern region in contrast show a morphological variability when embryonic development took place at comparable temperature ranges. Again, larvae reared at colder temperatures were larger indicating that development that is mainly based on protein depletion may be more sensitive to temperature changes. Differences in the morphological characteristics are especially pronounced in TL, the rostral spine, and the r-d distance. All these parameters include the rostral spine. Shirley et al. (1987) showed that a subtraction of the rostral spine from parameters measured prohibit significant differences. This is not the case in the present study but nonetheless the characteristic of the rostral spine seems to be sensitive to temperature.

A differentiation of two different species of *Pachygrapsus* was not possible by means of morphological traits in larvae, but through adult morphology and genetic analyses (Schubart et al. 2005). Larvae in this study from different oceans and coasts (East Pacific, West Atlantic east Atlantic) but from similar temperature regimes showed highest similarity in morphometric traits. These examples may suggest that within limits temperature has a more pronounced effect on larval morphology than genetic deviation. This is supported by the circumstance that *C. setosus* exhibits very little genetic diversity along its entire distribution range (Gomez-Uchida et al. 2003).
The comparison between Zoea II larvae reared at different temperatures showed that warmer temperatures lead to significantly smaller larvae, independent of the instar, at 24 °C, a temperature typically found during strong El Niño events (SHOA 2009). These observations coincide with the approximate upper thermal tolerance threshold suggested for larvae of the species (Weiss et al. 2009b).

Regarding the r-d distance measured by Quintana and Saelzer (1986) from larvae of the region of Concepción it is apparent that although Concepción is 13° south of Antofagasta and water temperatures are lower (11 -14 °C), larvae measured

in that study seem to be generally smaller. This might be due to the measurement technique used in their study. In the present study the r-d distance was measured along the curvatures, whereas Quintana and Saelzer (1986) measured the straight distance between both spine tips (see Quintana 1981).

A comparison with cancrid decapods from the North Sea, *Cancer pagurus*, shows that larvae of this congener hatch with much larger sizes (~2.5 mm r-d distance) (Ingle 1981; Hartnoll and Mohamedeen 1987) than larvae of *C. setosus* over its distributional range, but the final zoeal instar (Zoea V) of larvae reared at 20 °C in the present study has nearly the same size in both species (~ 4.83 mm). This is also reflected in the cumulative growth rates measured in carbon gain (from ZI to ZV) (Weiss et al. 2009a, b). Those are exceptionally high for *C. setosus* (2940%) and comparably low for *C. pagurus* (876%).

Morphological variability in decapod larvae has been frequently discussed as an ecological strategy and adaptation to changing environmental conditions (Anger 2001). Those energy saving traits of producing larger eggs and early larval instars at higher latitudes (here = colder temperatures) once more support the controversially discussed but often supported “Thorson’s rule” (Mileikovsky 1971, Clarke 1993, Thatje and Bacardit 2000) that appears to be particularly useful for the evaluation of energetic trait-offs in decapods (Thatje et al. 2003). Fischer et al. (2009b) found more energy rich fatty acids in the southern than in the northern population eggs, independent of the incubation temperature. This might indicate a genetic adaptation to colder temperatures and might be a selection in the direction of non feeding larvae and hence towards lecithotrophic development, which is indicating the beginning of an abbreviated development (Thatje et al. 2005). It is widely accepted, that a development with many larval stages represents a phylogenetically ancestral state, while an abbreviation of the larval stage is said to be derived (Strahmann 1978, Anger 2001). Especially in an unfavourable habitat for planktotrophic, extended larval phase (short period of food availability, temperatures close to the lower limit, high predation pressure) an abbreviated larval development would enhance survival rates. Quintana and Saelzer (1986) showed that zoeal development of *C. setosus* larvae of a population from Coliumo Bay (Concepción, central Chile) takes about 60 days under natural temperature conditions (13.5 - 14.6 °C), while larvae from the Antofagasta region have a 1.5 times exceeded zoeal development at 16 °C and show higher survival rates (Weiss et al. 2009b), and larvae from Concepcion are capable to

develop through all zoeal instars at lower temperatures. These findings indicate a better cold adaptation of larvae originating from a southern population (see also Fischer and Thatje 2008, Fischer et al. 2009a) and from an evolutionary point of view could be considered ancestral to further abbreviated larval developments (Strathmann 1978), which is favourable for larvae from colder regions.

Although acclimation to colder temperatures leads to a relatively short larval development, the pelagic phase might still be longer in the southern population, where larvae are subject of predation for a longer period of time. However, a synergetic effect of a larger total length and elongated spines in colder regions might be that larvae stand better chances for survival by being protected from certain predators.

The herein reported phenotypic plasticity in early zoeal larvae enables this species to locally respond to some extend to the inter-decadal warming induced by El Niño, but it should be underlined that strong EN events exceed the upper temperature threshold in this species and are lethal for larval instars.

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2.4 Publication IV

Physiological capacity of *Cancer setosus* larvae – adaptation to El Niño Southern Oscillation conditions

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Physiological capacity of *Cancer setosus* larvae - adaptation to El Niño Southern Oscillation conditions

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Abstract

Temperature changes during ENSO challenge the fauna of the Pacific South American coast. In many ectotherm benthic species pelagic larvae are the most important dispersal stage, which are, however, particularly vulnerable to such environmental stress. Thermal limitation in aquatic ecotherms is hypothesized to be reflected first in the aerobic scope of an animal. Here we present results on whole animal oxygen consumption and on the activity of two metabolic key enzymes (citrate synthase (CS) and pyruvate kinase (PK)) of *Cancer setosus* zoeal larvae, acclimated to different temperatures. Larvae acclimated to cooler temperatures (12 & 16 °C) were able to compensate for the temperature effect as reflected in elevated mass specific respiration rates (MSR) and enzyme activities. In contrast, warm acclimated larvae (20 & 22 °C) seem to have reached their upper thermal limits, which is reflected in MSR decoupling from temperature and low Q10 values (Zoea I: 1.4; Zoea III: 1.02). Thermal deactivation of CS in vivo, close to habitat temperature (between 20 and 24 °C) was detected. The capacity of anaerobic metabolism, reflected by PK, was not influenced by temperature, but increased with instar, reflecting behavioural changes in larval life style. Functioning of the metabolic key enzyme CS was identified to be the possible key for larval limitation in temperature tolerance.

KEY WORDS: Brachyura, hairy crab, early ontogeny, oxygen consumption, citrate synthase, pyruvate kinase

Introduction

Temperature is often referred to as the main factor determining biogeographical distribution of marine organisms. The physiological background of temperature dependent latitudinal ranges of marine ectotherms has been subject of numerous studies and during the last decades our knowledge on temperature limitation and acclimation processes has expanded. However, most of these studies refer to adult animals. More recently, attention focused on early life stages, which are generally hypothesized to be more vulnerable to stress conditions than juveniles or young adults (Anger 2001). Early life stages are important for species distribution and recruitment especially in benthic coastal ectotherms (Cowen & Sponaugle 2009).

Temperature affects all levels of biological organization ranging from cellular to organismal level (Guderley & St-Pierre 2002). A mismatch between the oxygen demand of tissues and the supply by the circulatory and ventilatory system is the first mechanism restricting survival at unfavourable temperatures (Pörtner 2001; Heilmayer et al. 2004; Pörtner et al. 2004; Storch et al. 2009). The thermal tolerance thresholds (pejus and critical limits) presented by Pörtner et al. (2005) describe the shortage of aerobic capacity due to unfavourable temperatures. These limits can be compensated for by different (acclimation) mechanisms, e.g. changes in the kinetic characteristics of enzymes (Sokolova & Pörtner 2001; Heilmayer et al. 2004).

Two temperature sensitive key enzymes regulating energy provision in the form of ATP were chosen as proxies of thermal acclimation and limitation at an enzymatic level: citrate synthase (CS) as an indicator of animal aerobic metabolism (Hochachka *et al.* 1970) and pyruvate kinase (PK), which represents the potential for glycolytic flux (Johnston et al. 1977; Childress & Somero 1979; Lemos et al. 2003). Investigations on metabolic enzymes in larval stages are scarce and studies in shrimp larvae show that CS activity is inversely correlated with growth during ontogeny (Lemos et al. 2003).

The model species, *Cancer setosus* (Molina 1782; synonymous *C. polyodon* Poeppig 1836), studied herein covers a wide latitudinal cline of about 44° (Garth and Stephenson 1966, Fischer & Thatje 2008). The commercial value of this species for the Chilean and Peruvian artisanal fishery increased during the last decades (Wolff & Soto 1992, SERNAPESCA 2006, Thatje et al. 2008). The El Niño Southern Oscillation (ENSO), with drastically changing water temperatures between La Niña

and El Niño, strongly affects the abundances and distribution range of this commercially important crab. Increasing temperatures during El Niño events are discussed to be one of the main factors causing mass mortalities of this species (Arntz et al. 1988). The early ontogeny, which in *C. setosus* consists of five planktotrophic zoeal stages (zoea I – V = Z I – V) and one megalopa before reaching the first crab stage (Quintana & Saelzer 1986), is considered as the most delicate part within the life cycle of brachyuran and in particular cancrid crabs (Anger 2001, Weiss et al. 2009a). A unique physiological plasticity to respond to latitudinal and seasonal changes in temperature, has been observed in early egg traits of *C. setosus* (Fischer et al. 2009). The relationship between environmental temperature and metabolic acclimation has been discussed in a broad variety of studies, but knowledge of temperature effects on the physiology of crab larvae, especially concerning the ontogenetic development, is scarce. Furthermore, it remains speculative how larval instars are capable of physiological responses to drastic temperature changes as caused by ENSO.

The present paper examines the influence of temperature on metabolism (oxygen consumption and enzyme activities) of larval *C. setosus* from the Antofagasta region in order to evaluate their capacity to respond to fluctuations in temperature as encountered by larvae during ENSO.

Materials and Methods

Sampling and maintenance of adults

Ovigerous *Cancer setosus* (carapace width: female A = 12.8 cm CW, female G = 14.0 cm CW) were caught in February 2007 by fishermen of the “Caleta Colosso” (23°45’ S, 70°27’ W) by scuba diving and immediately transferred to the laboratory of the Instituto de Investigaciones Oceanológicas of the Universidad de Antofagasta, Chile. Animals were maintained individually in flow-through seawater aquaria (12 l) at ambient temperature ~16.0 °C and salinity 34 psu in a 12:12-h light/dark cycle and fed *ad libitum* with living *Perumytilus purpuratus*.

Experimental set-up

Freshly hatched larvae were collected in filters receiving water from the overflow of the aquaria. Since most larvae hatched at night, samples were taken every morning. Filters were cleaned every evening to ensure daily larval age did not

vary by more than 12 hours (after Lovrich et al. 2003). Solely actively moving larvae were transferred to 100 ml bowls with 16°C filtered seawater and afterwards were allowed to acclimate to the corresponding temperature in the corresponding temperature chamber for the experiments. Acclimation temperatures were chosen to simulate LN conditions (12 °C), normal conditions (16 °C) slight EN (20 °C) and EN conditions (22°C). For each acclimation temperature an initial number of 2000 larvae was cultured (maximum 10 individuals per bowl), water was changed daily and larvae were checked for moults or mortality and fed *ad libitum* with freshly hatched *Artemia* spp. nauplii. Larvae of female A were used for the oxygen consumption experiment and larvae of female G were used for the enzyme activity experiment.

Oxygen consumption

Randomly selected larvae of the same age from the midst of Zoea I (ZI), Zoea III (ZIII) and Zoea V (ZV) at each acclimation temperature were chosen (as available – see Table 1) for oxygen consumption measurements. The middle of each instar was determined by using the larval duration times described in Weiss et al. 2009a. 8 Zoea I instar larvae, 3 Zoea III larvae, and 2 Zoea V larvae per replicate were transferred to an acclimation bath, where they were allowed to acclimate to the measurement temperature (12, 16, 20 or 22 °C) for 20 minutes to avoid shock reactions and to exclude weak larvae (which died during the acclimation period) from further analyses. Afterwards larvae were transferred into 1.0 ml glass caps with fully aerated seawater and sealed with silicon membrane lids. Care was taken that no air bubbles were enclosed. Oxygen micro-optodes (needle-type, fiber-optic microsensor, flat broken tip, 140 µm) were inserted through the silicon membranes into the glass caps. Each measurement consisted of 3 replicates and 1 blank (glass cap filled with aerated seawater). Blanks were run in order to correct for bacterial oxygen consumption. Each combination of temperature and larval instar was tested 3 times (see Table 1). The caps and the optodes were fixed in a mounting rack, which was placed into the temperature controlled basin. Optodes were connected to a 4-channel microsensor oxygen meter (PreSens GmbH, Regensburg, Germany), and oxygen was continuously recorded once every 15 seconds until depletion occurred by a minimum of 5 % oxygen. During the experiments the experimental setup was gently shaken once every 5 minutes to avoid the development of oxygen gradients within the glass caps. Prior to experiments, optodes were calibrated with aerated seawater (see above) for 100 % oxygen, and with a saturated sodium dithionite ($Na_2S_2O_4$)

solution for 0 % oxygen. Experimental temperatures were kept constant (± 0.5 °C) in temperature controlled water baths.

Enzyme assays

Samples for the determinations of enzyme activities of citrate synthase (CS) and pyruvate kinase (PK) were taken in the post- (P) and premoult (A) period of the Zoea I (ZIP, ZIA), Zoea III (ZIIIP, ZIIIA) and Zoea V (ZVP, ZVA) instar of larvae reared at the four acclimation temperatures (12, 16, 20, 22 °C) and analyzed following a modified method of Sidell *et al.* (1987) (CS) and a modified method of Simpfendörfer et al. (1995) (PK), which were adopted for small sample size and measurement in a microplate reader. Three replicates were collected for each "instar x acclimation temperature" combination as available (see Table 1).

Frozen samples were homogenized in ~0.3 µl extraction buffer (75 mM Tris-HCl, 1 mM EDTA; pH 7.6) per 1µg larval DW (dry weight) to get a 1:10 (w/v) ratio with a Branson Sonifier 450 (0° C, output control 8, duty cycle 50%, 15 min). Cell debris was removed by centrifugation for 5 minutes at 7400 g and 0° C with an Eppendorf centrifuge 5810R.

A) Protein content

The concentration of soluble protein in the extracts was measured according to Bradford (Bradford 1976). The samples were diluted 1:5 with 0.9 % NaCl before being applied in duplicate (5 µl) on microplates. Subsequently 250 µl dye reagent (Biorad protein assay 500 0006, diluted 1:5 with aqua dest) were added and the optical density was measured at 620 nm in a microplate reader (FLUOstar Galaxy, BMG). Bovine serum albumine (BSA, 0 - 3.5 µg per well) was run in parallel as standard.

B) Citrate synthase

Citrate synthase (CS) (E.C. 4.1.3.7) is a key regulatory enzyme in the tricarboxylic acid (TCA) cycle and chosen as an indicator of aerobic capacity. Due to the limited volume of extract the assay according to Sidell et al. 1987 was adapted for use in a microplate reader and under controlled (below ambient) temperature conditions.

Table 1. Overview about conducted experiments. ZI, ZIII, ZV - Zoeal instars, P - postmoult, A - premoult, "+" - experiment conducted, "-" - experiment not conducted due to insufficient larval material.

Experiment	Acclimation Temperature [°C]	Instar	n/sample	Experimental Temperature [°C] 12	16	20	22
Oxy	12	ZI	8	+	+	+	+
		ZIII	3	-	-	+	+
		ZV	2	-	-	-	-
	16	ZI	8	+	+	+	+
		ZIII	3	+	+	+	+
		ZV	2	-	-	-	-
	20	ZI	8	+	+	+	+
		ZIII	3	+	+	+	+
		ZV	2	+	+	+	+
	22	ZI	8	+	+	+	+
		ZIII	3	+	+	+	+
		ZV	2	-	-	-	-
CS	12	ZIP	10	+	+	+	+
		ZIA	6	+	+	+	+
		ZIIIP	2	+	+	+	+
		ZIIIA	2				
		ZVP	1	-	-	-	-
		ZVA	1	-	-	-	-
	16	ZIP	10	+	+	+	+
		ZIA	6	+	+	+	+
		ZIIIP	2	+	+	+	+
		ZIIIA	2	+	+	+	+
		ZVP	1	+	+	+	+
		ZVA	1	+	+	+	+
	20	ZIP	10	+	+	+	+
		ZIA	6	+	+	+	+
		ZIIIP	2	+	+	+	+
		ZIIIA	2	+	+	+	+
		ZVP	1	+	+	+	+
		ZVA	1	+	+	+	+
	22	ZIP	10	+	+	+	+
		ZIA	6	+	+	+	+
		ZIIIP	2	+	+	+	+
		ZIIIA	2	+	+	+	+
		ZVP	1	-	-	-	-
		ZVA	1	-	-	-	-
PK	12	ZIP	70	+	+	+	+
		ZIA	35	+	+	+	+
		ZIIIP	15	+	+	+	+
		ZIIIA	10	-	-	-	-
		ZVP	3	-	-	-	-
		ZVA	2	-	-	-	-
	16	ZIP	70	+	+	+	+
		ZIA	35	+	+	+	+
		ZIIIP	15	+	+	+	+
		ZIIIA	10	+	+	+	+
		ZVP	3	-	-	-	-
		ZVA	2	-	-	-	-
	20	ZIP	70	+	+	+	+
		ZIA	35	+	+	+	+
		ZIIIP	15	+	+	+	+
		ZIIIA	10	+	+	+	+
		ZVP	3	+	+	+	+
		ZVA	2	+	+	+	+
	22	ZIP	70	+	+	+	+
		ZIA	35	+	+	+	+
		ZIIIP	15	+	+	+	+
		ZIIIA	10	+	+	+	+
		ZVP	3	-	-	-	-
		ZVA	2	-	-	-	-

Homogenates (2 µl / well) were assayed in 200 µl of 75 mM Tris-HCl buffer (pH 8.0), 0.25 mM DTNB (5.5'-dithio-bis-(2-nitrobenzoic acid)) and 0.4 mM acetyl-CoA. 0.4 mM Oxalacetate was added to start the reaction (omitted for the blanks).The microplates were incubated at the respective temperature (12, 16, 20, 22, 28 °C) on a thermostated aluminium block (constructed by E. Dunker, Alfred Wegener Institute Bremerhaven); and the development of free SH groups was measured quickly every five minutes for 30 minutes (6 measurements) in a microplate reader at 405 nm at room temperature after which time the plate was returned to the aluminium block for further incubation. Enzyme activity in units per mg protein (U $gprt^{-1}$) were calculated using a standard curve produced with dithiothreitol (DTT) corresponding to 25 to 200 µM SH groups.

C) Pyruvate kinase

Pyruvate kinase (PK) (EC 2.7.1.40) catalyzes the last step of the glycolytic pathway which is the transphosphorylation from phosphoenol-pyruvate and ADP to pyruvate and ATP. Hence, PK activity may represent the potential for anaerobic work in the glycolytic pathway (Johnston et al. 1977). Again, the assay according to Simpfendörfer et al. (1995) was adapted for use in a microplate reader below ambient temperature. Homogenates (10 µl / well) were assayed in 200 µl of assay buffer (pH 8.0) (6 mM Tris-HCl, 60 mM KCl, 6 mM $MgSO_4$ x 7 H_2O) 7 mM NADH, 0.5 mM PEP and 5.5 U/ml LDH. 1mM ADP was added to start the reaction (omitted for the blanks). Standards of 7 mM NADH (2 – 10 µl per well) were run in parallel. The microplates were incubated at the respective temperature (12, 16, 20, 22, 28 °C) on a thermostatted aluminium block; and the coupled oxidation of NADH was measured quickly every five minutes for 30 minutes (6 measurements) in a microplate reader at 340 nm and room temperature after which time the plate was returned to the aluminium block for further incubation. Enzyme activity in units per mg protein (U $gprt^{-1}$) was calculated using a standard curve produced from NADH (0.5 mM) and corresponding to 7 to 35 µM NADH groups.

Statistical analyses

All data were tested with the Jackknife distances test (Kezdi et al. 2002) to exclude outliers from analysis. The effect of acclimation temperature T_a, measurement temperature T_m (Kelvin) and body mass *DW* (µg dry mass) on

metabolic activity (oxygen consumption, CS activity, PK activity) was assessed by means of analysis of covariance (ANCOVA) according to the model

$$\ln(rate) = a + b_1 / T_m + b_2 * \ln(DW) + D_1 + D_2 / T_m$$

where variables D_1 and D_2 attain values specific for T_a (12, 16, 20, 22°C). Only the significant terms of each model are presented in the result chapter. The relationship between ln(CS activity) and the inverse of T_m was not linear over the whole temperature range, i.e. this model could not be applied (see results). Instead, we introduced T_m as a categorial variable to test for effects of W and of T_a.

Results

Oxygen consumption

Larval oxygen consumption is predicted from measurement temperature (T_m), acclimation temperature (T_a) and larval body mass (DW) by the model

$$\ln(Oxy) = 8.8781 - 4207.9393 * 1/T_m - 0.0885 * \ln(DW) + D_1 + D_2 /(T_m - 0.0034)$$

where D_1 = 0.0678, -0.0953;0.0153; 0.0122 and D_2 = -2154.3805; -1001.2125; 1102.7057; 2052.8873 for T_a = 12, 16, 20, 22 °C.

N = 256, F = 15.112, R^2 = 0.33, $p < 0.0001$ for the whole model and each term. Please note that $1/Tm$ is adjusted to mean = zero in the interaction term in order to make the test for the main effects independent of the test for interaction ("centered polynomials").

Note that the model (Figure 1) predicts larval oxygen consumption for just any combination of larval body mass and temperature; whereas our experiments indicate that the "body mass × temperature" space where larvae perform aerobically is limited (see Discussion). Outside the Pejus range larval oxygen consumption rates will level off.

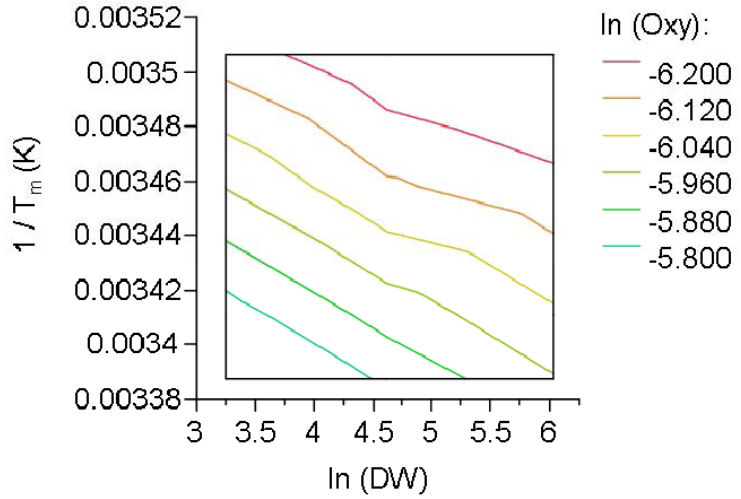

Figure 1. Model of oxygen consumption of larval *Cancer setosus* throughout their development. Equation for the model is:
$\ln(Oxy) = 8.8781 - 4207.9393 * 1/T_m - 0.00885 * \ln(DW) + D_1 + D_2/(T_m - 0.0034)$
($N = 256$, $F = 15.112$, $R^2=0.33$, $p < 0.0001$). T_m represents the measurement temperature in Kelvin, T_a represents the acclimation temperature of larvae and *DW* is the body mass in dry weight (µg). Isolines represent the oxygen consumption in ($\mu mol * day^{-1} * DW^{-1}$). For further details see text.

In general whole organism oxygen consumption of *C. setosus* larvae increased with progressing development. However, depending on acclimation temperatures the increase varied without any discernible pattern. Mass specific respiration rates (MSR) showed significant differences between acclimation temperatures ($p = 0.040$, $F = 2.81$, $df = 3$), measurement temperature ($p < 0.0001$, $F = 84.60$, $df = 1$), body mass ($p = 0.0002$, $F = 14.38$, $df = 1$) and in the interaction term of and "acclimation temperature x measurement temperature" ($p = 0.0086$, $F = 3.97$, $df = 3$). As expected, MSR of larvae increase with measurement temperature. Thermal sensitivity of oxygen turnover (expressed as Q10) decreased with increasing acclimation temperature from 2.3 at 12 °C acclimated Zoea I larvae to 1.4 in 22 °C acclimated larvae. This decrease is even more pronounced in ZIII larvae, showing a Q10 of 2.28 at an acclimation temperature of 16 °C and a Q10 of 1.02 at 22 °C acclimation temperature.

Citrate synthase

The citrate synthase (CS) activity of *C. setosus* larvae differed significantly depending on acclimation temperature ($p = 0.0003$, $F = 6.34$, $df = 3$), assay temperature ($p < 0.0001$, $F = 21.20$, $df = 1$) and body mass ($p = 0.0007$, $F = 11.56$, $df = 1$). CS activity was found to be highest at an acclimation temperature of 12 °C, and remained virtually unchanged at the higher acclimation temperatures. The CS activity increased with increasing assay temperature, but was significantly lower at the assay temperature of 28 °C (Figure 2) which is also reflected in different Q10 values for the lower (12-20 °C, Q10: 1.5 ± 0.36) and upper temperature range (20 - 28 °C, Q10: 0.45 ± 0.15).

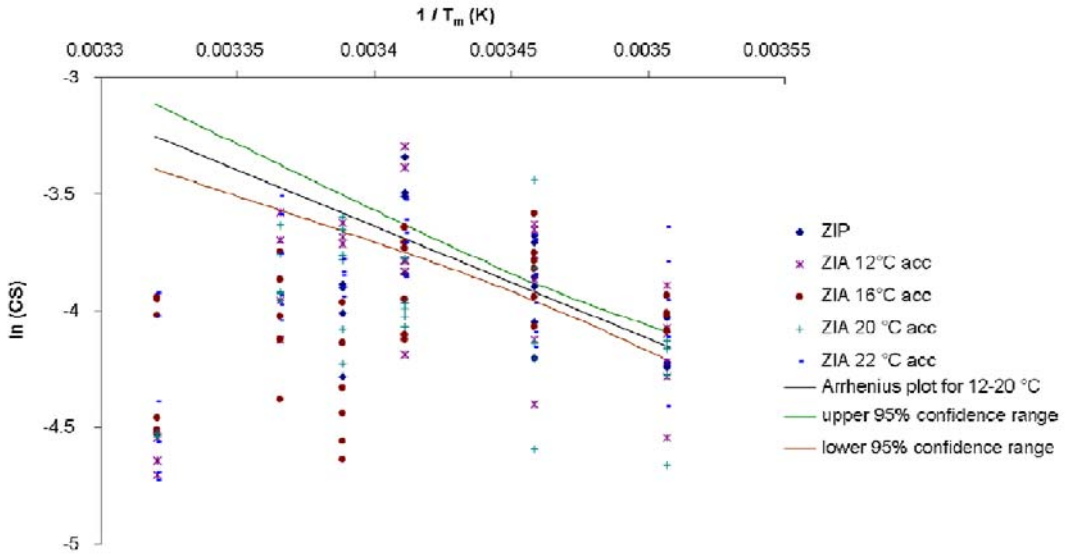

Figure 2. CS activity in units per mg protein (U $gprt^{-1}$) at different assay temperatures (12 - 28 °C) of Zoea I *Cancer setosus* larvae. Arrhenius equation (black line) only fits for assay temperatures 12 – 20 °C, indicating thermal inactivation of the enzyme at higher temperatures. Arrhenius equation: ln (CS) = 6.57 – 3035.05*1/T_m. N = 299, F = 55.49, R^2=0.16, $p < 0.0001$. Green and brown lines are indicating the upper and lower 95% confidence range.

The CS activity decreases towards larger larvae. The number of assay temperatures did not allow exact calculations of Arrhenius breakpoint temperatures (ABT) with acclimation temperature and instar. Nevertheless, the ABT of CS seems to be located between 20 and 24 °C, as seen in the Arrhenius plot (Figure 2).

Pyruvate kinase

Larval PK activity is predicted best from measurement temperature (T_m) and larval body mass (DW, µg) by the model

$$\ln(PK) = 11.8344 - 4158.7783 * 1/T_m + 0.1469 * \ln(DW)$$

N = 844, F = 548.09, R^2 = 0.57, p < 0.0001 for both slopes and for the whole model. PK activity showed a continuous increase with measurement temperature (Figure 3). The Q10 values were lower for freshly hatched larvae (ZIP, Q10: 1.17) than for ZI larvae in the premoult phase (ZIA: 1.83 ± 0.14), but in ZIII and ZV Q10 values are higher in the postmoult phase than during the premoult phase (ZIIIP, Q10: 1.86 ± 0.097; ZIIIA, Q10: 1.34 ± 0.722; ZVP, Q10: 1.41; ZVA, Q10: 1.39). PK activity increased continuously with body mass, with slightly higher values in the beginning of the ZIIIA and ZVA instar than at the end of the corresponding instar.

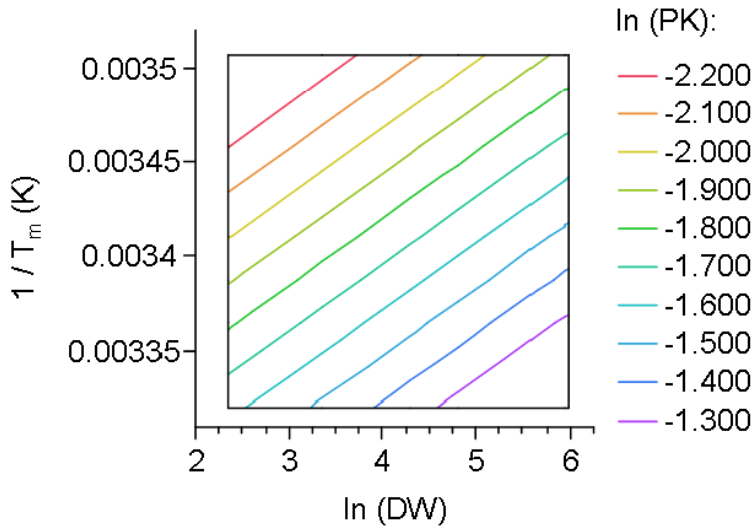

Figure 3. Model of PK activity of larval *Cancer setosus* throughout their development. Equation for the model is:
ln(PK) = 11.8344 – 4158.7783 * $1/T_m$ + 0.1469 * ln(DW)
N = 844, F = 548.09, R^2 = 0.57, p < 0.0001 for the whole model. T_m represents the measurement temperature in Kelvin and DW is the body mass in dry weight (µg). Isolines represent the PK activity in units per mg protein (U $gprt^{-1}$).

The CS/PK ratio was determined for 16 and 20 °C, temperatures which supported complete zoeal development. Here the CS/PK ratio was not dependent on acclimation temperature, but values decreased significantly from Zoea I to Zoea V ($p < 0.0001$; $N = 65$; $df = 5$; $F = 8.65$).

Discussion

Temperature limitations of *C. setosus* larvae were found to be reflected in the functioning and activity of the metabolic key enzyme CS. Our results indicate a thermal cold acclimation due to an activity increase of CS at an acclimation temperature of 12 °C. The onset of a thermal deactivation of the enzyme was detected to lie between 20 and 24 °C. Those temperature effects on the larval metabolism could also be found in the MSR, which show high values in cold acclimated larvae (12 °C)and a capacity limitation at higher temperatures (20 & 22 °C).

Standard metabolic rate of an organism typically increases exponentially with increasing temperature between critical temperatures, which border the passive thermal tolerance window (Pörtner et al. 2005). As Zoea larvae are actively swimming in the water column, larval oxygen consumption comprises standard metabolism and oxygen demand for swimming, and can be best described as routine metabolism. Although an exponential pattern of routine metabolism could not be observed in larvae of the congener *Cancer irroratus* (Sastry 1979) and other decapod larvae such as *Taliepus dentatus* (Storch et al. 2009), *C. setosus* larvae obviously display an exponential increase of their routine metabolism with increasing temperature.

As expected, mass specific oxygen consumption decreases with body mass. Interestingly the slope of this decrease is much lower (-0.08) than expected theoretically and as we know from empiric data (-0.25). An almost linear increase of individual respiration rates with DW normally is only found during periods of increased growth (Hoegh-Guldberg & Manhan 1995; Heilmayer et al. 2004). This finding is also reflected in the extremely high cumulative growth rates of *C. setosus* larvae (Weiss et al. 2009a) when compared to other decapod crustacean larvae (Anger 1995). When acclimated to higher temperatures (20 and 22 °C) larval oxygen consumption did not increase with measurement temperature, demonstrated in low Q10 values at higher acclimation temperatures (see Results). This indicates that

larval metabolism of warm acclimated animals is functioning at its upper limit and has no/only very limited capacities to adjust to further temperature increments. As stated by Hoegh-Guldberg & Pearse (1995) a lack of temperature compensation indicates that larval development occurs close to the upper possible temperature limit. In cold acclimated larvae a higher thermal plasticity was found, indicated by higher Q10 values. Zoea III acclimated at 12 °C show particularly high oxygen consumption rates and corresponding high levels of CS activity. An elevated CS activity at cold temperatures indicates metabolic cold compensation (Sokolova and Pörtner 2001; Lemos *et al.* 2003). CS activity measured at different assay temperatures increased with increasing temperature before declining at temperatures warmer than 20 °C (Figure 2), indicating onset of thermal deactivation of the enzyme which is manifested in reduced activities in all groups at 28°C assay temperature even when compared to 12°C assay temperature. This means that the temperature optimum for the functioning of CS lies close to the optimum temperature for larval development and growth (~20 °C) (Weiss et al. 2009a) in the Antofagasta region. Although denaturation temperatures are usually found far beyond the naturally experienced temperatures of (temperate and cold-water) ectotherms (Sokolova and Pörtner 2001) as supported by the present PK data, a similar discontinuity in CS activity has been described for *Littorina saxatilis*, where the deactivation temperature of the enzyme equally lies close to high ambient temperatures encountered by the animal (Sokolova & Pörtner 2001). This suggests that the failure of CS at high temperatures may substantially contribute to the thermal limitation of larvae. The functioning of CS is restricted in all larval instars. Our results also show that the ABTs of CS in higher instars of warm acclimated larvae probably would be shifted towards warmer temperatures. Further investigations with even more assay temperatures are needed to substantiate this finding. Such a shift in ABT with acclimation temperature may suggest that higher instars show a certain capability for warm acclimation and are therefore less vulnerable to elevated temperatures. This may correspond with the local oceanographic conditions in the experimental region, where younger larvae most likely drift into the Antofagasta bay and are retained within a cyclonic current at elevated temperatures for longer periods of time. According to the present data an acclimation of the CS properties occurs under the local conditions in the experimental area. This may support elevated instar dependent temperature optima of growth rates in *C. setosus* Zoea II+III instars (Weiss et al. 2009a).

In light of the temperature changes associated with ENSO Zoea I instars display very limited tolerance as indicated by the lack of thermal compensation in respiration rates and the low denaturation temperature of CS, (*sensu* Sokolova and Pörtner 2001) The shift in the ABT of CS to warmer temperatures would indicate a certain degree of warm acclimation. We may conclude that larvae hatched after the onset of EN have better chances for survival than cold acclimated larvae. The ABT of CS between 20 and 24 °C (Figure 2) (Table 5), indicates thermal inactivation of CS closely preceding the acute lethal limit of *C. setosus* Zoea I larvae, which lies between >24 and <30 °C (Weiss et al. 2009b and preliminary experiments). Thus, dysfunction of mitochondrial enzymes and more generally loss in aerobic capacity may contribute to mortality under acute heat stress, as during EN events, especially in warm water regions like the Antofagasta bight.

The moderate warm acclimation capacity of the larvae matches the slight seasonal temperature variations of ~4°C (SHOA 2009) in Antofagasta, more than the larger (up to 10 °C) temperature fluctuations associated with EN events. Oxygen consumption measurements and recent studies of elemental composition and phenotypic plasticity (Weiss et al. 2009a; Weiss et al. 2009b) indicate that *C. setosus* larvae already reach their thermal limits at ~22 °C.

CS activity is changing depending on the instar and is clearly declining towards the Zoea V (Table 5). A decline in CS activity through ontogenetic development can be related to the 450-fold increase in body size (Weiss et al. 2009a) and thus allometric effects from instar to instar. Another reason for this decrease may lie in the reduced requirement for locomotory activity and therefore aerobic capacity after transition from planktonic to benthic life style which occurs within the megalopa (Lemos *et al.* 2003).

In contrast to CS activities, pyruvate kinase (PK) activities were not affected by acclimation temperature indicating that PK is not contributing to thermal compensation or displays sufficient capacity at any acclimation temperature. PK activity increased continuously with assay temperature from 12 to 28 °C, with no signs of thermal inactivation (Figure 2). PK activity increased with increasing larval size, and thus the CS/PK ratios decreased with increasing instar, as would be expected from allometric relationships. Higher glycolytic over TCA (tricarboxylic acid) capacities in later larval instars indicate higher capacities for anaerobic metabolism.

These findings correspond with larval life styles, as they improve their capacity to hunt prey and escape from predators during their ontogeny (Lemos et al. 2003).

In higher instars (ZIII and ZV) PK activity is more pronounced in the beginning of the instar than in the end (also reflected by Q10 values), which may indicate low PK activity during ecdysis. During the premoult phase food uptake is stopped, which obviously weakens the larvae, as it is visible in the CHN values and the high mortality during ecdysis (Weiss et al. 2009a). Low PK activity during the energy consuming moult shows, that larvae can only revert to a limited anaerobic capacity, what might contribute to the high mortality of larvae during ecdysis.

Conclusion

C. setosus Zoeal instars show high temperature sensitivity in aerobic metabolism. Respiratory and CS capacities show compensation in the cold but are limited at warmer temperatures. Anaerobic capacities display no compensation, but a higher anaerobic than aerobic capacity may be associated with slowing larval life style.

The results of this study indicate that *C. setosus* larvae are able to display a certain thermal compensation in the cold, but larvae obviously already live at their upper tolerance limits. Limitation of aerobic pathways seems to be responsible for a restricted thermal tolerance of *C. setosus* larvae during EN events.

Acknowledgements

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2.5 Publication V

Effect of salinity changes during El Niño on the development of *Cancer setosus* Zoea I larvae

Weiss M; Heilmayer O

(2009)

In preparation

Manuscript in preparation

Effect of salinity changes during El Niño on the development of *Cancer setosus* Zoea I larvae

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Abstract

Large-scale environmental patterns in the Humboldt Current System (HCS) show drastic changes during strong El Niño (EN) events that might result in mass mortalities of endemic species. Besides intense temperature changes, a temporal intrusion of oceanic or equatorial water masses and a heavy precipitation in arid regions of Pacific South America result in varying salinities, especially in the coastal region. Salinity changes are known to influence the metabolic efficiency and the survival of marine invertebrates. The hairy crab, *Cancer setosus*, is one of the most important brachyuran crab species for the Peruvian and Chilean artisanal fisheries and it is known to suffer mass mortalities during strong EN events, but the influence of the abiotic factors on the larval part of *C. setosus* is unknown. The consequences of salinity reduction on the survival and elemental composition of *C. setosus* Zoea I larvae was determined in this study. We reared Zoea I larvae under six different hyposalin conditions (5, 10, 15, 20, 25, 30 and 35 PSU) and recorded daily the survival. Salinities of 5, 10 and 15 PSU were lethal for larvae within 1 – 3 days. A salinity of 20 PSU resulted in relatively high mortality, but survival rates at 25 – 35 PSU were generally high. The elemental analyses of Zoea I larvae reared at different salinities reveals that biomass and lipid accumulation decreased with decreasing salinity probably due to energy consuming active ion regulation. The results show that reduced salinities negatively influenced larval fitness, but nevertheless larvae were able to survive a quite wide range in salinity, allowing for larval surivival during weak EN events.

Keywords: Brachyura; ENSO; Chile; survival; elemental composition

Introduction

During El Niño (EN) intense precipitation in arid regions of Peru and Chile causes a significant increase of freshwater input, leading to reduced salinities in coastal waters, especially close to river estuaries (Arntz 1991; Thatje et al. 2008). A reduced salinity influences survival (Anger *et al.* 1998), metabolism and cumulative growth (Torres *et al.* 2002) of crustacean larvae. However, species-specific differences occur. Larvae of *Armases imersii*, for example, show an ontogenetic shift in salinity tolerance, which can be attributed to construction of organs that facilitate osmoregulation in higher stages (Megalopa and Crab I stage) (Anger *et al.* 2000). The ontogenetic shifts in salinity tolerance of *Chasmagnathus granulata* (Charmantier *et al.* 2002) in contrast can be explained as an adaptation to predictable changes occurring in the natural habitat. EN in contrast is not a predictable, regular event and salinity changes due to freshwater inflow from the continent or the intrusion of less saline water masses, hit the temperature and salinity stable habitat of the Humboldt Current System (HCS) and therefore present a drastic task for the biotic environment.

Different *Cancer* species (e.g. *C. irroratus*, *C. borealis*; Charmantier & Charmantier-Daures 1991) are known to be osmoregulators as adults, while their larvae are osmoconformers. Larvae of those sympatric species show differences in the salinity tolerance, due to acclimations to slight differences in the selected habitat for larval release. The influence of changes in salinity has been the subject of several estuarine and rocky shore species, but only little is known about the physiological responses of larvae of a stenohaline species. In this study survival rate and changes of the elemental composition of Zoea I larvae was examined under hyposaline conditions of *C. setosus* from a salinity and temperature stable region.

Material and methods

Sampling and maintenance of adults

Ovigerous *Cancer setosus* were caught in April 2008 by fisherman of the "Caleta Colosso" (23°45' S, 70°27'W) by scuba diving and were immediately transferred to the laboratory of the Instituto Investigaciones Oceanológicas of the Universidad de Antofagasta, Chile. Animals were maintained individually in flow-through seawater aquaria (12 l) at ambient temperature (~16.0 °C) and salinity (34

psu) in a 12:12-h light/dark cycle and fed with *ad libitum* with life *Perumytilus purpuratus*.

Experimental set-up

Freshly hatched larvae were collected in filters receiving water from the overflow of the aquaria. Since most larvae hatched at night, samples were taken in the morning. Filters were cleaned every evening to ensure daily larval age did not vary by more than 12 hours (Lovrich et al. 2003). Solely actively moving larvae were used for experiments. Freshly hatched larvae were kept for 4 days in an aerated 5 l container under conditions comparable with the natural habitat of 16 °C water temperature. Water was changed and larvae were fed *ad libitum* with freshly hatched *Artemia* spp. Nauplii daily. After 4 days larvae were transferred to the experimental tanks. Filtered seawater of 7 different salinities (5, 10, 15, 20, 25, 30, 35 PSU) was prepared. Herein 35 PSU represent the control salinity. Larvae were maintained in 400 ml glass bowls with a density of 30 ind/bowl, with a replicate number of 8 bowls per salinity. Bowls were placed into a 20 °C chamber afterwards to simulate slight EN temperatures. Water was changed, larval survival was checked and larvae were fed as mentioned above daily.

Elemental analyses (CHN)

On the first (day 0) and the last day (day 4) of the experiment samples were taken for CHN measurements. 30 larvae with a replicate number of 3 were taken for analyses at each salinity. Carbon (C), hydrogen (H), nitrogen (N) contents were determined following Anger & Dawirs (1982). In brief: Larvae were rinsed in distilled water, blotted on filter paper, placed into pre-weighed tin cartridges and stored at -80 °C. Afterwards samples were vacuum-dried for 48 hours in a Virtis Benchtop SLC freeze dryer at -70º C and a pressure below 0.01 mbar. Samples were transported to the home institute in airtight boxes with silica gel, where they were dried again, at 50 °C in a dry oven for 24 hours and weighed to the nearest 0.1 µg on a Sartorius M2P microbalance. CHN content was measured with a HEKAtech EURO EA 3000 CHNS-Analyzer using Acetanilid as a standard.

Statistics

All data were tested with the Mahalanobis distances test (Mahalanobis 1936) to exclude outliers from analysis. The effects of salinity and experimental day on

larval survival were analysed with a full interaction analysis of covariance (ANCOVA). The effect of salinity on elemental composition was tested with an analysis of variance (ANOVA). Normal distribution of data was tested with a Saphiro-Wilk W test. All post hoc comparisons were conducted using a student's t-test. The C/N ratio was transformed with an arcsin transformation prior analysis.

Results

Survival and elemental composition of Zoea I larvae were affected by reduced salinities. Salinity ($p<0.0001$, $F=537.84$, $DF=4$), duration of the experiment ($p<0.0001$, $F=97.45$, $DF=3$) and the interaction term ($p<0.0001$, $F=6.09$, $DF=12$) had a significant influence. All larvae died during the first day of the experiment at 5 and 10 PSU. At 15 PSU only 8 larvae survived the first day. The first day of the experiment larvae reared at 20, 25, 30 and 35 PSU showed no differences in survival. Survival at salinity of 20 was reduced to 15 larvae after 4 days, being significantly lower than at 25, 30 and 35 PSU (Figure 1).

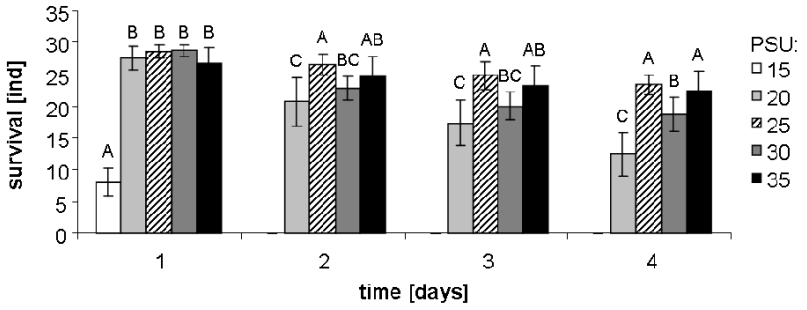

Figure 1. *Cancer setosus*. Survival of Zoea I larvae reared under different hyposalin conditions at a constant temperature of 20 °C. Letters indicate significant differences.

Results of the CHN analyzes (Figure 2) stresses those findings. Highest W, C and N were measured at a salinity of 35 and values decreases significantly with salinity. The C/N ratio decreases with decreasing salinity, meaning that the carbon content decreases stronger than nitrogen content with decreasing salinity, which indicates an increased degradation of lipids.

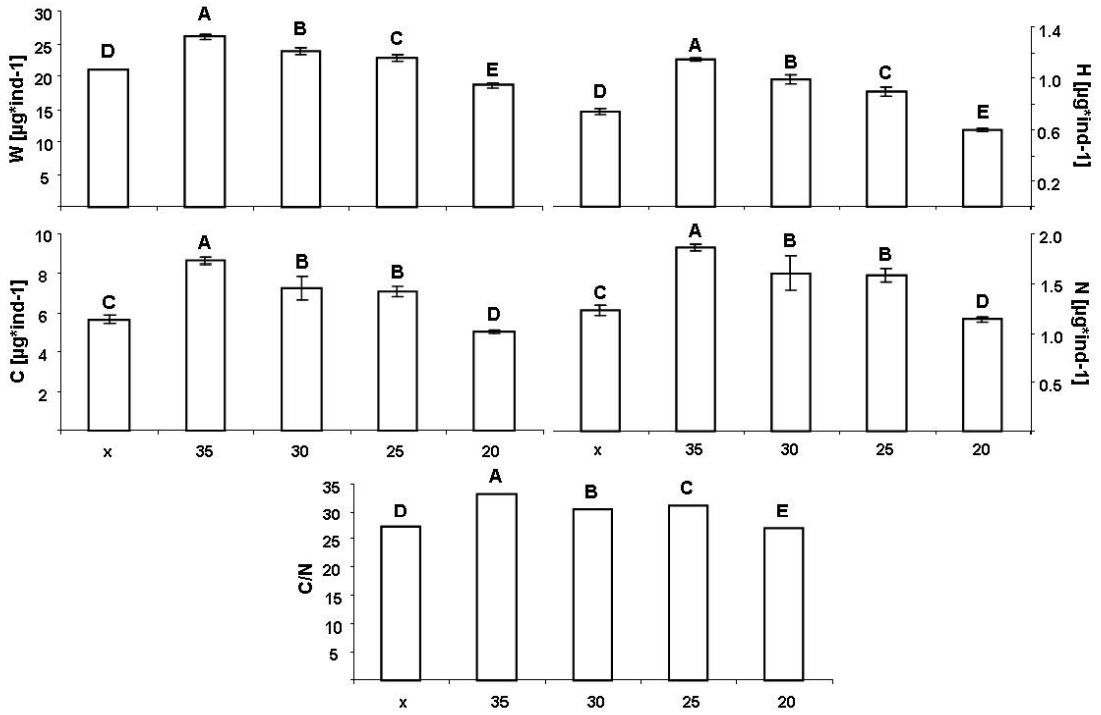

Figure 2. *Cancer setosous*. Influence of different hyposalin conditions on dry weight (W) ($p<0.0001$, $F=167.45$, $DF=4$) and the elemental composition (C – carbon ($p<0.0001$, $F=62.24$, $DF=4$), H – hydrogen ($p<0.0001$, $F=190.46$, $DF=4$), N – nitrogen ($p<0.0001$, $F=34.71$, $DF=4$), C/N – carbon to nitrogen ratio ($p<0.0001$, $F=201.72$, $DF=4$)) of Zoea I larvae. x – control: samples taken at day 0 of the experiment.

Discussion

The present study, which simulates the spontaneous salinity reductions during El Niño, shows that decreased salinities strongly affect the survival and the elemental composition of *C. setosus* Zoea I larvae. The salinities of 5, 10 as well as 15 PSU are lethal for larvae. At higher salinities (35, 30, 25, 20) the survival rate and the cumulative zoeal growth decreased with salinity (Figure 1). The survival rates correspond with the tolerable salinities of other cancrid species (Charmantier & Charmantier-Daures 1991). A reduction of growth at lower salinities can be interpreted as a consequence of energy expenditure to adjust osmolarity. Larvae of congeners are known to be osmoconformers (Charmantier & Charmantier-Daures 1991): they do not actively maintain osmolarity that is different from the surrounding seawater, but osmoconformers as well have to invest energy to keep their body fluids isotonic to the external environment by actively regulating their internal concentration of amino acids, ions, and proteins to match the osmolarity of the environment. The

decrease in lipids, which are the most important energy reserves for crustacean larvae, shows that hypoosmotic stress causes enhanced metabolic demands or a reduced capacity for assimilation. This is either due to a decrease in food uptake or to a decrease in conversion efficiency (Anger *et al.* 1998), as it can also be found under unfavourable temperatures (Weiss *et al.* 2009a).

In osmoconforming species like *H. araneus*, *H. gammarus* as well as *C. irroratus*, *C. borealis* and most probably *C. pagurus* the sensitivity to salinity changes is high (Charmantier & Charmantier-Daures 1991, Torres *et al.* 2002). The response we found in our study leads us to the conclusion, which has to be testified by further studies, that *C. setosus* larvae are osmoconformers as well. As stated by Riascos et al. 2009, the temperature change during ENSO is the most striking factor setting limits for survival and affecting the metabolism, also of *C. setosus* larvae (Weiss et al. 2009 a,b). The combination of diverse abiotic and biotic factors that underlie changes during ENSO enforce the effects. Although *C. setosus* Zoea I larvae survive a remarkable range of salinities, effects on higher instars stay unknown. The combination of reduced salinities with additional changes in for example temperature and food resources during EN events most likely lead to a mass mortality of whole larval cohorts.

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3 Synoptic discussion

ENSO is threatening the marine ecosystem of the HCS and therewith affects marine resources and human livelihood. However, still the knowledge on the effects of climate change (in the sense of global warming) on ENSO and the affected ecosystem is restricted.

To study the effects of climatic changes, an adequate model organism is required. *C. setosus* has been reported to suffer mass mortalities during strong EN events (Arntz & Fahrbach 1991), but this phenomenon never gained more attention. Since *C. setosus* has a wide latitudinal distribution and is an ecologically and economically important species, it is an ideal model organism to study temperature effects. The main focus of the present thesis was to reveal the ecophysiological effects of temperature changes, as they occur during ENSO, on the early life stages of *C. setosus* (Figure 11).

Cancer setosus facts:

- Broad latitudinal distribution.
- Key predator in the HCS.
- Adults suffer mass mortalities during strong EN events.
- Important target species for the artisanal fisheries.

➢ **Ideal model organism.**

The first step to study the effect of temperature changes on *C. setosus* larvae was to determine survival rates and instar duration of larvae reared at simulated LN (12 °C), "normal" (16 + 20 °C) and EN (22 °C) temperatures. At 12 and 22 °C mortality rates were exceptionally high and larvae only reached a Zoea IV instar. The instar duration generally decreased with increasing temperatures. Only at 16 and 20 °C zoeal development could be completed (Publication II).

To reveal temperature induced changes in the metabolic rate of larvae, we measured the oxygen consumption and determined the aerobic and anaerobic capacity by means of enzyme measurements (citrate synthase (CS) and pyruvate kinase (PK)). At low rearing temperatures (12 °C) an increase of the metabolic

activity in terms of elevated CS activity and high oxygen consumption rates, pointed at a thermal metabolic compensation. At warm acclimation temperatures low Q10 values of the oxygen consumption indicated that no/only scarce further metabolic compensation was possible. The PK activity did not reveal any contribution to thermal acclimation. The CS/PK ratios decreased with increasing instar, indicating better options for fast movements, accepting an anaerobic metabolism at higher instars which allows faster hunting and predator avoidance (Publication IV).

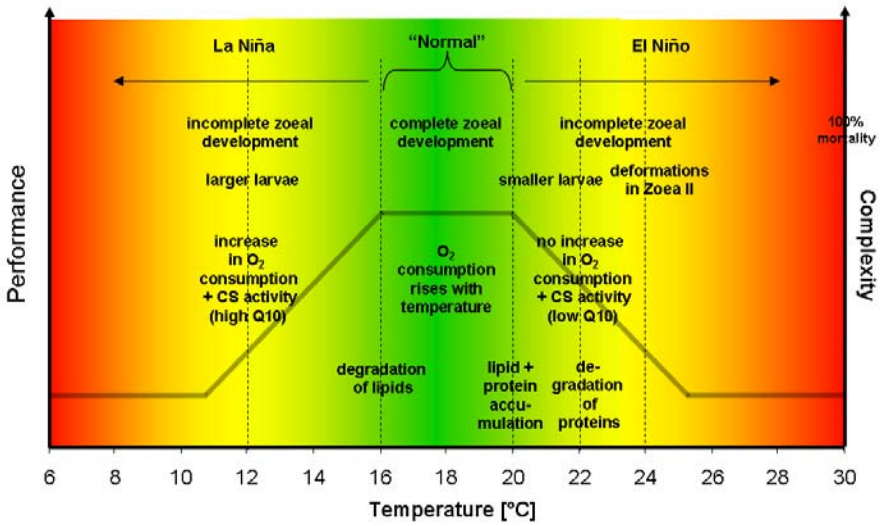

Figure 11. Ecophysiological response of *C. setosus* larvae
at different hierarchical levels on temperature changes during ENSO fitted into a potential temperature tolerance model for zoeal *C. setosus*.

A further step to reveal temperature induced metabolic changes was to analyse the elemental composition of larvae reared at different temperatures. The C, H, N content is a precise reflection of the nutritional condition of larvae. Typical fluctuations of C, H, N during the moulting cycle (Publication II) and as well variations in the elemental composition known from other crustaceans under similar stress conditions were detected. At 20 °C, lipid and protein accumulation pointed at optimum growth conditions. At 16 and 22 °C a mismatch between energy supply and demand resulted in reduced growth rates.

The pattern of growth (increment in carbon in each instar) pointed at an instar dependent temperature tolerance with a changing optimum growth between 20 and 22 °C, which might be an adaptation to larval migrations. A growth model has been developed that describes larval growth (µg C) as a function of time and temperature.

A further step to analyse the capability of larvae to react and adjust to changing temperatures, the phenotypic plasticity in morphometric traits of larvae from different latitudes that experienced different temperature regimes during embryogenesis and during larval development was studied. Results showed that larvae generally display phenotypic plasticity. At colder temperatures larger larvae with longer spines develop (Publication III). This can be regarded as an adaptive response to gradual (latitudinal) or sudden (ENSO) temperature changes. The results indicate that a metabolism based on fatty acids might produce larger larvae, since more fatty acids are known to be consumed during embryonic and larval development at colder temperatures and eggs from a southern population receive a better fatty acid maternal provisioning (Fischer et al. 2009b). Fatty acids also seem to serve as a protection shield against the impact of temperature, since cold temperatures do not evoke size changes in larvae from the southern region (Publication III).

During ENSO not only the temperature is changing, but also other biotic and abiotic factors are influencing the community. To address at least one more important abiotic factor, the impact of hyposalin conditions on larval survival and metabolism was determined. Reduced salinities have an unfavourable effect on the survival of Zoea I larvae that is also reflected in their elemental composition. Biomass and lipid accumulation decreased with decreasing salinity, which is probably due to energy consuming active ion regulation. Thus, reduced salinities are negatively influencing larval fitness, but nevertheless Zoea I larvae are able to survive within a quite wide range of salinities. It can be assumed that the negative effect of elevated temperature on larval survival during EN events would be enforced by reduced salinities (Publication V).

Since not only intraspecific information about a variety of traits is required to explain ecosystem functioning but also information about interspecific differences is needed, a comparison with a species from a cold temperate region with high seasonal changes in temperature and food availability was conducted. *C. pagurus* from the North Sea has a limited temperature tolerance window of 14 ± 3 °C, the temperature optimum of 14 °C is also reflected in the maximum activity of the enzyme CS at an assay temperature of 14 °C. No thermal compensation could be detected in the CS analyzes.

- 20 °C are the optimum temperature of *C. setosus* Zoea larvae from the Antofagasta region during austral summer – autumn.
  At 20 °C larvae show:
    - Complete zoeal development with highest survival rates
    - Increase in lipids and proteins
    - moderate oxygen consumption
    - no body deformation
- Optimum salinity of *C. setosus* larvae is 35 PSU.
- A combination of changing parameters like changing temperatures and salinity during ENSO enforces the negative effect on of the endemic ectothermal fauna.

3.1 Cancer pagurus versus *Cancer setosus*

Cancer pagurus and *C. setosus* have a broad geographical distribution (Table 1), which indicates a high thermal tolerance of both species. They display shifts in their distributional range towards higher latitudes. *C. pagurus* is shifting its biogeographical range further northwards (Woll et al. 2006), due to rising SST (Wiltshire & Manly 2004), as it has been shown for a large number of other species (e.g. Perry et al. 2005). The increase in temperature due to global change is a steady but relatively slow process.

In contrast to *C. setosus* which is reproducing irrespective of season, the reproductive cycle of *C. pagurus* follows, as typical for *Cancer* species from boreal and cold temperate waters, the seasonal changes. One egg mass per year is produced in autumn (Table 1), incubated during the winter period and larval hatching occurs in spring when temperatures are favourable and sufficient food is provided by the plankton bloom (Wilson 1999). The speed of embryonic development and the larval release are closely connected to environmental temperature. Therefore it can be assumed that larval hatching occurs within a very narrow temperature frame. The total lack of acclimation capacities to temperature changes underlines this assumption. For example, the aerobic key enzyme CS does not show any temperature compensating changes in larvae reared at different temperatures (see Publication I) (Table 2). Larval cohorts of some populations

might die during summer, because larval thermal tolerance in this species is very restricted (14 ± 3 °C) (Table 2) and the thermal limits are exceeded during hot summers, e.g. the SST near Helgoland frequently reaches >20 °C, (Wiltshire & Manly 2004).

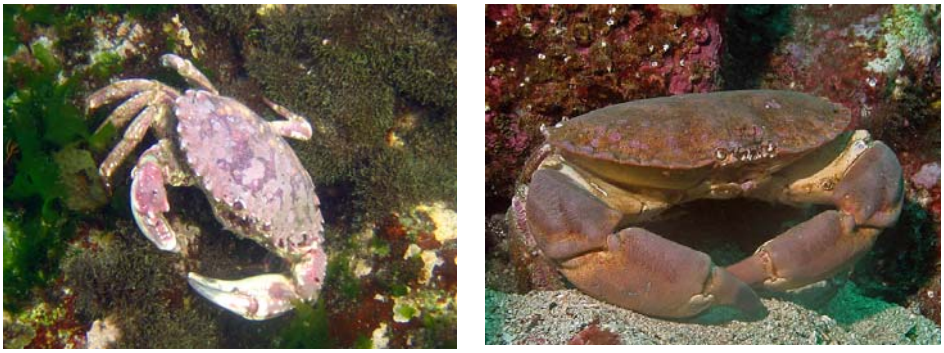

Figure 12. Adult *C. setosus* (left) and *C. pagurus* (right)

Adults are able to avoid warm waters (behavioural thermoregulation) (Lagerspetz & Vainio 2006) by submergence to deeper waters or by shifting the whole range of their habitat towards higher latitudes as it has been observed for a large range of terrestrial and aquatic species (Parmesan & Yohe 2003) including invertebrates (Southward et al. 1995) and fishes (Perry et al. 2005). Additionally, adults are probably less sensitive to elevated summer temperatures in general, and are able to produce a new generation of larvae in the following year.

Favourable temperatures with only weak seasonal changes (Urban 1994) and high food availability during “normal” (non EN/LN) periods allow a year-round reproduction of *C. setosus* from Central Southern to Northern Chile. A year-round reproduction has also been found for *Cancer antennarius* and *Cancer anthony* from the Southern California Current, where stable temperature and food conditions prevail as well (Caroll 1982; Shields et al. 1991). Only close to the extremes of its biogeographic range, reproduction in *C. setosus* is restricted to one annual egg mass, probably due to elevated metabolic costs of living close to the lower and upper temperature thresholds (Fischer & Thatje 2008). Despite a weak seasonality in central distributional regions, slight temperature changes can be measured during the year. In Antofagasta, the temperature ranges between ≤16 °C in August and ≥20 °C in February (Figure 7). *C. setosus* larvae from the Antofagasta region have the capability to enforce their CS activity under low temperatures. Additionally, there is evidence that in warm acclimated larvae an in increase of the thermal

stability of the enzyme at elevated temperatures occurs (Publication IV). The capability of enzyme regulation might be an adaptation to the seasonal changes in temperature of ≥4 °C (Guderley 1990; Segal & Crawford 1994; Seebacher et al. 2003) encountered by year round larval release, but could also be an adaptation to survive the restricted time period that larvae potentially are exposed to varying temperatures during their path of distribution (Figure 5) (see paragraph 1.2.4).

Table 2. Comparison of zoeal *C. setosus* and *C. pagurus*

	C. setosus	*C. pagurus*	Publication No.
Temperature window	≥16 - <22 °C	14 ± 3 °C	I + II
estimated optimum temperature	20 °C	14 °C	
Duration of zoeal phase	35 – 85 days	30 - 40 days	I + II
Cumulative growth (% W)	2940 %	876 %	II
W [µg] Zoea I, freshly hatched	10.6 ± 0.39	15.6 ± 0.5	I + II
C [µg] Zoea I, freshly hatched	3.4 ± 0.05	5.0 ± 0.09	I + II
C/N Zoea I, freshly hatched	4.22 ± 0.04	4.18 ± 0.01	I + II
W [µg] Zoea V, day 3/4	400.4 ± 50.54	129.0 ± 8.50	I + II
C [µg] Zoea V, day 3/4	132.9 ± 57.60	45.7 ± 3.70	I + II
C/N Zoea V, day 3/4	4.15 ± 0.05	4.43 ± 0.23	I + II
CS activity	thermal metabolic compensation in cold + warm conditions	no acclimation processes	IV
CS optimum temperature	20 °C	14 °C	IV

It is particularly striking that the cumulative growth of *C. setosus* is extraordinarily high, not only in comparison with *C. pagurus*, but for decapod crustacean species in general (see Table 2) (Anger 1995). Those high growth rates result from small freshly hatched *C. setosus* (smaller W and C values than in *C. pagurus*) and relatively large (high W and C values) Zoea V larvae, which are much larger than *C. pagurus* Zoea V larvae (see Table 2). The high cumulative growth

rate is afforded by an elongated pelagic larval phase of *C. setosus* that allows for an extended food uptake. An abbreviation of the pelagic larval phase, accompanied by a sufficient maternal energy provisioning in general is regarded as a derived feature in reproduction (Strathmann 1978; Anger 2001). This could imply that the Atlantic *Cancer* species are younger and more derived than the South Pacific species. Since the timing and geography of dispersal between the Atlantic and Pacific oceans is still not fully unravelled and the genetic marker COI (DNA sequence of the mitochondrial cytochrome oxidase I gene) and fossil investigations are not consistent (Harrison & Crespi 1999a), the question of the evolutionary history of *Cancer* crabs can not be answered accurately.

- Reproduction of both *Cancer* species is adapted to the corresponding climatic properties (low seasonality *vs*. high seasonality).
- At the enzymatic level *C. setosus* larvae show a thermal acclimation, whereas in *C. pagurus* no acclimation could be detected.
- Smaller hatchlings and longer pelagic larval phase in *C. setosus* could be regarded as evolutionary ancestral.

3.2 Larval dispersal in small and large spatial and temporal scale

The larval phase is the most important dispersal stage for the majority of coastal marine species, offering a wide variety of means to disperse individuals within and among populations in the fluid environment (Cowen & Sponaugle 2009). Generally, patterns of larval dispersal show a broad coincidence with currents and ocean circulation (Fernandez-Alamo & Faerber-Lorda 2006). Larval dispersal is not only influencing the biology, ecology and evolution of a species (Kinlan & Gaines 2003), but the understanding of population recruitment and the underlying mechanisms also have important applications for species management and conservation (Cowen & Sponaugle 2009). To determine the optimal location and size of potential protected areas, assumed dispersal kernels, mean dispersal distances as well as the knowledge about advective and diffusive processes should be considered, thus ensuring the connectivity among local sub-populations and their capacity for self-replenishment (Cowen et al. 2006).

Larvae of coastal benthic organisms which develop in the plankton for an extended period of time (weeks to months) are suggested to control their path of distribution due to behavioural adaptations (e.g. Sulkin 1984; Shanks 1986). They display vertical migrations in dependence of the prevailing current direction to return into their estuary of origin (Anger et al. 1994) or to travel close to the shore for settlement or to remain in the region of their origin (Shanks 2000). It is known that cancrid larvae off Chile (~36° - 37°S) do not display vertical migrations and that they reside in the uppermost water layer, mainly in the depth of ~10 m (Yannicelli et al. 2006), where they are subject to surface currents. In this paragraph we tried to reveal the consequential option of *C. setosus* larval fate in large scale and in a small local region based on the known facts.

As stated by Gomez-Uchida et al. (2003), there is no distinct differentiation between single stocks of *C. setosus* throughout a wide range of distribution, pointing at a pronounced gene flow between different populations, most likely due to larval dispersal. It is assumed that this exchange takes place in a northerly direction via the Humboldt Current itself, and also in a pole-ward direction by means of the Peru – Chile Counter Current (Gomez-Uchida et al. 2003) (Figure 6). Probably the costal Chile current with an equator-wards direction plays the most important role in the distribution of planktonic organisms. It occurs closest to the shore (Silva 1983) (Figure 6), where the highest abundances of larvae originating from coastal benthic animals can be assumed. In the large scale, the distance of the offshore distribution depends on the westward directed flow of water that determines in which specific current and how long larvae will flow south or north and with which probability they will be able to return to the shore for settlement. During upwelling events, advection generally enforces transport of surface waters away from the coast. Maximum velocities of 30 m/s have been found in front of the Mejillones Peninsula (Giraldo et al. 2002). The dispersal distance is directly dependent on the larval pelagic duration, which is positively correlated with temperature (Shanks et al. 2003).

Larvae that are transported into the Iquique – Antofagasta anticyclonic current (Figure 6), that has an assumed maximum velocity of 28 cm/s and a North – South extension of ~800 km (Silva 1983), would take ~40 days to travel back to their place of origin. A time period of 40 days makes a trip within the cyclone a realistic scenario. Generally, the currents display high maximum velocities in both directions. Assuming that the time for complete zoeal development takes 35 days

at an ambient temperature of 20 °C (see publication II), it would be possible for larvae to travel up to 850 km under “normal” (no EN or LN) oceanographic conditions (Figure 6).

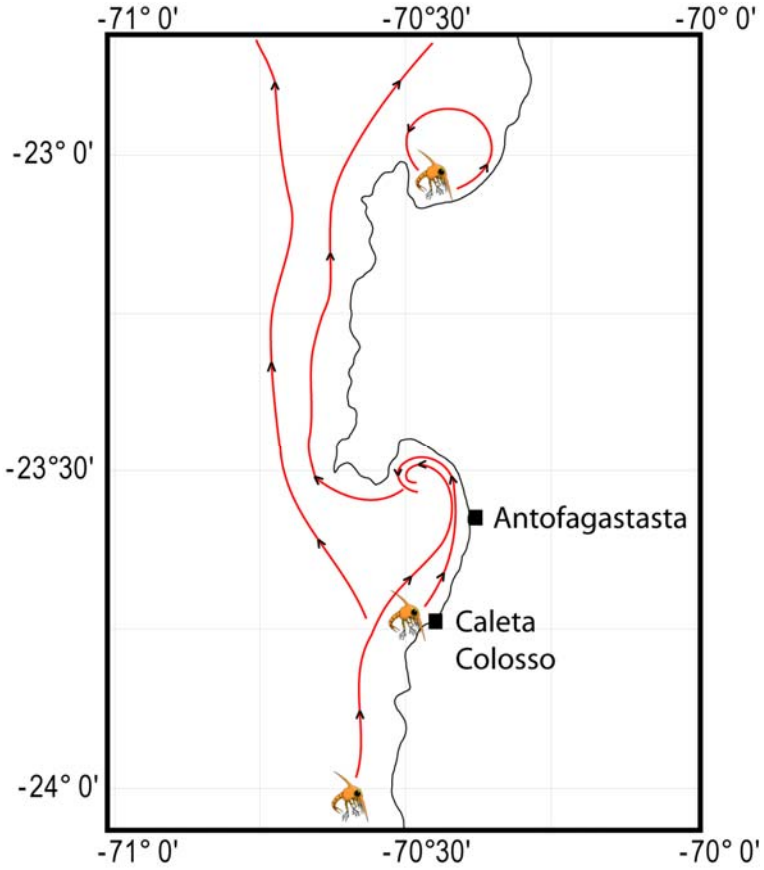

Figure 13. Larval dispersal in the Antofagasta region.

Larval flow is strongly dependent on seasonal changes and local oceanographic conditions such as eddy generation or flow reversals. Modelling the larval distribution would require high-resolution long-time surface current and wind direction data sets (Aiken et al. 2007). In the Antofagasta region, unique oceanographic conditions with high heterogeneity in temperature and currents prevail. Northwest of the Peninsula Mejillones (Figure 5) a local pole-ward flow was detected, which in interaction with a strong upwelling plume in front of the Peninsula generates a narrow (20–40 km) and shallow, re-circulating environment (Marín et al. 2001) with a cold retention zone in the Mejillones Bay (Figure 13). Due to these oceanographic conditions, it is most likely that planktonic organisms from that region retain on their place of origin and are adapted to the temperatures they encounter (see paragraph 3.3).

Larvae from the experimental site of the present study (Caleta Colosso) or nearby hatch into waters of intermediate to cold temperatures (see Figure 5). Due

to the prevailing conditions of the region described in paragraph 1.2.4 (north-ward directed winds) larvae that live in the uppermost layer are transported into the bay, where water temperatures are significantly higher (2 - 3°C) than in the adjacent waters (Piñones et al. 2007). The reported retention time of the water of up to two weeks in the bay indicates that larval development would then take place at those elevated temperatures. The path of the water masses is not fully revealed but it is likely that a certain quantity of larvae remains in their bay of origin due to the cyclonic feature (Figure 13). The temperature distribution might also suggest that water masses that are leaving Antofagasta bay disperse westward in regions of intermediate to warm waters (~20°C) (Figure 5).

This path during development perfectly corresponds to the finding reported in Publication II: zoeal *C. setosus* larvae display an ontogenetic shift in their instar (zoeal stage) specific temperature optimum concerning their growth rates. Zoea I larvae show the highest growth rates an intermediate temperature of 20 °C (hatching in waters of intermediate or cold temperatures), and Zoea II and III show the highest growth rates at higher temperatures of 22°C (retention time in the bay), while the Zoea IV again has higher growth rates at the intermediate temperature of 20 °C (movement out of the bay). Regarding the growth rates, it is also evident that the temperature tolerance decreases with instar, which corresponds with the fact that larvae that hatch south of Antofagasta might encounter a quite wide range of temperatures within a restricted area (Figure 5). During the transition of the bay, temperatures vary in the higher temperature ranges, while the westward directed oceanic region shows a wide distribution of a water mass of a constant temperature.

A shoreward transport to allow the transition as a megalopa to the benthic habitat, where juvenile *C. setosus* can be found (Wolff & Soto 1992) is necessary for successful recruitment. The finding that cancrid larvae off Chile do not display vertical migrations might be biased by the fact that the corresponding publication (Yannicelli et al. 2006) did neither differentiate between different instars nor the four endemic *Cancer* species. A possible scenario might be that older instars (ZV or megalopa) migrate to shoreward moving water masses (Shanks 1986 and publications therein; Aiken et al. 2007). Another scenario might be that during upwelling relaxation periods, surface residing larvae are concentrated on the shoreward side of the upwelling front and are transported in high abundances over the inner shelf close to the coast (Shanks 2000). The latter scenario was designed

for an upwelling region off North Carolina, where the characteristics of the upwelling front seem to be more pronounced than in the Antofagasta region, where coastal water masses show weaker density differences. Here, the high homogeneity of coastal water masses (density variation from δ = 25 – 25.5 within > 200 nautical miles (Silva 1983)), reduce the probability that water masses of different densities form fronts that present barriers for larval distribution. Nevertheless the stratification of coastal waters of course is more pronounced during upwelling events (Piñones et al. 2007). The local process of shoreward transport of larvae in the Antofagasta region could not be clearly identified and might be one or a combination of the above mentioned processes.

Finally, it is likely that the larval recruitment is composed of larvae that stay in their region of origin (self-recruitment) and larvae that recruit from a non-local population source (subsidy) (Cowen & Sponaugle 2009). This composition secures genetic heterogeneity of the population and would ensure recolonization after local extinction events.

- Larval dispersal is crucial for species biology, ecology and evolution and is dependent on ocean currents.
- Larval dispersal is dependent on local characteristics such as coastline topography and resulting particularities like eddies or cyclones.
- *C. setosus* larvae off Antofagasta seem to be acclimated to the oceanographic small scale variations of the region.
- Local recruitment is a combination of self-recruitment and subsidy, as it can be assumed by combining the detected larval retention time in the pelagic environment and small and larger scale oceanographic conditions.

3.3 Recolonization theories

C. setosus especially in its northern distributional area is confronted with the sudden and drastic changes of ENSO. This study shows that larvae cannot survive temperatures ≥24 °C (Publication III, IV), but during strong EN events temperatures might rise >10 °C in comparison with non EN years. Most likely, larvae would already die during intermediate EN events (Pubication II) and supposably elevated

precipitation and freshwater inflow could increase mortality rates (Publication V). Also mass mortalities of adults have been observed during strong EN events (Arntz & Fahrbach 1991). Therefore, this cold-water species is one of the numerous species that are exposed to a continuous local extinction - recolonization process, like e.g. in *Mesodesma donacium* (Riascos et al. 2009).

As it has been described for the rocky inter- and subtidal zone in central Peru by Arntz & Fahrbach (1991), the ecosystem already starts to recover after the onset of temperature equilibration after strong EN events: during the first months the algal assemblage recovers. After one year sessile organisms like barnacles and mytilids settle again and their populations recover quickly, before the populations of predators like seastars and *Cancer* crabs recolonize. The succession of the recolonization process is not totally clear. Reproduction and recruitment are key processes controlling long-term variability and persistence of populations (Beukema & Dekker 2007), but the connectivity of subpopulations is subject to variable biophysical processes which require integrated interdisciplinary approaches (Cowen & Sponaugle 2009). Three different theories of how recolonization of *C. setosus* would be possible after strong EN events are discussed below:

1. Due to behavioural thermal adaptation, adult *C. setosus* might migrate into deeper waters, where conditions are favourable for survival during EN events (oxygen minimum zone is reduced, food availability is sufficient due to more benthic life during EN) (Figure 13, Theory I). Most likely, the following larval generations will fail – they hatch in deeper waters, but decapod larvae are known to migrate to the surface (Sulkin 1984) where temperatures would exceed the thermal threshold (Publication II), but after the recovery of the adults' habitat and complete thermal equilibration, larval recruits could settle successfully. One factor contradicting this hypothesis is the mass mortality of adult *C. setosus* during strong EN events, most likely caused by mass mortality of the main prey items and the exceeded expanses due to higher metabolic rates, resulting in an extinction of the species in this particular area. Arntz & Fahrbach (1991) reported that mortality was species dependent: while *C. setosus* suffered high mortality rates, *C. porteri* and *C. coronatus* retreated into deeper waters and occurred again in artisanal catches half a year later, while *C. setosus* re-occurred after 3 years. Nevertheless, a small number of adults would probably survive in deeper waters and due to the

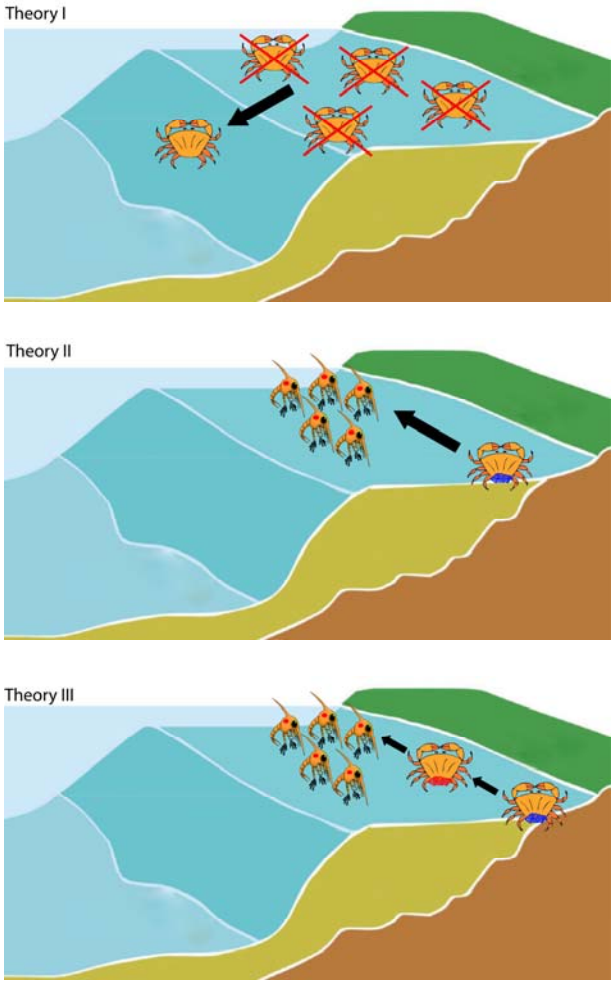

Figure 14. Three theories of recolonisation after mass mortalities of *C. setosus* due to catastrophic EN events. Red – warm acclimated. Blue – cold acclimated

remarkably high reproductive output of *C. setosus* is (Fischer & Thatje 2008), already a low number of adults could sustain the recolonization. Based on the species growth rates (Wolff and Soto 1992) (see chapter 1.3.1) those recruits might contribute to artisanal catches again after 3 years.

2. Recolonization after mass mortalities of adult *C. setosus* in the northern areas after strong EN episodes, supposedly takes place by means of larvae from southern populations (Figure 13, Theory II). Here, specimens are likely adapted to slightly cooler waters (Publication II, III, IV). But in comparison to other ecosystems the latitudinal temperature gradient is not pronounced (Camus 2008). Larval dispersal can be of an extraordinary wide range due to the characteristics of the Humboldt Current, which is underlined by low genetic diversity of *C. setosus* populations along a wide range of the Chilean coast (Gomez-Uchida et al. 2003). *C. setosus* reaches its size of maturity after 2 years (Wolff and Soto 1992) what supports a relatively fast recruitment of the northern distributional area. The question, whether larvae are able to settle in a habitat that provides unfavourable temperatures, thus remains. As reported in Publication II, the temperature tolerance window of larvae spans only ~ 4 °C, and the metabolism responds very sensitively to temperature changes (Publication II + IV). A dispersal of only a limited range could result in temperature changes that might already have unfavourable effects on the larval metabolism. The adaptive responses found in the CS (Publication IV) might be an

indicator that *C. setosus* in fact could be adapted to long dispersal distances that allow the frequently necessary recolonization processes. But the question if and to what extent larvae are able to recolonize the northern area and which adaptations are necessary remains unravelled.

3. Assuming that larval thermal sensitivity is too high and temperature conditioning might probably take place during embryogenesis ("environmental imprinting") as it has been found for the mummichog, *Fundulus heteroclitus* (Tay & Garside 1975), recruitment might take place via migrations of adult specimen. Females of *Cancer* species are known to conduct wide migrations during their life (Wolff & Soto 1992; Bennett 1995). Adults or at least females with stored sperm in the spermatheca might migrate into a habitat with a lower density of *C. setosus* after EN events. Female *C. setosus* are known to be able to produce at least three egg masses without mating (Fischer & Thatje 2008). If the habitat has already recovered (food and shelter by algae) they will find optimum feeding and reproduction conditions and contribute to a fast recovery of the population.

- Due to ENSO *C. setosus* appears to be subject to local *extinction – recolonization processes* in its northern distributional range.
- Recolonization of *C. setosus* might take place via
  1. thermal behavioural adaptation of adults (migration into deeper waters during warm periods)
  2. larval recruitment from southern populations
  3. migration of adults from southern populations or
  4. a combination of those theories.

3.4 Future ENSO scenarios

Based on the knowledge about past and future climate change, models simulating different future scenarios of ENSO have been introduced (1.2.3) (Figure 14). In the following paragraph possible implications of different future scenarios on the distribution of *C. setosus* will be discussed.

The consequence of permanent EN like conditions (e.g. Cane 1998) would be a south-ward displacement of the distributional range of the species as it also

has been shown for a variety of molluscs (Arntz & Fahrbach 1991). In general, under permanent warm conditions, it could be assumed that the vast majority of species of the northern HCS ecosystem would shift further south and a more tropical ecosystem will form in the region of North Chile and Peru. The cold Antarctic water masses still present at the southernmost distributional border might result in a narrowed distributional range of the species. Introduced models simulating more frequent EN events (Timmermann et al. 1999) (Figure 4) and stronger cold and/or warm events in the tropical Pacific Ocean (Timmermann et al. 1999; Huber & Caballero 2003), most likely would result in severe disturbances of the permanent populations of cold-water species in their northern distributional area. Strong EN events would still cause mass mortalities of adults and recruits and stronger LN events would inhibit recruitment success (Publication II, IV). Recolonization due to one or a combination of the 3 above mentioned options is still possible, but would probably not be a long-term establishment. The consequence could again be a south-ward displacement of the distributional range of the species. *Cancer setosus* might then only be a short time visitor in regions where it is a permanent target for artisanal fisheries nowadays.

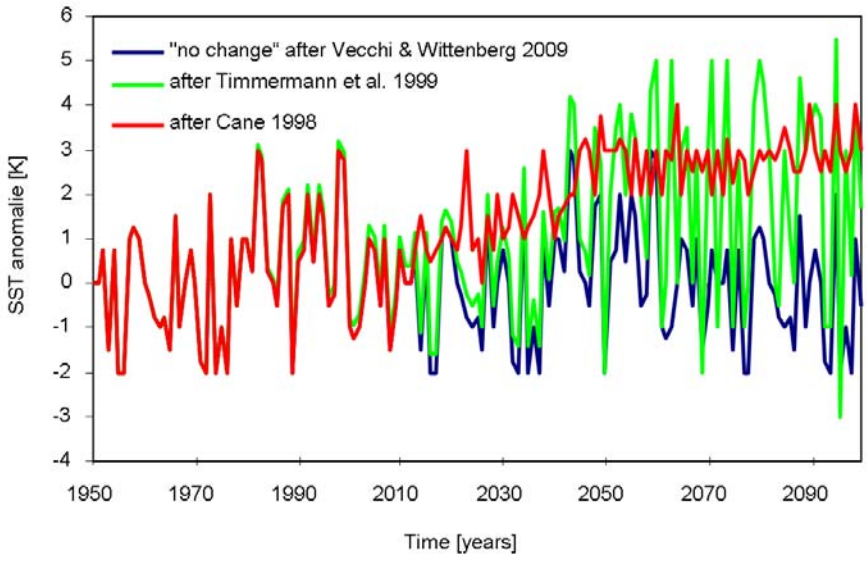

Figure 15. Future ENSO under influence of global warming.
Schematic diagram of different models. Blue = no changes in ENSO (Vecchi & Wittenberg 2009), green = more frequent EN, stronger LN events (after Timmermann et al. 1999), red = shutdown of ENSO, permanent EN like conditions (after Cane 1998).

Eventually population dynamic depends on the characteristic of realistic future changes and more reliable consistent models of future ENSO scenarios would allow biologists to conclude to which extent a stable ecosystem can be established in a region with drastic oscillating changes.

Furthermore, it is not sure whether predicted and recorded changes really base on global warming (Vecchi & Wittenberg 2009), or rather are caused by the "chaotic behaviour" of the tropical Pacific climate. Nevertheless, EN and LN events will likely continue to occur and there will continue to be variation in the character of EN and LN events on different timescales. This will affect the species distribution of a great variety of cold adapted marine invertebrates, but since they might find a cold refuge in higher latitudes, and since populations seem to have been able to survive the extinction – recolonization events for thousands of years, it is likely that those species will continue to exist.

- Under the scenario of permanent EN like conditions, cold adapted species will most likely be shifted southwards.
- Assuming more frequent and stronger EN/LN events, northern populations would underlie severe disturbances and permanent maintenance of the population would be questionable.

3.5 Future perspectives

C. setosus has been the subject of a wide range of investigations during the last years and certainly is one of the best-studied brachyuran crabs off Chile and Peru. Vast knowledge about its ecological and economical importance, about reproductive strategies, behaviour and its life cycle has been collected. Within the present thesis the thermal tolerance window, the capabilities of acclimation processes as well as the phenotypic plasticity of zoeal instars have been investigated for the population of the Antofagasta region.
Based on the results of this thesis the following open questions emerge.

(i) In future studies, the knowledge achieved within this thesis should be extended to the species level, by collecting more comparable data along

the species latitudinal distribution. Those data will improve our knowledge about phenotypic and physiological plasticity and acclimation processes that allow such a wide geographical species distribution.

(ii) *C. setosus* larvae are able to acclimate to changing temperatures only within a very limited range with slight temperature changes already affecting the larval metabolism. Regarding the species distribution and the connected mechanisms of species dispersal, it is of importance to reveal if temperature conditioning of larvae occurs during embryonic development as shown for other marine ectotherms (e.g. fishes; Tay & Garside 1975) or if it is genetically defined.

(iii) Within this thesis, possible scenarios of larval dispersal and recolonization have been discussed. Larval dispersal and population recruitment provide important information for a sustainable species management. Further investigation should be based on previous studies from the marine realm:

a. Larval dispersal of fishes and molluscs can be determined by means of stable isotopes or geochemical signatures of hard parts (e.g. otholits and shells) (Cowen & Sponaugle 2009). Those methods are inapplicable for frequently moulting crustacean larvae; hence genetic markers (e.g. mitochondrial DNA (mtDNA), microsatellites) could be utilized for future labelling experiments in high sampling resolution over the whole range of species distribution.

b. Larval drift can be calculated by high- resolution biophysical models. This approach has been successfully applied modelling the dispersal of fish larvae (e.g. James et al. 2002) and larvae in general (Aiken 2007), but is dependent on high resolution data sets of surface currents, local oceanographic features and wind direction.

c. Further investigation of the behaviour of ovigorous females will provide further information about locations of hatching events, which would improve the precision of models of larval drift.

(iv) HSPs and other molecular chaperones stabilize denaturing proteins, refold reversibly denatured proteins, and facilitate the degradation of irreversibly denatured proteins (Tomanek & Sanford 2003 and publications herein) and might be regarded as an additional acclimation feature. In addition to the metabolic reactions on temperature changes

described in this thesis heat shock proteins (HSPs) provide a deeper insight in the reaction of *C. setosus* larvae on temperature stress.

(v) While studying the properties of the metabolic key enzyme, it has been revealed, that the tolerance of warm temperatures might be restricted by the functioning of CS. Further studies should investigate (a) if this thermal inactivation also occurs in adults and (b) if the thermal restriction of the enzyme cause the general thermal limitation of Cancrid species in general (≤24°C) and (c) what processes allow closely related brachyuran species to live in warmer waters.

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Bremen, den 07. Januar 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.

Monika Weiß