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ECOPHYSIOLOGICAL ADAPTATIONS TO SALINITY VARIATION IN EARLY LIFE-HISTORY STAGES OF DECAPOD CRUSTACEANS

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¿Y por qué están ahí, los cangrejos?

Tim Andersson

“And why are the crabs there?” asked Tim, my three-years-old nephew.

“I am trying to find out”, I answered

CONTENTS

SUMMARY

Estuaries and coastal waters, with their characteristic variations in salinity, represent transitory habitats between the limnic and marine environments. Therefore, the ability to cope with reduced or varying salinities was one prerequisite to invade physically harsh or unstable environments. Adaptations at various levels of organization were necessary. At the populational level, larval export strategies allow vulnerable early ontogenetic stages to avoid stressful environments, while physiologically stronger stages stay at the parental habitats. Larval retention in estuarine habitats requires an ontogenetically early appearance of adaptations at the physiological level. This includes the acquisition of hyper-osmoregulatory capabilities at hatching, based on active ion uptake through the modulation of ion-transporters. At the cellular level, a proliferation of ionocytes and further differentiation takes place in ion-transport tissues in hyper-osmoregulators. At the biochemical and molecular level, modulations of the activity of a key enzyme, the Na^+-K^+-ATPase, must have played a decisive role.

In the present work, I identify effects of osmotic stress on physiological, biochemical and molecular processes in larval and early juvenile decapod crustaceans. These effects influence, directly or indirectly, also populations and communities. At the population level, I experimentally evaluated the potential importance of osmotic gradients that must be passed during the upstream migration of the Megalopa stage of a freshwater-inhabiting crab, *Armases roberti*. A direct exposure to low salinities (<5 ‰) caused high mortality rates and greatly delayed metamorphosis. By contrast, a stepwise acclimation to low salinities allowed for lower mortality rates and even metamorphosis in freshwater. The speed of this ontogenetic migration is limited by physiological constraints in the capability of osmoregulation. On the other hand, it is stimulated by increasing levels of odours from conspecific adults. At the physiological level, I evaluated the relationships between biomass variations in response to short- and long-term exposure to reduced salinities, and species- or stage-specific osmoregulatory capacity. Differences in the ontogeny of osmoregulation in *Armases miersii*, *A. roberti*, and *A. ricordi* reflect differential reproductive strategies in these euryhaline species. By contrast, biomass in stenohaline larvae (*Liocarcinus pusillus*, *Nephrops norvegicus*, *Hyas araneus*) is more sensitive to hypo-osmotic stress. In general, detrimental effects of reduced salinities on growth decreased with increasing osmoregulatory capacities. Hence, the ability to buffer the internal medium stabilizes metabolic processes and allows for larval growth also at reduced salinities. The metabolic costs for osmoregulation in the early life-history stages of decapod crustacean may thus offset by the capacity of maintaining high

growth rates in variable environments. After a long-term exposure to hypo-osmotic stress since hatching, however, the hyper-osmoregulating Megalopa of *Chasmagnathus granulata* was not able to reverse the cumulative negative effects of continued osmotic stress. Such effects may influence the number and quality of larvae reaching metamorphosis, and therefore may be important also for population dynamics.

The hyper-osmoregulatory capacity is directly related to the modulation of the activity of Na^+-K^+-ATPase. Therefore, at the tissue and biochemical levels, I studied the response of this enzyme activity to salinity variations. In juvenile *Eriocheir sinensis* exposed for ca. 1 year to a reduced salinity (5 ‰), the enzyme activity was higher than in crabs kept in 25 ‰. This enhancement was achieved by differentiation of the ion-transport tissues (i.e. an enlargement of the lamellae in the posterior gills and a proliferation of ionocytes), which in turn allows for successful development and growth at reduced salinities.

At the molecular level, investigations with *Armases miersii* showed that, the increase in the activity of the sodium-pump in early life-history stages is achieved through *de novo* synthesis of Na^+-K^+-ATPase molecules.

In conclusion, successful invaders of habitats characterised by hypo-osmotic conditions must show strong hyper-osmoregulatory capacities, stable growth at reduced salinities, strongly differentiated ion-transporting tissues, high activities of Na^+-K^+-ATPase, and an enhanced expression of mRNA encoding for the Na^+-K^+-ATPase α-subunit. In addition, ontogenetic patterns of osmoregulation must reflect characteristic changes in the environmental conditions that each stage must face in nature.

ZUSAMMENFASSUNG

Ästuarien und Küstenwässer stellen aufgrund ihrer charakteristischen Salzgehaltsschwankungen Übergansbereiche zwischen Süß- und Salzwassersystemen dar. Die Fähigkeit, bei niedrigen bzw. schwankenden Salinitäten zu bestehen, ist deshalb eine wichtige Voraussetzung für die Besiedlung solcher Habitate. Dies erfordert Anpassungen auf verschiedenen Organisationsebenen. Auf der Populationsebene ermöglichen larvale Exportstrategien den frühen, physiologisch meist besonders empfindlichen Entwicklungsstadien eine Vermeidung osmotischer Stressbedingungen, während spätere, weniger empfindliche Stadien im Lebensraum der Adulten verbleiben können. Ein Überleben von Larvenstadien in Brackwasser erfordert spezielle physiologische Anpassungen, insbesondere eine schon zum Zeitpunkt des Schlüpfens ausgeprägte Fähigkeit zur Hyper-Osmoregulation. Diese basiert auf einer aktiven Ionenaufnahme, die durch Modulation von Ionentransportern erreicht wird. Auf histologischer und zellulärer Ebene erfolgt hierzu eine Vermehrung der Ionozyten sowie eine Ausdifferenzierung der Ionen-Transportgewebe. Auf biochemischer und molekularer Ebene spielt eine Regulation des Schlüsselenzyms Na^+-K^+-ATPase bei der Besiedlung osmotisch variabler Lebensräume eine bedeutende Rolle.

In der vorliegenden Arbeit beschreibe ich Auswirkungen osmotischen Stresses auf physiologische, biochemische und molekulare Prozesse bei larvalen und juvenilen Entwicklungsstadien dekapoder Krebse. Derartige Effekte können sich direkt oder indirekt auch auf Populationen und Gemeinschaften auswirken. In diesem Zusammenhang untersuchte ich in Laborexperimenten die potenzielle Bedeutung osmotischer Gradienten, die während flussaufwärts gerichteter Wanderungen im Megalopa-Stadium der Süßwasser-bewohnenden Krabbe *Armases roberti* durchlaufen werden müssen. Wurden die Larven unmittelbar einer geringen Salinität (<5 ‰) ausgesetzt, dann war die Mortalität hoch und die Metamorphose stark verzögert. Eine schrittweise Akklimatisierung an geringe Salinitäten hingegen erhöhte die Überlebensrate und ermöglichte sogar eine Metamorphose in Süßwasser. Die Geschwindigkeit der Rückwanderung wird demnach durch physiologische Prozesse begrenzt, während von artgleichen erwachsenen Krabben abgesonderte chemische Signalstoffe stimulierend auf die Einwanderung der Megalopen wirken.

Auf physiologischer Ebene untersuchte ich den Zusammenhang zwischen stressbedingten Veränderungen des Larven-Wachstums (ermittelt anhand der Biomasse) unter kurz- oder langzeitig herrschenden Bedingungen niedriger Salinität einerseits und den art- bzw. stadienspezifischen osmoregulatorischen Fähigkeiten andererseits. Unterschiede in der Ontogenie der Osmoregulation zwischen den Krabben *Armases miersii*, *A. roberti* und *A.*

ricordi spiegeln unterschiedliche Reproduktionsstrategien dieser euryhalinen Arten wider. Das Wachstum stenohaliner Larven (*Liocarcinus pusillus*, *Nephrops norvegicus*, *Hyas araneus*) hingegen reagierte empfindlicher auf hypo-osmotischen Stress. Allgemein sank der negative Einfluss niedriger Salinitäten auf das Wachstum mit steigenden osmoregulatorischen Fähigkeiten. Die Fähigkeit, die Ionenkonzentration im Innenmedium mit Hilfe von Osmoregulation gegen Schwankungen im Außenmedium zu puffern, stabilisiert somit den Stoffwechsel euryhaliner Arten und ermöglicht ein Larvenwachstum auch bei reduzierten Salzgehalten. Wirkt hypo-osmotischer Stress jedoch längerfristig, seit dem Zeitpunkt des Schlüpfens, dann kann auch eine relativ stark hyper-regulierende Megalopa die kumulativen Effekte des bis dahin anhaltenden Stresses nicht mehr kompensieren, wie ich in Experimenten zum Wachstum der Larven der Krabbe *Chasmagnathus granulata* unter kontinuierlichem Einfluss geringer Salinität zeigen konnte. Solche kumulativen Effekte können die Anzahl und die Fitness metamorphisierender Larven mindern und sich so auch auf die Populationsdynamik auswirken.

Hyper-osmoregulatorische Fähigkeiten hängen direkt mit der Regulierung der Na^+-K^+-ATPase-Aktivität zusammen. Auf der biochemischen und zellulären Ebene habe ich deshalb Änderungen der Enzymaktivität bei verschiedenen Salinitäten gemessen. Juvenile *Eriocheir sinensis*, die für ca. ein Jahr einer geringen Salinität ausgesetzt waren, wiesen eine erhöhte Enzymaktivität auf. Diese Steigerung wurde durch eine Differenzierung der Ionentransportgewebe erreicht (d.h. Verdickung der Lamellen und Vermehrung der Ionozyten in den hinteren Kiemen), wodurch eine erfolgreiche Entwicklung und ein Wachstum auch bei geringen Salinitäten ermöglicht wurde. Auf molekularer Ebene zeigten Untersuchungen an den Larven von *A. miersii*, dass, ähnlich wie bei adulten Tieren, der Anstieg der Na^+-K^+-ATPase-Aktivität durch *de novo* Synthese der Na^+-K^+-ATPase-Moleküle erreicht wird.

Zusammenfassend lässt sich sagen, dass erfolgreiche Besiedler von Lebensräumen mit hypo-osmotischen Bedingungen ausgeprägte hyper-osmoregulatorische Fähigkeiten, ein stabiles Wachstum auch bei niedrigen Salinitäten, stark differenzierte Ionen-Transportgewebe, eine hohe Na^+-K^+-ATPase-Aktivität sowie eine erhöhte Expression der die Na^+-K^+-ATPase-α-Untereinheit codierenden mRNA aufweisen. Ontogenische Änderungen in der Fähigkeit zur Osmoregulation spiegeln charakteristische Änderungen in den Umweltbedingungen wider, denen die aufeinander folgenden Entwicklungsstadien in der Natur ausgesetzt sind.

I. GENERAL INTRODUCTION

All organisms interact with the external medium, being in permanent exchange of matter and energy through physiological processes (i.e., feeding, respiration and excretion), and of information through the sensory organs. Simplified, they may be described as an aqueous solution contained inside a semi-permeable membrane, the body surface. In order to ensure survival, an adequate functioning must be ensured through the stabilisation of the internal medium. Therefore, variations in the surrounding medium must somehow be compensated (Vernberg and Vernberg, 1975).

In oceanography, salinity is defined as the total concentration of dissolved inorganic salts, expressed as g/kg water, or ‰ (=ppt; also referred to as “Practical Salinity Units, PSU). In the marine environment, both total salinity (33-36 ‰) and the relative ionic composition show only slight variations caused mainly by seasonal ice melting in Polar Regions and evaporation in the equatorial zones (Kalle, 1971). By contrast, coastal and estuarine waters do not show such a physical stability; salinity fluctuates here regionally, locally and seasonally. Since organisms require an internal steady state in the osmotic and ionic medium to ensure the adequate functioning of metabolic and physiological processes, salinity plays a key role for survival and development of species inhabiting coastal zones (Anger, 2003). Salinity variation influences biological processes on different levels of organisation: (1) populational, (2) physiological, (3) biochemical, and (4) molecular (Newell, 1976; Anger, 2003, Cieluch et al., 2004; Luquet et al., 2005).

There is a large amount of information available concerning salinity effects on adult stages of aquatic invertebrates, in particular crustaceans (reviewed in Péqueux, 1995; Lucu and Towle, 2003). However, much less is known about effects of osmotic stress on the early life-history phase of crustaceans, namely the embryonic, larval, and early juvenile stages. Planktonic larval stages of benthic decapod crustaceans may differ considerably from the adults in morphology, anatomy and physiology, because of differential adaptations to pelagic and benthic environments, respectively. Therefore, the study of effects of salinity variation on larval and juvenile stages has recently become a major subject of intensive and stimulating research efforts in

larval biology (for review see Charmantier, 1998; Anger, 2001, 2003). This is the topic of the present work.

The general objective of this thesis is to identify and explain effects of osmotic stress on physiological, biochemical and molecular processes in larval and early juvenile decapod crustaceans. All those effects may eventually affect also, directly or indirectly, populations and communities. In the section below, I give a brief overview of effects of exposure to salinity variation on decapod crustaceans, according to the different levels of organization, as an introduction to individual chapters and particular objectives.

I.1. Effects of salinity variation on population processes in decapod crustaceans

The influence of salinity at the population level occurs through variation in mortality, reproduction and migrations. For example, stenohaline decapod crustaceans (*Hyas araneus*, *Cancer pagurus*, *Nephrops norvegicus*), unable to cope with osmotic stress, occupy osmotically stable oceanic environments. Other species show different levels of euryhalinity, at least during the adult phase, for instance the shore crab *Carcinus maenas*, estuarine crabs such as *Chasmagnathus granulata*, or tropical mangrove crabs including *Armases ricordi*, *Armases roberti* and *Armases miersii*. These are found in a variety of physically less stable habitats ranging from coastal waters with little osmotic stress, estuaries with moderate variability in osmotic conditions, to highly stressful environments including rivers or supratidal rock pools (Christiansen, 1982; Anger, 1995a; Torres et al., 2006; Anger et al., in press).

The larval stages do not always show the same patterns as the conspecific adults. In euryhaline species, the conquest of variable environments such as estuaries involved the evolution of two main strategies (Strathmann, 1982): (a) larval retention in the parental habitat, (b) larval export to physically more stable waters. The retention strategy is successful only in those species, whose early stages are able to survive osmotic stress. It is therefore considered as a step towards an invasion of freshwater and terrestrial habitats (Anger, 2001). If the larval stages are

not able to survive in physically harsh environments, they cannot possibly develop in the habitats of the euryhaline conspecific adults. The early stages must thus be exported from freshwater or estuaries to coastal marine waters and only later stages can return to the parental habitat.

During their re-immigration into the parental habitat, the late stages experience an increasing osmotic stress in salinity gradients. In addition, they might receive signals (maternal cues) that could stimulate the migration process by accelerating the metamorphosis (for review, see Gebauer et al., 2003). In an experimental study of the survival and development of the megalopae of an exporting crab, *Armases roberti*, I evaluated the effects of exposure to gradual salinity reductions and increments in adult odours. The Megalopa and the early juveniles of this species are physiologically able to begin the upstream migration towards the parental freshwater habitats and may thus be an ideal model organism to explore effects of salinity gradients on the survival and development during the re-immigration process.

I.2. Effects of osmotic stress on physiological processes in larvae

Variations in salinity may affect the volume of water and the concentration of soluble compounds in an organism, and in consequence, alter the performance of its internal systems. Physico-chemical laws regulate the exchange of water and solutes across membranes. On the one hand, solutes move passively by diffusion or through channels, depending on electrical and chemical gradients between the intra- and extracellular medium (Nelson and Cox, 2000). On the other hand, active pumps may transport solutes, independent of existing gradients. This active transport leads to a steady state and to the establishment of a permanent gradient between the internal medium and the environment.

To ensure a correct performance of the cells, the composition of the intra- and extracellular fluids must be near the optimum. The maintenance of a stable cellular composition is achieved through osmoregulatory strategies (for review see Péqueux, 1995). In osmoconformers, the concentrations of the principal osmotic effectors (mainly Na^+ and Cl^-) in the extracellular fluid remain isosmotic with the environment.

Within relatively narrow limits, variations in the osmotic pressure of body fluids can be compensated by means of intracellular regulation (see section below). By contrast, osmoregulators are able to maintain the extracellular fluid constant, independent of environmental changes in salinity (Mantel and Farmer, 1983). This mechanism belongs to the principal issues of this comparative study.

Most of the marine crustaceans (e.g. *Cancer pagurus*, *Hyas araneus*) are osmoconformers (Péqueux, 1995; Pfaff, 1997). Since they do not possess mechanisms to compensate great changes in salinity, their hemolymph is isosmotic with the external medium (Fig. 1). These species are therefore restricted to habitats with little variability in salinity (Péqueux, 1995). A smaller group of crustaceans can occupy also environments with variable salinity conditions, because they are osmoregulators. They are classified in three groups: weak hyper-osmoregulators, strong hyper-osmoregulators, and hyper-hypo-osmoregulators (Fig. 1). The first two types are hyper-iso-regulating organisms. They maintain their extracellular fluid hyper-osmotic (i.e. more concentrated than the surrounding media), when they are exposed to a dilute environment, but remain isosmotic, when they are confronted with highly concentrated media. Weak hyper-osmoregulators (for example *Homarus gammarus*; Charmantier, 1999) are able to cope with a hypo-osmotic environment only for a restricted time, and they are not able to migrate to freshwater (Felder, 1978). By contrast, strong hyper-osmoregulators like *Eriocheir sinensis* (Gilles, 1975; Péqueux and Gilles, 1978; Cieluch et al., in press) are capable of maintaining their extracellular fluids even in freshwater hyper-osmotic. Some of these species (e.g. *Pseudothelphusa jouyi*, *Metapaulias depressus*; Thompson, 1970) are fully adapted to freshwater and incapable to return to seawater. Hyper-hypo-osmoregulators (e.g. *Armases roberti*, *A. ricordi*, *A. miersii*; Schubart and Diesel, 1998; Charmantier et al., 1998) have the ability to maintain their extracellular fluid almost constant in a wide salinity range from concentrated sea water (> 35 ‰) to freshwater.

The difference in osmotic concentration (osmolality) between the hemolymph and the external media is termed osmoregulatory capacity (OC). It can be used as a comparative measure to evaluate the strength of osmoregulation in different species or developmental stages exposed to various salinity conditions. It also provides information about the direction of the response to osmotic stress, i.e. positive OC values indicate hyper-osmoregulation, negative values hypo-osmoregulation (for

reviews see Péqueux, 1995; Charmantier, 1998; Charmantier and Charmantier-Daures, 2001).

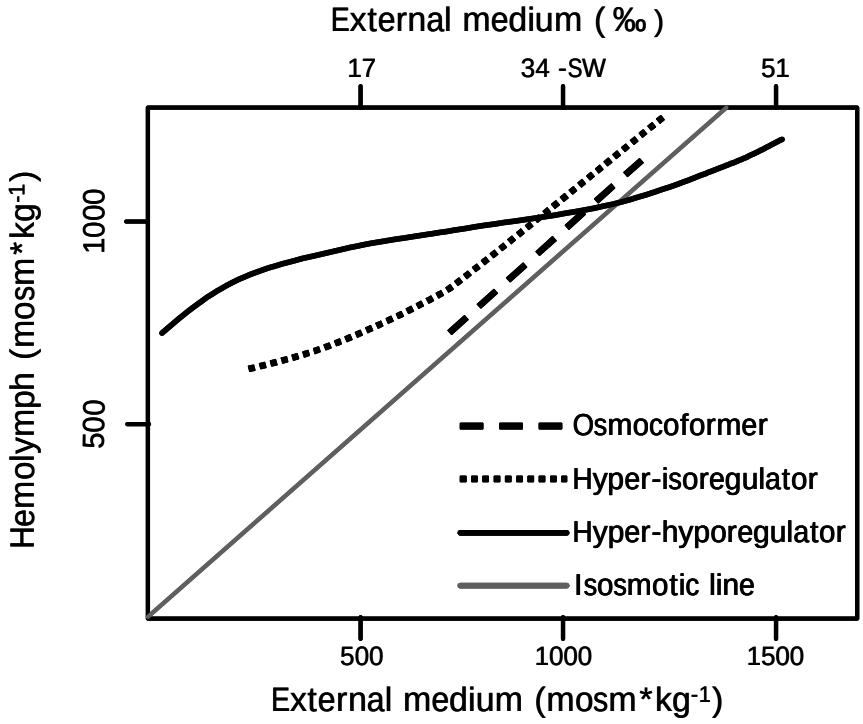

Fig. 1. Patterns of hemolymph osmoregulation in aquatic crustaceans (After Péqueux, 1995). Hemolymph and external medium osmotic concentration given as osmolality (SW: seawater).

The pattern of osmoregulation may vary during the ontogeny of an organism. Species with a larval export strategy (see section II.2 for details) inhabit during their life-cycles different environments, which is commonly reflected in the ontogeny of osmoregulation (reviewed in Charmantier, 1998).

Associated with species-specific or ontogenetic changes of osmoregulation patterns, there should be changes in the effects of salinity variation on larval biomass and growth. In my Diploma thesis (Torres et al., 2002), I explored such relationships using the Zoea-I stages of four species of decapod crustaceans. This work suggested that an exposure to low salinities caused weaker effects on biomass and growth of osmoregulating larvae, especially in the lipid content, as compared to osmoconformers. In the present thesis, I tested this hypothesis by evaluating effects of salinity on biomass and growth (1) in Zoea-I larvae of three additional osmoconforming species (*Hyas araneus*, *Nephrops norvegicus*, *Liocarcinus pusillus*), and of stronger osmoregulators (*Perisesarma fasciatum*, *Sesarma curacaoense*) compared to those used by Torres et al. (2002); (2) during the ontogeny of species,

which differ in their ontogenetic patterns of osmoregulation (*Chasmagnathus granulata*, *Armases ricordi*, *A. roberti* and *A. miersii*). These new data may aid to a model, linking osmoregulatory capacity with effects of salinity on growth in decapod crustacean larvae.

I.3. Biochemical responses to reduced, optimal and enhanced salinities

The influence of salinity variation is related to changes in the concentrations of single osmotic effectors such as Na^+, K^+ and Cl^-, which can affect the functions of enzymes, hormones and other vital molecules. For example, in the lobster *Homarus gammarus* and the crayfish *Astacus astacus,* changes in the activities of the lactate-dehydrogenase and 3-glycerophosphat dehydrogenase were related to the concentration of NaCl (reviewed in Schoffeniels and Gilles, 1970). However, the most important aspects of biochemical responses to salinity variations are those closely related to osmoregulation. This involves the “pumps and leaks” system through limiting and compensatory processes (Péqueux, 1995). *Limiting processes* are mostly used by osmoconformers, acting on the permeability properties of the boundary structures (e.g. body wall) in order to minimize the diffusive movements of osmotic effectors (Schoffeniels and Gilles, 1970). *Compensatory processes* involve active movements of solutes to compensate diffusive fluxes between spaces inside and outside the cell. Florkin (1960) defined two mechanisms of compensation: isosmotic cellular regulation and anisosmotic extracellular regulation (reviewed in Péqueux, 1995). *Isosmotic cellular regulation* implies that the intracellular osmotic pressure is maintained isosmotic with the extracellular pressure regulating the level of intracellular osmotic effectors (e.g. amino acids and phosphoric compounds; Schoffeniels and Gilles, 1970).

Here, I will focus on *anisosmotic extracellular regulation*, where the osmotic concentration of the extracellular fluid is maintained constant. This occurs (a) passively due to the Donnan effect (*extra osmotic pressure of protein solutions* caused by impermeable protein molecules resulting in uneven distribution of small, permeant cations and anions in blood plasma); upon the dissolved proteins; and

upon a modification of the permeability of the membranes for salts and water; (b) actively as an ion transport through enzymes such as Na^+-K^+-ATPase. The Donnan effect and changes in the permeability play only a small role in the regulation in euryhaline species (reviewed in Péqueux, 1995).

Na^+-K^+-ATPase plays the central role in active osmoregulation. The active absorption of ions counteracts salt losses through the cellular membrane. Na^+ tends to enter or leave the cell depending on the extracellular fluid concentration, which in turn depends on the concentration of the external medium. When an animal is exposed to dilute media, the Na^+ concentration should decrease in the apical extracellular fluid (i.e. the fluid between the cuticle and the apical membrane) causing a passive loss of Na^+ from the cell through the apical Na^+ channels. To counteract this loss, Na^+-K^+-ATPase actively transports Na^+ outside the cell, passing through the basolateral membrane towards the hemolymph (i.e. into the basolateral extracellular fluid; Fig. 2). Therefore, the Na^+ concentration inside the cell will decrease and Na^+ ions will tend to enter the cell through the apical channels using the electrochemical gradient as driving force.

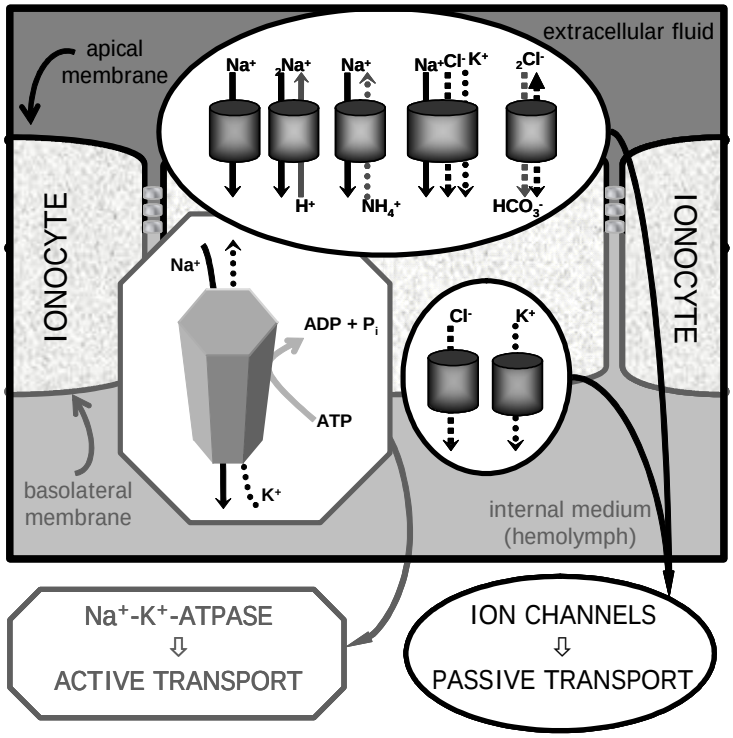

Fig. 2. Conceptual model of an ion-transporting cell. Na^+ movements during exposure to dilute media: Na^+ leaves the cell towards the hemolymph ACTIVELY (through Na^+-K^+-ATPase activity) reducing the Na^+ concentration inside the cell and generating a new concentration gradient; then Na^+ from the diluted environment enters the cell PASSIVELY through the ion channels, following the newly established concentration gradient (After Torres, 2001)

The activity of this enzyme is highly dependent on the salinity experienced by an organism, increasing during exposure to reduced salinity (e.g. *Carcinus maenas*: Siebers et al., 1982, 1983; *Eriocheir sinensis*: Péqueux et al., 1984; *Homarus gammarus*: Flick and Haond, 2000). By contrast, the activity decreases during an increase of salinity, i.e. transfer from fresh to brackish water (e.g. *Procambarus clarkii*: Sarver et al., 1994; *Macrobrachium rosenbergii*: Wildera et al., 2000).

In order to investigate the relationships between salinity and biochemical changes in early life-history stages of crustaceans, I studied changes in the localisation and in the activity of Na^+-K^+-ATPase of early juvenile stages of *Eriocheir sinensis* in response to prolonged exposure to reduced, optimal, and enhanced salinities. This species shows a larval export strategy; the strongly osmoregulating juveniles re-immigrate into the riverine parental habitat, facing an increasing osmotic stress. This special characteristic makes this species an interesting model for the investigation of changes in ion-transporting tissues and enzymes caused by salinity variation.

I.4. Differential expression of mRNA encoding for transport enzymes

The physiological and biochemical mechanisms by which euryhaline organisms respond to osmotic stress are little explored at the molecular level, in particular regarding gene expression in crustaceans (Towle et al., 2001). The ion-transporting enzyme Na^+-K^+-ATPase plays a major role in osmoregulation, and therefore in the response to osmotic stress. Hyper-osmoregulating organisms exhibit an increased Na^+-K^+-ATPase activity when exposed to dilute media. However, classical biochemical activity measurements do not elucidate whether the observed increase in activity is due to a regulation of pre-existing Na^+-K^+-ATPase molecules (e.g. post-translational processes, membrane trafficking or cell signalling), or the result of enhanced gene transcription and/or translation (e.g. *de novo* mRNA synthesis). Newly developed molecular techniques, namely reverse-transcription-PCR (RT-PCR) and quantitative real-time-PCR (Q-PCR), allow for the acquisition of information concerning the relationship between activity and gene transcription-translation.

Studies conducted on gills of adult specimens of *Chasmagnathus granulata* and *Scylla paramamosain* showed an increase in the mRNA quantity after exposure to diluted media, suggesting a *de novo* synthesis of the ion-transporting enzyme (Luquet et al., 2005, Chung and Lin, 2006).

The scarce information existing on this topic was obtained exclusively from adult crabs, while no information concerning the early life stages has been available. This is the theme of the last paper of my thesis, focussing on effects of osmotic stress on the expression of mRNA coding for Na^+-K^+-ATPase in strongly hypo-hyper-osmoregulating early stages of a tropical crab, *Armases miersii*.

SUMMARY OF OBJECTIVES AND OUTLINE OF THE THESIS

Throughout this thesis, I study effects of salinity variation on larval and early juvenile crustaceans at various levels of biological organization:

CHAPTER III: POPULATION LEVEL

Survival and development during experimentally simulated upstream migration of megalopae

- Effects of salinity

  Model species: *Armases roberti*

  PUBLICATION 1

- Effects of adult odours

  Model species: *Armases roberti*

  PUBLICATION 2

CHAPTER IV: PHYSIOLOGICAL LEVEL

Larval growth and biomass variation during exposure to variations in salinity

- Comparison of effects throughout the larval development in species with different life histories and ecophysiological characteristics

  Model species: *Armases miersii*, *A. roberti* and *A. ricordi*

  PUBLICATION 3

- Relationships between biomass variations in response to salinity changes and species- or stage-specific osmoregulatory capacity

  Model species: *Hyas araneus*, *Liocarcinus pusillus*, *Nephrops norvegicus*, *Perisesarma fasciatum*, *Sesarma curacaoense*, and additional species from other studies: *Cancer pagurus*, *Homarus gammarus*, *Carcinus maenas*, *Chasmagnathus granulata* (Torres et al., 2002), *Armases miersii*, *A. roberti*, *A. ricordi* (PUBLICATION 3).

  PUBLICATION 4

- Cumulative effects of long-term exposure to low salinities (throughout larval development) on biomass

  Model species: *Chasmagnathus granulata*

  PUBLICATION 5

CHAPTER V: BIOCHEMICAL LEVEL

Location and activity of Na^+-K^+-ATPase

- Effects of long-term exposure to reduced, optimal and enhanced salinities in juvenile crabs

  Model species: *Eriocheir sinensis*

  PUBLICATION 6

CHAPTER VI: MOLECULAR LEVEL

Expression of mRNA coding for the Na^+-K^+-ATPase α-subunit in early life-history stages

- Effects of exposure to reduced, optimal and enhanced salinities

  Model species: *Armases miersii*

  PUBLICATION 7

LIST OF PUBLICATIONS

This cumulative thesis includes seven publications as listed below. In addition, my contribution to each study is detailed.

CHAPTER III: POPULATION LEVEL

- PUBLICATION 1: Torres G, Anger K, Giménez L, 2006.

  EFFECTS OF REDUCED SALINITIES ON METAMORPHOSIS OF A FRESHWATER-TOLERANT SESARMID CRAB, *ARMASES ROBERTI*: IS UPSTREAM MIGRATION IN THE MEGALOPA STAGE CONSTRAINED BY INCREASING OSMOTIC STRESS?

  Journal of Experimental Marine Biology and Ecology 338, 134-139.

I performed the experiments. The analysis and interpretation of the data was done in joint cooperation with the third author. I wrote the manuscript. The final version was achieved considering revisions by both coauthors.

- PUBLICATION 2: Anger K, Torres G, Giménez L, in press.

  METAMORPHOSIS OF A SESARMID RIVER CRAB, *ARMASES ROBERTI*: STIMULATION BY ADULT ODOURS VS. INHIBITION BY SALINITY STRESS

  Marine and Freshwater Behaviour and Physiology

I performed the experiments. The analysis and interpretation of the data was done in joint cooperation with all authors. The first author wrote the manuscript and the final version was discussed with all coauthors.

CHAPTER IV: PHYSIOLOGICAL LEVEL

- PUBLICATION 3: Torres G, Giménez L, Anger K, in prep.

  EFFECTS OF REDUCED SALINITIES ON LARVAL GROWTH AND PROXIMATE BIOCHEMICAL COMPOSITION ARE RELATED TO LIFE-HISTORIES: THE GENUS *ARMASES* AS A MODEL

I developed the scientific idea, performed the experiments, did the analysis and interpretation of data and wrote the manuscript. The manuscript was improved in cooperation with both coauthors.

- PUBLICATION 4: Torres G, Giménez L, Anger K, in prep.

  RELATIONSHIPS BETWEEN GROWTH, EURYHALINITY AND OSMO-REGULATION IN DECAPOD CRUSTACEAN LARVAE

I performed the experiments. The analysis and interpretation of data, as well as writing the manuscript were done in cooperation with the second author. The final version was discussed with both coauthors.

- PUBLICATION 5: Torres G, Giménez L, Anger K, in prep.

  CUMULATIVE EFFECTS OF LOW SALINITY ON LARVAL GROWTH AND PROXIMATE BIOCHEMICAL COMPOSITION IN AN ESTUARINE CRAB, *CHASMAGNATHUS GRANULATA*

I developed the scientific idea, performed the experiments, did the analysis and interpretation of data and wrote the manuscript, which was improved in cooperation with both coauthors.

CHAPTER V: BIOCHEMICAL LEVEL

- PUBLICATION 6: Torres G, Charmantier-Daures M, Chifflet S, Anger K, submitted.

  EFFECTS OF LONG-TERM EXPOSURE TO DIFFERENT SALINITIES ON THE LOCATION AND ACTIVITY OF NA^+-K^+-ATPASE IN THE GILLS OF JUVENILE MITTEN CRAB, *ERIOCHEIR SINENSIS*

  Comparative Physiology and Biochemistry A

I developed the scientific idea and performed the experiments. I measured the enzymatic activity, and analysed and interpreted these data. The enzymatic activity method was adapted in cooperation with Dr. S. Chifflet. The histological data were obtained in cooperation with Dr. M. Charmantier-Daures. I wrote the manuscript and the final version was discussed with all coauthors.

CHAPTER VI: MOLECULAR LEVEL

- PUBLICATION 7: Torres G, Boulo V, Spanings-Pierrot C, Cucchi-Moulliot P, Anger K, Charmantier G, submitted.

  EFFECTS OF SALINITY ON GENE EXPRESSION OF NA^+-K^+-ATPASE IN THE EARLY LIFE-HISTORY STAGES OF A EURYHALINE CRAB

  Journal of Experimental Biology

I developed the scientific idea and performed the experiments. I was introduced into the molecular procedures in the laboratory of Prof. Dr. Guy Charmantier. The design of the specific-primers, as well as the Q-PCR measurements was achieved with the collaboration of Dr. Viviane Boulo. I did the analysis and interpretation of data and wrote the manuscript. The manuscript was improved with the cooperation with all coauthors.

For all studies, the adult population was maintained by Uwe Nettelmann. Identification and sorting of larval stages was done by myself, Dr. Luis Giménez and Uwe Nettelmann.

II. MODEL SPECIES

The following model species were chosen based on their different ecological and physiological characteristics and their diverse responses to osmotic stress.

II.1. Marine species (stenohaline)

Fully marine species are characterised by their stenohalinity, i.e. they are incapable to cope effectively with variations in salinity. Therefore, all life cycle stages must develop in osmotically stable marine habitats, so that the larval stages share the environment of the conspecific adults (Fig. 3).

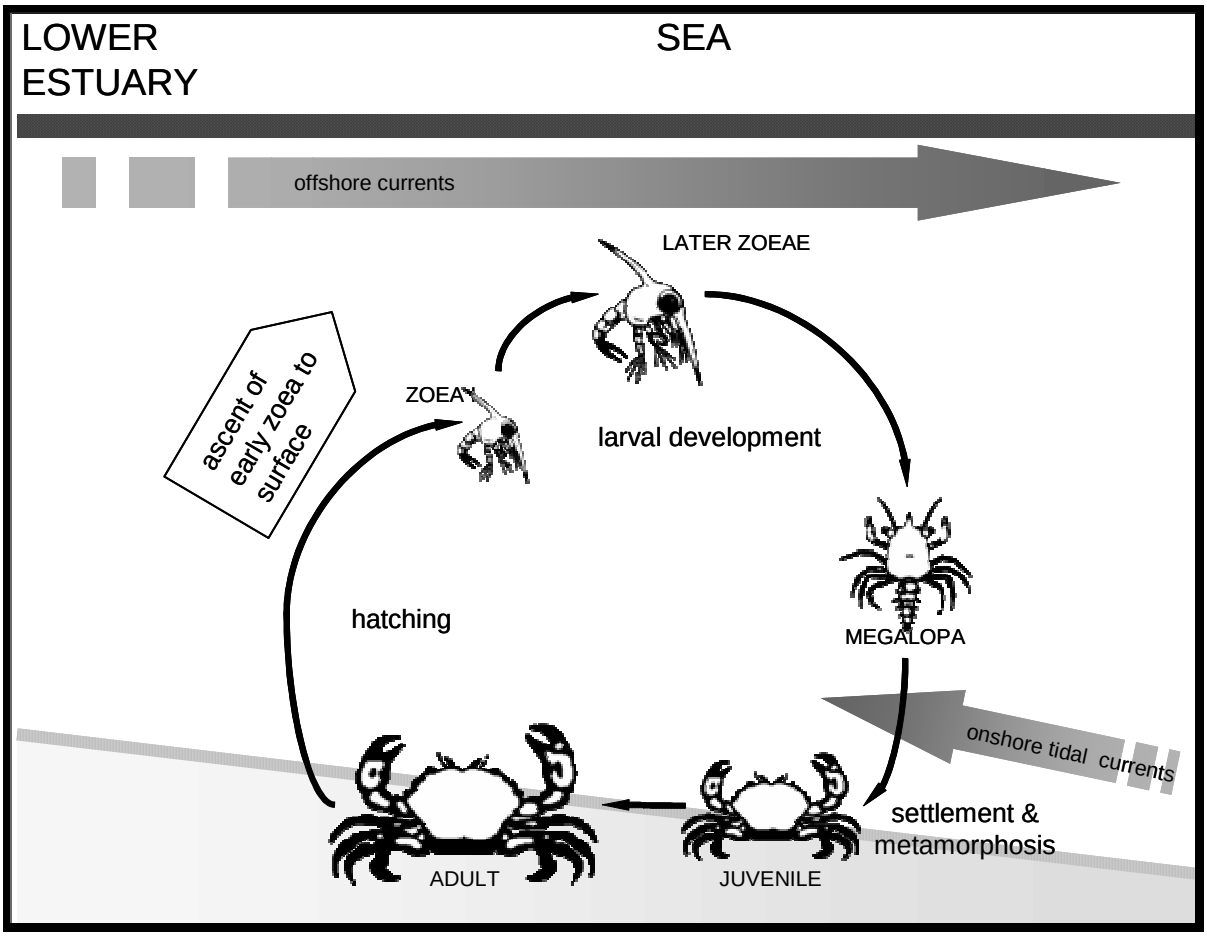

Fig. 3. Conceptual model of the life cycle of fully marine species (After Anger, 2001)

Nephrops norvegicus (Linnaeus)

The Norway lobster is a commercially exploited decapod, widely distributed from the Mediterranean Sea to Iceland (Sardà, 1995). The adults are found in marine muddy substrata. The larval development occurs through three planktonic mysis stages, followed by a decapodid and the juvenile stages. All larval and juvenile stages inhabit the physically stable marine environment of the adults (Jorgensen, 1925; Sardà, 1995). All life-cycle stages are stenohaline; larvae reared at 33-40 ‰ showed high survival (Figueiredo, 1971; Figueiredo and Vilela, 1972).

Hyas araneus (Linnaeus)

The spider crab *H. araneus* is distributed in the North Atlantic from the northern coast of Spitzbergen to the northwest coast of France, and from Rhode Island (USA) to Kiel Bight (Baltic Sea; Christiansen, 1982). Adult crabs are found on various substrates from the shallow subtidal to 350 meters depth (Christiansen, 1969). Its larval stages are pelagic, developing in oceanic waters. In the area around Helgoland, the zoeae are released between the end of January and the beginning of April (Anger, 1983). The larval development consists of two zoeal stages and a Megalopa (Christiansen, 1973; Anger and Harms, 1988). As a consequence of its ecology, this fully marine species exhibits a narrow range of salinity tolerance (from 20-35 ‰) for all life-cycle stages (Anger, 1985; Pfaff, 1997).

Liocarcinus pusillus (Leach)

The portunid crab *Liocarcinus (Macropipus) pusillus* occurs in the shallow subtidal zone of the North European coast (Türkay, 2001). The larval development takes place through five zoeal instars and a megalopa (Rice and Ingle, 1978). The information on aspects of the larval development of this species is very scarce.

II.2. Estuarine, semiterrestrial and freshwater-inhabiting species (euryhaline)

Marine species live throughout their life cycle under stable environmental conditions, so that they do not tolerate major variations in the physico-chemical factors. Likewise, fully limnic species inhabit stable habitats, with low concentration of ions. By contrast, estuarine and coastal species, show physiological and behavioural adaptations to environmental variability, especially euryhalinity. However, their larvae remain vulnerable to osmotic stress, which requires evolutionary adaptations to harsh conditions prevailing in the adult habitat. These adaptations include endogenous rhythms of egg hatching, ontogenetic changes in the physiological tolerance of stress factors, larval migrations, and behavioural responses which are to some extent determined genetically, that is, transmitted from generation to generation (Zeng and Taylor, 1996).

Estuarine and coastal species evolved two principal strategies. (a) Larval retention in the parental habitat; this implies that all life-cycle stages must be able to cope with environmental variability (Fig. 4); (b) larval export: physiologically fragile larval stages are transported to coastal or offshore marine areas, where they face more favourable conditions (Fig. 5; Strathmann, 1982).

II.2.1. Larval retention strategy

This strategy is considered as a transitional stage in the evolutionary path from marine to fully limnic and terrestrial species. All larval stages develop in the planktonic environment, without showing ontogenetic changes in their horizontal distributions (for review see Anger, 2001). To avoid irreversible advection from the parental habitat, the larvae show vertical migrations, swimming downwards during ebb tide to avoid the outflowing surface waters (Fig. 4a) and upwards during flood tide to escape from inflowing near-surface waters (Fig. 4b).

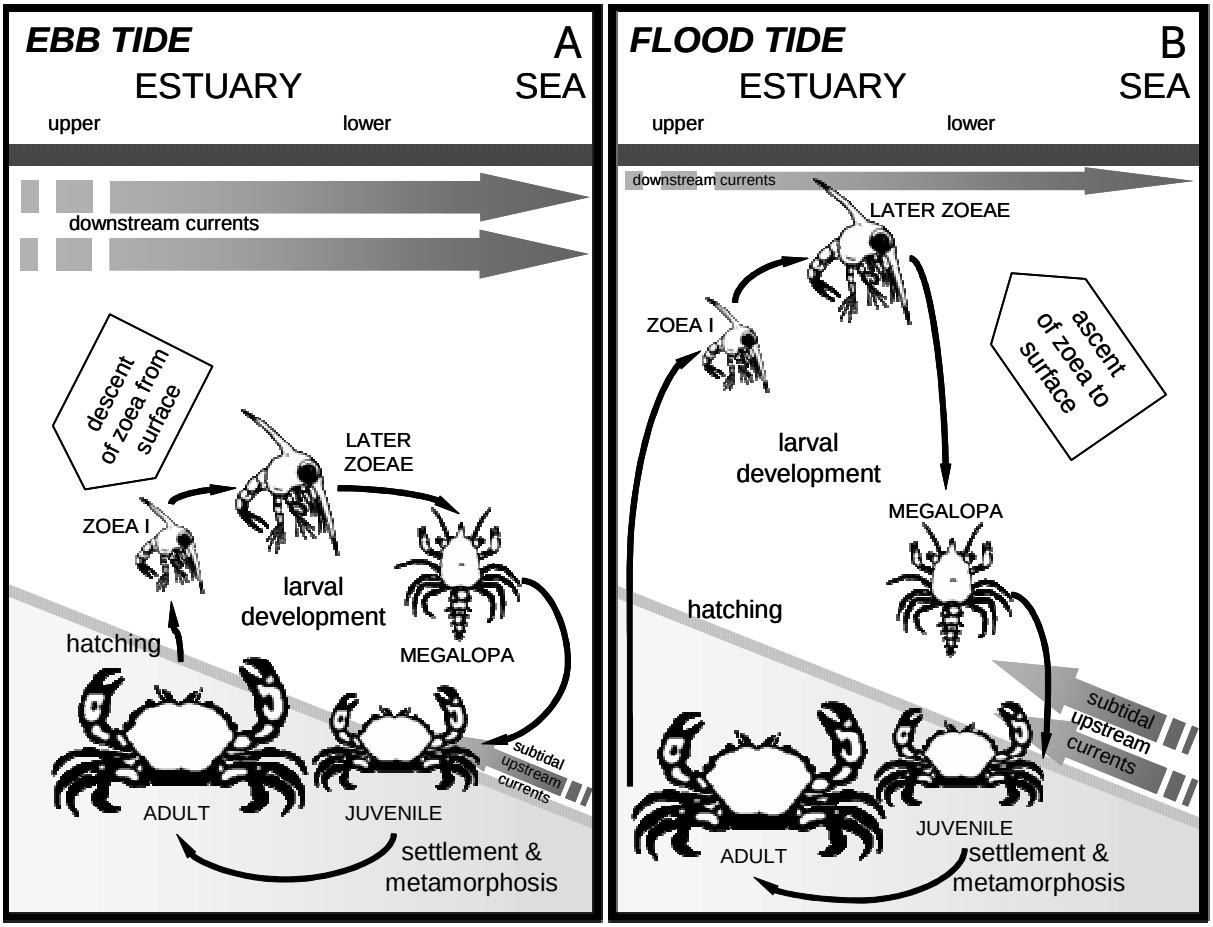

Fig. 4. Conceptual model of the retention strategy showing vertical migrations of the larvae, (a) downwards at ebb tide, (b) upwards at flood tide (modified, from Anger, 2001).

II.2.2. Larval export strategy

Species inhabiting estuarine or freshwater environments have frequently a physiologically sensitive larval phase, which cannot be successfully completed within the habitats where the adults live. These vulnerable early life-history-stages must therefore be exported to more favourable regions, where the salinities are, on average, higher and more stable (Anger 2001, 2003). In order to achieve this transfer, in some species the ovigerous females migrate towards the sea (e.g. Forward et al. 2005; Rudnick et al. 2005), in others the first planktonic stage may be released within the adult habitats, subsequently transported downstream utilizing the outflowing surface currents or tidal mechanisms of downstream transport (Fig. 5, Forward and Tankersley, 2001; for recent review, see Queiroga and Blanton, 2005). These export strategies permit a successful larval development in environments with

lower osmotic stress, e.g. in lower estuaries or coastal marine regions. In some species, the Megalopa stage attains the capability to cope with osmotic stress (Forward et al., 2003; Cieluch et al., 2004), and thus, is able to begin the re-immigration into the adult habitat. In other species, this ability is achieved only in the subsequent benthic juvenile stages (see Charmantier, 1998; Anger, 2001, 2003; Cieluch et al., in press).

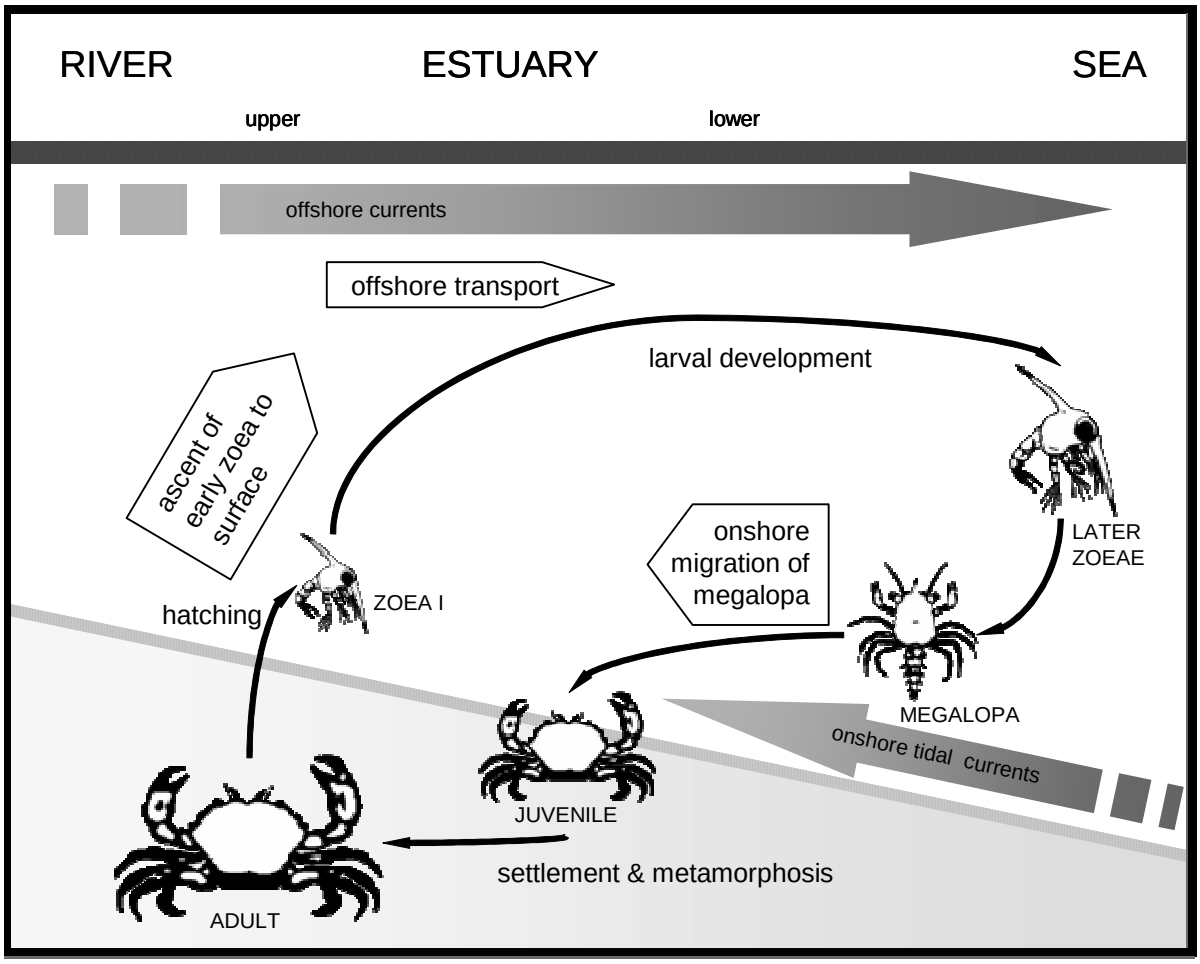

Fig. 5. Conceptual model of the export strategy (After Anger, 2001).

II.2.3. Estuarine species

Armases ricordi (H. Milne Edwards)

This highly terrestrial species has been reported to live between Bermuda, Surinam and the Gulf of Mexico (Abele, 1992). It occupies typical dry coastal habitats within mangroves as well as rocks and debris in the supralittoral fringe up to ca. 200 meters inland (Hartnoll, 1965; Chace and Hobbs, 1969; Abele, 1992; Diesel and Schuh, 1998). The ovigerous females migrate to the sea to release the first zoeae,

clinging to rocks to shed the larvae into the coastal waters (Diesel and Schuh, 1998). The larval stages (four zoeae and a Megalopa) develop in the coastal plankton (Díaz and Ewald, 1968; Alvarez and Ewald, 1990, Diesel and Schuh, 1998). All larval stages show a narrow salinity tolerance range, with high survival at a salinity range from 15 to 35 ‰ (G Torres, preliminary experiments). The Zoea I tolerates a wide salinity range from 5 ‰ to 55 ‰ (Diesel and Schuh, 1998).

Perisesarma fasciatum (Lanchester)

The known distribution range of this semiterrestrial species reaches from Thailand, Malaysia, Singapore and Indonesia to Hong Kong. Similar to *Armases ricordi*, it is found on the upper, often dry fringes of mangroves on relatively hard and sandy substratum. Its larval phase comprehends four zoeal stages and a Megalopa, probably developing in adjacent brackish and/or marine waters (Guerao et al., 2004). Little is known about the osmoregulatory characteristics of this species, but it can be assumed that all life-cycle stages should be at least weak osmoregulators to survive in such varying environments.

Sesarma curacaoense (Rathbun)

This semiterrestrial crab, the closest relative of the coastal marine ancestor, which gave rise to the adaptive radiation of endemic freshwater and terrestrial sesarmid species on the island of Jamaica (Schubart et al., 1998), occurs from southern Florida to north-eastern Brazil and the West Indies (Abele, 1992). Its natural habitat is found in coastal mangrove swamps where the larvae are released in stagnant, temporary water puddles; larval development consists of two zoeal stages and a Megalopa (Anger and Schultze, 1995; Schuh and Diesel, 1995; Anger et al., 1995; Montú et al., 1995). All life-cycle stages are able to survive in harsh environments, based on the early appearance of osmoregulatory functions (Anger and Charmantier, 2000).

Armases miersii (Rathbun)

The distribution range of this semiterrestrial crab includes the Caribbean islands, Florida and the Bahamas (Abele, 1992). On the northern shore of Jamaica, it breeds

in coastal mangrove swamps and in land-locked supratidal rock pools (Schuh and Diesel, 1995; Anger, 1995a). The larval development consists of three zoeal stages and a Megalopa, which are retained in physically highly variable pools until metamorphosis to the first juvenile stage occurs. Semiterrestrial juvenile and adult crabs hide in crevices between mangrove roots or inhabit subtidal rocks as well as coastal limestone caves with brackish or fresh water (Anger and Schultze, 1995; Anger, 1995b, c; Schuh and Diesel, 1995). The conquest of this harsh environment was possible due to the ability of osmoregulation already present from the early larval stages, which hyper-osmoregulate at low salinities and isoconform at salinities higher than 25 ‰ (Charmantier et al., 1998). The Megalopa and the subsequent juvenile stages exhibit a slightly developed capability of hyper-hypo-osmoregulation, i.e. hyper-osmoregulation at low salinities and slight hypo-regulation at salinities higher than 25 ‰. The adults show a strong capacity of hyper-hypo-osmoregulation in conditions ranging from freshwater to concentrated media (45 ‰).

Chasmagnathus granulata (Dana)

This temperate estuarine species inhabits South American salt marshes and lagoons along the Brazilian, Uruguayan, and Argentine coasts (Boschi 1964; Spivak 1997, Giménez 2003). Its life history is characterised by an export strategy (Anger et al., 1994). Soon after hatching, the Zoea-I larvae are carried by outflowing tidal currents out of the brackish parental habitats towards the open sea, where the subsequent planktonic development (comprising four or five zoeal stages and a Megalopa) occurs (Boschi et al., 1992; Pestana and Ostrensky, 1995). The megalopae re-immigrate into the adult environment (estuaries and lagoons), where they settle and metamorphose to the first juvenile (Luppi et al., 2001). Juvenile growth, reproduction and embryonic development take place in the estuarine environment. Consequently, the adults show the typical traits of intertidal and estuarine species (reviewed in Mantel and Farmer, 1983), being strong hyper-hypo-osmoregulators (Mañé-Garzón et al., 1974; Luquet et al., 1992; Nery and Santos, 1993). The patterns of osmoregulation change conspicuously during larval development, from a weakly hyper-osmoregulating Zoea I, through hyper-osmoconforming zoeal stages II-IV, to a weakly hyper-hypo-osmoregulating

Megalopa (Charmantier et al., 2002). This reflects the environmental osmotic conditions that each of these stages has to face. After the metamorphosis to the crab I, the adult pattern of hyper-hypo-osmoregulation increases gradually throughout the successive juvenile stages.

Armases roberti (H. Milne Edwards)

This little known tropical species is found in the West Indies, but not on the American mainland (Abele, 1992). The adult crabs live on the banks of rivers and streams from the mouth to several kilometres (ca. 10km) upstream (Chace and Hobbs, 1969; Abele, 1992). According to Diesel and Schuh (1998), the Zoea I is released directly into freshwater near the semiterrestrial habitat of the adult population, from where the freshly hatched larvae are carried downstream by river currents, so that they soon reach estuarine or coastal waters. The exported larvae develop in osmotically more stable waters through four zoeal stages to the Megalopa, which is presumed to migrate back into the estuary and probably further upstream into the riverine parental habitat (Torres et al., 2006; Anger et al., in press). In agreement with this proposed migration pattern, the Zoea I shows a wide range of salinity tolerance (0-50 ‰), while the following larval stages have a narrower range of salinity tolerance (15-45 ‰; Torres et al., in prep.). Only after metamorphosis to the Megalopa, the tolerance to salinity variation increases again (Diesel and Schuh, 1998).

Eriocheir sinensis (H. Milne-Edwards)

The Chinese mitten crab is a holo-euryhaline brachyuran originating from the Chinese Pacific coast. It colonised central Europe almost 100 years ago (Panning, 1939), and more recently also North America and southern Europe (Cohen and Carlton, 1997; Cabral and Costa, 1999; for recent reviews see Herborg et al., 2003; Rudnick et al., 2003). The adults live in rivers and other inland waters. With about 4-5 years of age, they migrate downstream to brackish estuaries, where they reach maturity, mate, and release planktonic larvae. Larval development, consisting of five (occasionally 6) zoeal stages and a Megalopa (Kim and Hwang, 1995; Montú et al.,

1996), takes place in estuarine and coastal marine regions; subsequently, the benthic juveniles begin to re-immigrate upstream, towards the parental limnic habitat (Anger, 1991; Rudnick et al., 2003). The successful dispersal of this species in brackish coastal lagoons, rivers and land-locked inland waters is based on the adults' ability to cope equally well with freshwater, brackish water and seawater (Cieluch et al., in press). In contrast, the zoeal stages show only slight hyper-iso-osmoconforming capacities, which would not allow survival in freshwater. A change in this osmoregulation pattern occurs after the metamorphosis to the Megalopa, which is capable of weak hyper-hypo-regulation. This ability increases with the second metamorphosis to the first juvenile crab stage (Cieluch et al., in press). The appearance of a strong osmoregulatory capability enables the early juveniles to rapidly re-invade the riverine habitat of the adults.

III.POPULATION LEVEL

III.1. Effects of reduced salinities on re-immigration

PUBLICATION 1

EFFECTS OF REDUCED SALINITIES ON METAMORPHOSIS OF A FRESHWATER-TOLERANT SESARMID CRAB, *ARMASES ROBERTI*: IS UPSTREAM MIGRATION IN THE MEGALOPA STAGE CONSTRAINED BY INCREASING OSMOTIC STRESS?

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Effects of reduced salinities on metamorphosis of a freshwater-tolerant sesarmid crab, *Armases roberti*: Is upstream migration in the megalopa stage constrained by increasing osmotic stress?

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Abstract

Numerous species of estuarine and freshwater-tolerant crabs show an "export strategy", i.e. an early larval downstream transport towards coastal marine waters, later zoeal development at higher salinities, and a return of the last larval stage, the megalopa, into estuaries or rivers. The speed and extent of the upstream migration of the megalopa through strong salinity gradients may be constrained by increasing hypo-osmotic stress. In an experimental laboratory study with *Armases roberti*, a freshwater-inhabiting sesarmid crab from the Caribbean region, we studied in the megalopa stage (after zoeal rearing at 25‰) the tolerance of reduced salinities.

In the first experiment, the larvae were exposed directly to various constant salinities (1–25‰). For the second experiment, they were transferred stepwise to strongly diluted media (within 6 days from 25‰ to ≤3‰), simulating differential scenarios of upstream migration into brackish or freshwater habitats.

When postmoult megalopae were exposed directly to salinities ≤3‰, they all died within 24 h. A slightly higher salt concentration (5‰), however, allowed for considerable survival (46%) through metamorphosis to the first juvenile crab stage. In treatments with continuous exposure to 10–15‰, as well as in a control group (25‰), survival to metamorphosis was significantly higher (83–96%), and the average duration of development was shorter compared to 5‰ (12–13 vs. 16 days). In the second experiment, with stepwise salinity reductions, gradual acclimation to decreasing osmotic pressures permitted a successful development to metamorphosis at ≤3‰ and even in freshwater (<0.2‰).

This strong physiological adaptability enables the megalopa of *A. roberti* to cross during its upstream migration, within a short time (6 days), strong osmotic gradients, so that metamorphosis is possible also in freshwater habitats where the conspecific adult crabs live. The speed of migration appears to be limited by physiological constraints related to changes in the capability for osmoregulation occurring during the course of the moulting cycle.

Keywords: Crustaceans; Estuarine; Larval biology; Limnic; Metamorphosis; Ontogenetic migrations; Salinity tolerance

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1. Introduction

Many animal species migrate to exploit additional food sources, to escape from predation, or to reproduce. These migrations may be constrained, however, by ecological barriers such as aquatic-terrestrial transitions, or by gradients in food availability, temperature, or salinity (Alerstram et al., 2003; Henningsson and Alerstram, 2005). Depending on the strength of such gradients and on the speed of their transition, the crossing of barriers can affect survival and individual performance.

Numerous aquatic animals living in coastal, estuarine, and adjacent freshwater environments, for example many species of crabs and other decapod crustaceans, perform ontogenetic migrations, because their early life-history stages are unable to tolerate highly variable and mostly reduced salinities typically occurring in the habitats of the adults (Anger, 2001, 2003; Forward and Tankersley, 2001). The newly hatched larvae of such species are rapidly transported out of the parental habitat, towards lower estuarine or coastal marine waters with more stable and on average higher salinities. In some species, e.g. in the blue crab, *Callinectes sapidus* (Forward and Tankersley, 2001), or in the Chinese mitten crab, *Eriocheir sinensis* (Rudnick et al., 2005), this "export strategy" (Strathmann, 1982) includes downstream migrations of the ovigerous females, which mitigate the exposure of newly hatched larvae to hypo-osmotic stress. Other species release their planktonic larvae into the habitat of the adult population, from where they are rapidly transported downstream by outflowing surface currents (for recent review, see Queiroga and Blanton, 2004). After zoeal development in near-shore or continental shelf waters, the megalopa and/or the early benthic juvenile stages migrate back to the parental habitat. This implies that the returning developmental stages of estuarine and riverine species are during their upstream migration exposed to increasingly diluted media, which requires an increasing tolerance of hypo-osmotic conditions.

Armases roberti (H. Milne Edwards), a freshwater-dwelling, semiterrestrial crab from the Caribbean region, may be a particularly suitable and extreme example for a riverine species with an export strategy. Being strong hyper-osmoregulators (Schubart and Diesel, 1998), the adult crabs live on riverbanks up to about 10 km away from the sea (Chace and Hobbs, 1969; Abele, 1992; Diesel and Schuh, 1998). Laboratory observations (Diesel and Schuh, 1998) suggest that the zoeae are released in freshwater, where they can survive for up to two days. Subsequently, however, the larvae must reach lower estuarine or coastal marine waters to develop at higher salinities through four zoeal stages. However, since only preliminary laboratory data (with constant salinities from hatching; Diesel and Schuh, 1998) and no field data are available, it has remained unknown, whether already the megalopa stage or only the subsequent benthic juveniles of this species are physiologically capable of migrating back into estuaries or rivers, crossing horizontal salinity gradients. In an experimental laboratory study, we therefore evaluated the salinity tolerance of the megalopa stage of *A. roberti* and, in particular, its capability to acclimatize to conditions of decreasing osmotic pressure during simulated upstream migrations, so that it can successfully develop to metamorphosis near to or in freshwater habitats, where the conspecific adults live.

2. Materials and methods

2.1. Obtaining and rearing of larvae

Ovigerous *A. roberti* were collected in March 2003 from the lower Rio Bueno River, northern Jamaica. At the collection site, the following water characteristics were measured with a "Combo pH and EC" (Hanna Instruments, Kehl, Germany) apparatus: water temperature: 23.8 °C, conductivity: 0.38 mS/cm, total dissolved matter: 0.19‰, pH: 8.10. Thereafter, the crabs were transported to the Helgoland Marine Biological Station, Helgoland, Germany, and maintained in aquaria with freshwater (<0.2‰) kept under constant conditions of temperature (24 °C) and a 12:12-h photoperiod. Food (frozen isopods, *Idotea* sp.) and water were changed daily. After hatching in freshwater, zoeae were mass-reared in bowls with 400 ml water (initial density 50 zoeae per bowl) at the same conditions of temperature and light, but at a salinity of 25‰ (considered as an optimal condition for zoeal development; Diesel and Schuh, 1998), until they reached metamorphosis to the megalopa stage. In daily intervals, water was changed and the larvae were fed *ad libitum* with freshly hatched brine shrimp (*Artemia franciscana*) nauplii. Different experimental salinities were obtained by diluting filtered seawater from the North Sea (ca. 1 μm filter pore size; 32‰) with appropriate quantities of freshwater (total dissolved substances: 0.17 mg/l, conductivity: 0.41 mS/cm), and checked with a WTW (Weilheim, Germany) Cond-330i salinometer to the nearest 0.1‰.

2.2. Experiments

The experiments were started the first day after moulting from the zoea-IV stage. In the first experiment, megalopae were transferred from the salinity of previous zoeal rearing (25‰) to treatments with constant 15‰, 10‰, 5‰, 3‰, or 1‰ (n=24 megalopae each; for

experimental design, see Fig. 1B–F). The mode of transference in these treatments is considered as "direct", because it was done within a single day (salinity decrements of ≤5‰, intervals of 2 h). One group (A, Fig. 1; $n=23$ megalopae) remained as control at constant 25‰. The purpose of this set of treatments was to estimate the limits of tolerance of various levels of continued hypo-osmotic stress in the megalopa stage.

In a second experiment, we tested the megalopal tolerance of rapidly increasing hypo-osmotic stress, with treatments G–J (Fig. 1) simulating various scenarios of rapid upstream migration from the lower part of an estuary (moulting to the megalopa stage at 25‰) into rivers or upper parts of an estuary. During the first 6 days of their moulting cycle, the megalopae were in these treatments transferred stepwise to increasingly dilute media (decrements of ≤5‰; intervals of 1–2 days; final salinities: 3‰, 2‰, 1‰, or 0.17‰).

In all treatments, the megalopae were reared individually in 80-ml vials with a piece of nylon gauze provided as substrate. Otherwise, rearing conditions (feeding, water change, temperature, light) were the same as in the zoeal stages (see above). The experiments were terminated when all individuals had either died or passed through metamorphosis to the first juvenile crab stage.

2.3. Statistical analysis

Data were analysed with separate factorial designs (Zar, 1996). The first design included the treatments A, B, C, and D, i.e. without decreases in salinity after day 1;

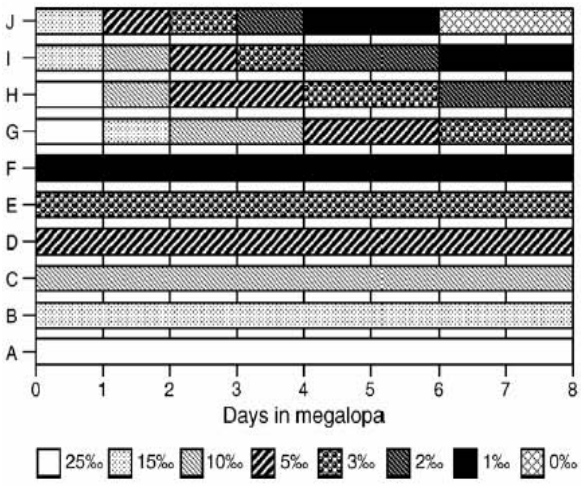

Fig. 1. Tests for salinity tolerance of the megalopa stage of *Armases roberti*, experimental design. (A) Control condition (constant salinity of 25‰); (B–F) treatments with direct transfer of megalopae from 25‰ to constantly reduced salinities (15‰, 10‰, 5‰, 3‰, 1‰); (G–J) treatments with stepwise reductions of salinity, simulating various speeds of megalopal upstream migration into estuaries and rivers.

the treatments E and F, with salinities 1‰ and 3‰, were not included in this analysis since mortality reached 100% within the first 24 h (see Results). The second design evaluated effects of treatments A, G, H, I, and J. Survival data were analyzed with log-linear analyses, while duration of development to the first juvenile stage with one-way ANOVA. For log-linear analyses, the significance of differences between the control treatment A and treatments G–J were adjusted with the sequential Bonferroni method (Rice, 1989). For the ANOVAs, normality was checked with normal plots of residuals, heterogeneity of variance with Cochran's test.

3. Results

3.1. Survival

After direct exposure to very low salinities (1–3‰; treatments E, F; Fig. 1), all larvae died within 24 h. An exposure to 5‰ (treatment D) caused significantly higher mortality compared to all treatments with higher salinities ($\chi^2_3=17.2$, $p<0.001$). Treatments A–C (10–25‰) allowed for 80–95% survival, while only 45% survived in treatment D (5‰). In the latter group, mortality was particularly high during the first 72 h of the megalopal moulting cycle (Fig. 2D). At 10–25‰, mortality occurred mostly after about 9 days or thereafter (Fig. 2A–C).

Exposure to different rates of decrease in salinity (treatments G–J) also affected survival ($\chi^2_3=11.0$, $p<0.05$). It exceeded 80% when the decrease in salinity was slow (treatment G, Fig. 2G), while minimum survival was observed in treatment J (42%, Fig. 2J), where the decrease in salinity was faster. Mortality began mostly to increase conspicuously when salinity was decreased below 3‰ (Fig. 2G,H). After performing a Bonferroni correction, the survival in the control group A was not significantly different from that in treatments G ($\chi^2_2=0.18$, $p>0.05$) and I ($\chi^2_2=3.96$, $p>0.025$), but it was higher than in treatments H and J ($\chi^2_2=11.46$ and 17.32, respectively, all $p<0.001$).

3.2. Duration of development

When salinity was either maintained constant at 25‰ (control, A) or rapidly reduced during the first 24 h after moulting to the megalopa stage (treatments B–D), the duration of development increased with decreasing salinity (one-way ANOVA: $F_{3,\ 69}=11.09$, $p<0.0001$). Individuals reared at 5‰ showed a significantly longer duration of development than those reared at conditions of 10–25‰ (Fig. 3A).

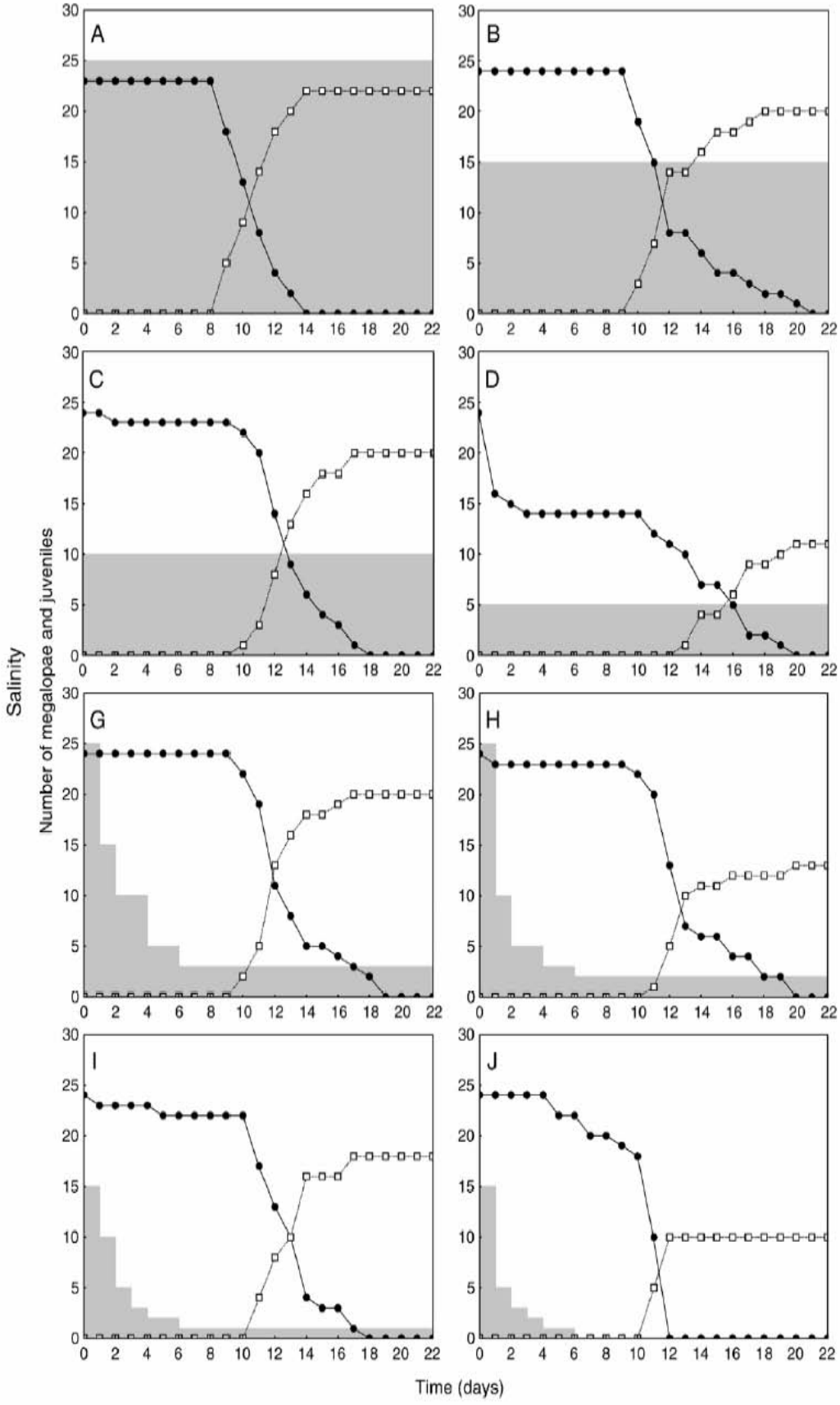

Fig. 2. *Armases roberti*, survival and development through the megalopa stage, during or after reductions of salinity (for experimental design, see Fig. 1); shaded areas, salinity (‰); filled circles, number of megalopae; open squares, numbers of first-stage crabs.

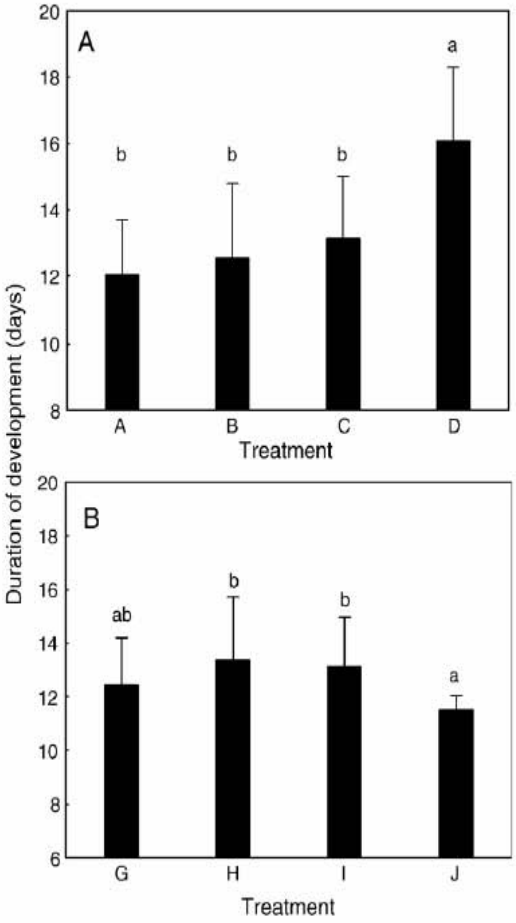

Fig. 3. *Armases roberti*, duration of development through the megalopa stage, during or after reductions of salinity (for experimental design, see Fig. 1); different letters indicate significant differences between treatments ($p<0.05$; Student–Newman–Keuls tests).

The duration of development during a stepwise reduction of salinity was ca. 1 day longer in treatments with a slower rate of decrease (G, H, I) compared to the treatment with the fastest rate of decrease (J, Fig. 3B). One-way ANOVA and a SNK post hoc test, including also the control group A, revealed that these differences were statistically significant ($F_{4,\ 78}=2.66$, $p<0.05$), although the variances remained heterogeneous also after data transformation. A *t*-test with separate variance estimates, performed to test for differences between treatments G, H, I vs. treatment J, showed also significant differences ($t=4.31$, $p<0.0001$). The development time in the control group A was not significantly different from that in the other treatments with decreasing salinities (cf. Fig. 3A,B).

4. Discussion

Our results suggest that megalopal upstream migration and recruitment to freshwater-inhabiting populations of *A. roberti* may be limited by physiological or developmental constraints. When the last larval stage was directly transferred from 25‰ to 5‰, it showed an increase of mortality and a longer duration of development. Most megalopae died in this treatment within the first few days, suggesting an osmotic shock. In treatments with a direct transfer from 25‰ to ≤3‰, complete mortality occurred within only 24 h.

Our experiment with stepwise reductions of salinity, by contrast, revealed that the megalopae are capable of gradual acclimation to low salinities. In treatment G, 80% of the megalopae were able to survive for an extended period (6–12 days) at a salinity as low as 3‰, followed by successful metamorphosis under this condition. In treatment J, ca. 50% of the larvae survived even in freshwater through metamorphosis to the first juvenile crab stage. However, mortality increased during a fast decrease of salinity (compare treatments G, J), suggesting physiological limitations to the possible speed of upstream migration. Surprisingly, mean duration of development was shorter in the apparently stressful treatment J than in those with slower reductions of salinity (cf. H, I, Fig. 3B). This result might have been caused by selective mortality of physiologically weaker individuals, so that only the strongest and fastest developing larvae may have survived though metamorphosis, reducing the average value.

Our experimental observations suggest that migrations may initially be carried out at a low speed, allowing for gradual acclimation. During the early postmoult phase, decapod larvae are osmo-conformers rather than osmo-regulators, mainly due to high permeability of the initially thin cuticle (Charmantier, 1998). During this period, the capacity for osmoregulation may therefore be too weak to compensate for passive losses of ions and intrusion of water in dilute media and, as a consequence, the megalopae may initially be more vulnerable to osmotic stress than during later stages of the moulting cycle (intermoult, premoult). The pace of megalopal upstream migrations into oligohaline and freshwater environments, where the adult populations live, may thus be physiologically limited by constraints related to the moulting cycle.

Acknowledgements

We thank U. Nettelmann and K. Boos for help in the experiments. G.T. acknowledges the Deutscher Akademischer Austausch dienst, DAAD (Bonn, Germany) for funding this study as a part of her doctoral dissertation, and L.G. the Alexander-von-Humboldt Foundation (Bonn, Germany) for a postdoctoral research grant.

The experiments comply with the current German animal and manipulation laws. [SS]

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III.2. Effects of reduced salinities and increasing adult odours on re-immigration

PUBLICATION 2

METAMORPHOSIS OF A SESARMID RIVER CRAB, *ARMASES ROBERTI*: STIMULATION BY ADULT ODOURS VS. INHIBITION BY SALINITY STRESS

Klaus Anger, Gabriela Torres, Luis Giménez

Marine and Freshwater Behaviour and Physiology, in press.

ABSTRACT

In an experimental laboratory study with the megalopa stage of *Armases roberti*, a freshwater-inhabiting species of crab from the Caribbean region, we evaluated the combined, potentially antagonistic effects of odours from conspecific adults and of stepwise salinity reductions (simulating upstream migration, reaching within one week conditions of 2 ‰ or freshwater). Neither of these treatments affected the rate of survival, but the duration of development to metamorphosis was significantly (by about 25%) shortened, when odours from conspecific adult crabs were present, regardless of the salinity conditions. Our results indicate that the metamorphosis-stimulating effect of chemical cues from an adult population of *A. roberti* is far stronger than the potentially retarding effect of increasing hypo-osmotic stress. This suggests that the final phase of larval development, including the processes of settlement and metamorphosis, occurs in this species in freshwater habitats, where conspecific populations live.

Keywords: crustaceans, larval biology, ontogenetic migrations, salinity tolerance, chemical cues, recruitment

INTRODUCTION

Numerous species of estuarine and freshwater-inhabiting animals pass through a physiologically vulnerable larval phase, which cannot successfully be completed within the brackish or limnic environments where the adults live. The early life-history-stages must therefore be “exported” to more favourable regions, where the salinities are, on average, higher and more stable (Anger, 2001, 2003). This spatial transfer of the larval phase can either be achieved by reproductive migrations of the ovigerous females towards the sea (e.g. Forward et al., 2005; Rudnick et al., 2005), or hatching may occur within the adult habitat, followed by a rapid passive downstream transport of the first planktonic larval stage, utilizing the outflowing surface currents or tidal mechanisms of downstream transport (for recent review, see Queiroga and Blanton, 2005).

Although such “export strategies” (Strathmann, 1982) allow for successful larval development in the lower (more saline) parts of estuaries or in coastal marine regions, it remains generally an open question, which life-history stage attains the physiological competence for re-immigration and recruitment to adult populations. Upstream migrations imply the crossing of strong salinity gradients, requiring tolerance of an increasing hypo-osmotic stress. It is assumed that the re-immigration into estuaries or rivers begins in some cases during the final larval stage, the decapodid or megalopa (e.g. Forward et al., 2003), while other species may attain this competence only in subsequent benthic juvenile stages (for references, see Charmantier, 1998; Anger, 2001, 2003). The question of the ontogenetic timing of the return to brackish or freshwater habitats can be resolved either with studies of larval distribution in the field or with an experimental approach, testing late larval or early juvenile stages in the laboratory for their salinity tolerance. The latter approach was chosen in the present study.

Besides physical stress occurring during ontogenetic migrations, benthic species with complex life cycles must face another problem: the time spent as larvae in the plankton confers several risks of mortality due to pelagic predation and other adverse factors (for review, see Morgan, 1995). This selection pressure should limit the duration of the planktonic larval phase, and hence, also the duration and extent of ontogenetic migrations. In some species, this is achieved through megalopal responses to chemical cues that originate from habitats of the conspecific adults (including adult odours), accelerating the development to metamorphosis (Forward et al., 2001; Gebauer et al., 2003). On the other hand, physiological constraints imposed by weakly developed capabilities of osmoregulation may limit the possible rate of larval upstream migration through salinity gradients (Charmantier, 1998; Anger, 2003). This implies a trade-off between various risks of mortality during the larval phase.

In the present study, we used *Armases roberti* (H. Milne Edwards), a little known, freshwater-inhabiting and semiterrestrial crab from the Caribbean island of Jamaica to study the potentially antagonistic effects of metamorphosis-stimulating chemical cues from conspecific adults, in combination with an increasing hypo-osmotic stress. Adult populations of this “river crab” live in freshwater up to ca. 10 km from the sea (Chace and Hobbs, 1969; Abele, 1992; Diesel and Schuh, 1998). Previous laboratory

observations (Diesel and Schuh, 1998) showed that the zoeae of this species hatched in freshwater, where they could survive for up ca. 2 days. However, when the zoeae were continually exposed to reduced salinities below 15 ‰, they revealed a stenohaline response, which would not allow for larval development near the limnic habitats of conspecific adult populations. In conclusion, this species must follow an export strategy. The experimental data by Diesel and Schuh (1998) suggested an optimal development at about 25 ‰ rather than under fully marine or hypersaline conditions. On the one hand, this might indicate that zoeal development normally takes place in lower estuarine zones, where moderately reduced salinities prevail. On the other hand, a wide geographic distribution of this species throughout the Caribbean region (Abele, 1992) indicates that there must be also at least occasional larval transport with fully marine coastal and oceanic water currents.

These life-history patterns of *A. roberti* raise the question, in which ontogenetic stage its re-immigration into brackish and freshwater habitats becomes physiologically possible, and how those recruitment migrations are triggered. A recent study by Torres et al. (2006) showed that the megalopa stage of this species is very euryhaline and probably capable of immigrating into estuaries and rivers. During their presumable upstream migration, however, the megalopae not only are increasingly exposed to reduced salinities, but also to attracting chemical cues from the riverine habitats of conspecific adults. Such cues have been shown to stimulate metamorphosis in several species of sesarmid crabs (for review, see Gebauer et al., 2003), but is has remained unknown if this response occurs also in the megalopa stage of *A. roberti*. We tested this in the present study, evaluating combined (theoretically antagonistic) effects of an exposure to adult odours and reduced salinities.

MATERIAL AND METHODS

Habitat characteristics

As only little is known about the ecology of *Armases roberti* (Chace and Hobbs, 1969; Abele, 1992), we provide here a short description of presumably typical habitats, where we collected these crabs in March 2003 and 2004, and in April 2006.

Most material originated from the lower Rio Bueno River, near the town of Rio Bueno, northern Jamaica (Figs. 1A, B). On 11 April, 2006, the following water characteristics were measured at the collection site, using a "Combo pH and EC" apparatus (Hanna Instruments, Kehl, Germany): temperature 23.8°C; conductivity 0.38 mS/cm; total dissolved matter 0.19‰ (or ppt); pH 8.10. The flow velocity (roughly estimated with plastic floaters and a graded measuring rod) was ca. 100 cm/s. According to frequent previous observations, these conditions are typical for this site, at least during spring. Both juvenile crabs (minimum ca. 3 mm carapace size) and adults (above 1 cm) were observed on the riverbanks above the water level, mostly in or near rock crevices, especially on shady places under terrestrial vegetation (Figs. 1C, 1B). When crabs were disturbed, they jumped into the water, but soon they crawled up again, showing semiterrestrial rather than aquatic behaviour. Additional crabs of this species were collected from the Dunns River Falls (ca. 45 km east of Rio Bueno), where similar water characteristics were measured: temperature 24.9°C; conductivity 0.29 mS/cm; total dissolved matter 0.14‰; pH 8.48; flow velocity ca. 120 cm/s.

Obtaining and rearing of larvae

Ovigerous females were transported to the Helgoland Marine Biological Station (Germany), and thereafter maintained in aquaria with freshwater (0.41 mS/cm; total dissolved matter 0.20‰; 0.18‰; pH 8.00), kept under constant temperature (24°C) and a 12:12h photoperiod. Food (frozen isopods, *Idotea* sp.) and water were changed daily. After hatching in freshwater, zoeae from one female (originating from the Rio Bueno River) were mass-reared in bowls with 400 ml (initial density 50 zoeae per bowl) at the same conditions of temperature and light, but at a salinity of 25‰, until they reached metamorphosis to the megalopa stage. Water was changed daily, and the larvae were fed *ad libitum* with freshly hatched brine shrimp (*Artemia* franciscana) nauplii (ca. 10/ml).

Different experimental salinities were obtained by diluting filtered seawater from the North Sea (ca. 1 µm filter pore size; 32‰) with appropriate quantities of freshwater. For experiments with an exposure to odours from conspecific adults, salinities were adjusted by diluting seawater with filtered freshwater taken from aquaria in which adult crabs had been placed for 48 h previous to the experiment

(one adult male and one female crab per 4 L freshwater). The adults (ca. 2 cm carapace width) were not fed during this period in order to avoid potentially confounding effects of mixed odours originating from the adult crabs and their food.

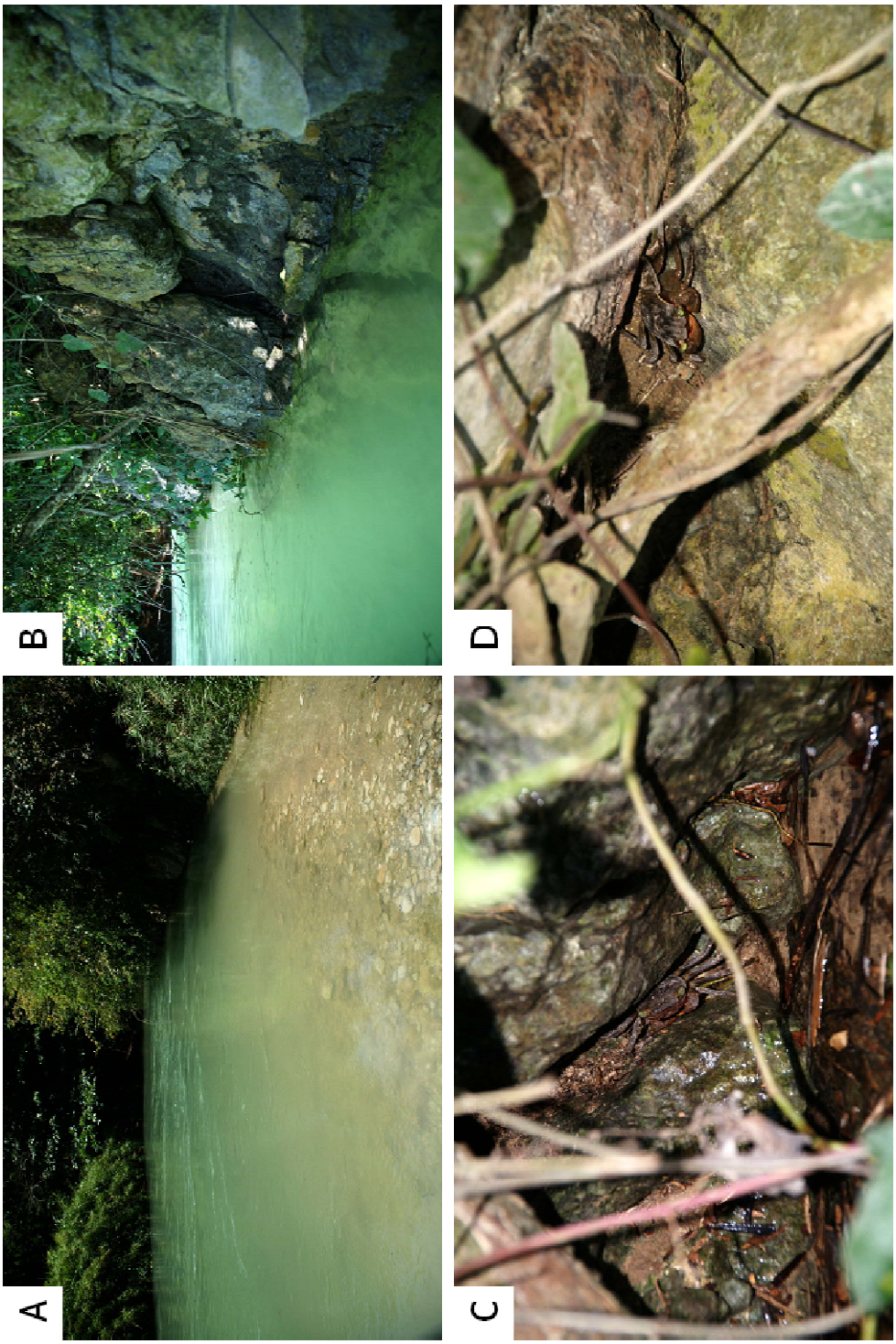

Fig. 1. *Armases roberti*, typical habitat. A, Rio Bueno River near the town of Rio Bueno, northern Jamaica; B, same site, river bank with typical habitats (shaded rock crevices, vegetation, roots of trees and other plants; above the water level); C, male; D, female.

Experimental design and statistical analysis

Megalopae were divided in four groups (factorial design) to test for effects of (a) the presence (denoted with + in Fig. 3) or absence (-) of chemical cues (conspecific adult odours); (b) of two different speeds of stepwise salinity decrease (from hereon referred to as “fast” and “slow”, respectively; or F and S, Fig. 3). All experiments began with an immediate postmoult transfer of megalopae from 25 to 15 ‰. In S treatments, salinity was thereafter reduced every 2d, reaching after 6d a condition of 2 ‰, while F treatments implied daily reductions, reaching freshwater conditions after 6d. In all treatments, the salinity reached after 6d remained constant until the megalopae either died or metamorphosed to juvenile crabs. In each of these four treatments, the megalopae were group-reared in 11-14 replicate bowls (a few replicates were accidentally lost), with initially 20 individuals per replicate (i.e. in total 960 megalopae), and a piece of nylon gauze provided as a substrate. Conditions of water change, feeding, temperature, and light were the same as for zoeal rearing. Additionally, we recorded in daily intervals the occurrence of moults or deaths. The duration of megalopa development (or time to metamorphosis) was defined as the time span between the moult from the zoea IV to the megalopa and that between the megalopa and the first juvenile crab stage.

Data on survival and duration of development were analysed with two-way ANOVA (Zar 1998) with factors “chemical cues” (two treatments, with or without adult water) and “salinity decrease” (two treatments, fast or slow).

Photographs

Photographs adult crabs and their habitat were taken with a Konica Minolta Dynax 7D (6.1 Megapixel) digital camera. Micrographs of larvae and juveniles were taken with an Olympus Color View Imaging System attached to a SZX12 stereo microscope.

RESULTS AND DISCUSSION

Larval development and chromatophore patterns

While a detailed morphological description of all larval stages and of the first juvenile crab instar of *Armases roberti* will be published elsewhere, the present paper provides a preliminary account of the pattern of larval development in this species. Consistent with a previous study (Diesel and Schuh, 1998), development from hatching to metamorphosis comprised in our experiments invariably four zoeal stages (at 24°C lasting 3-4 d each) and a megalopa (Fig. 2). The latter stage was reached, on average, 15 d after hatching. The duration of development through the megalopa stage varied from 10-18 d, depending on the rearing conditions (see below).

The larvae showed a characteristic pigmentation, which may become useful as an additional criterion for their identification in plankton samples, as soon as comparable descriptions are provided for other species of *Armases* co-occurring with *A. roberti* in the same region (cf. Abele 1992). From hatching, the zoeae showed on the second and third pleonal body segments two pairs of large white to bright yellow ventro-lateral chromatophores (Figs. 1A-C). They were present throughout the zoeal stages I-III, but disappeared in the zoea IV stage (Fig. 1D). Moreover, all zoeal stages showed on all pleonal somites as well as on the carapace and maxillipedes scatters of small orange chromatophores. The same type of chromatophores occurred also in the megalopa stage, especially latero-ventrally and dorsally on the carapace and, although less, dorsally on all pleonal somites. A third type of chromatophores, intermediate in size and dark red or brown in colour, was observed dorso-laterally and distally on the carapace, and on the pleonal somites of the zoeal stages, increasing during development in number and size. In the megalopa, pairs of brown chromatophores occurred ventrally on all pleonal somites, near the feeding appendages, and dorsally on and between the eye stalks, as well as in the anterio-median carapace region (Fig. 2E).

The first-stage juvenile crabs are characterized by numerous small bright yellow chromatophores on the dorsal sides of the carapace and of the eye stalks (Fig. 2F). The same type of chromatophores occurs also on all walking legs and on the chelae, but with a slightly lower density. In addition, the pereiopods show also orange chromatophores, which are slightly larger and occur in higher numbers. Four large

brown, strongly ramified chromatophores are distributed along the median line of the dorsal carapace surface. The same type of chromatophores is also scattered over the ventral carapace surface and on the ventrally bent pleon (not visible in Fig. 2F).

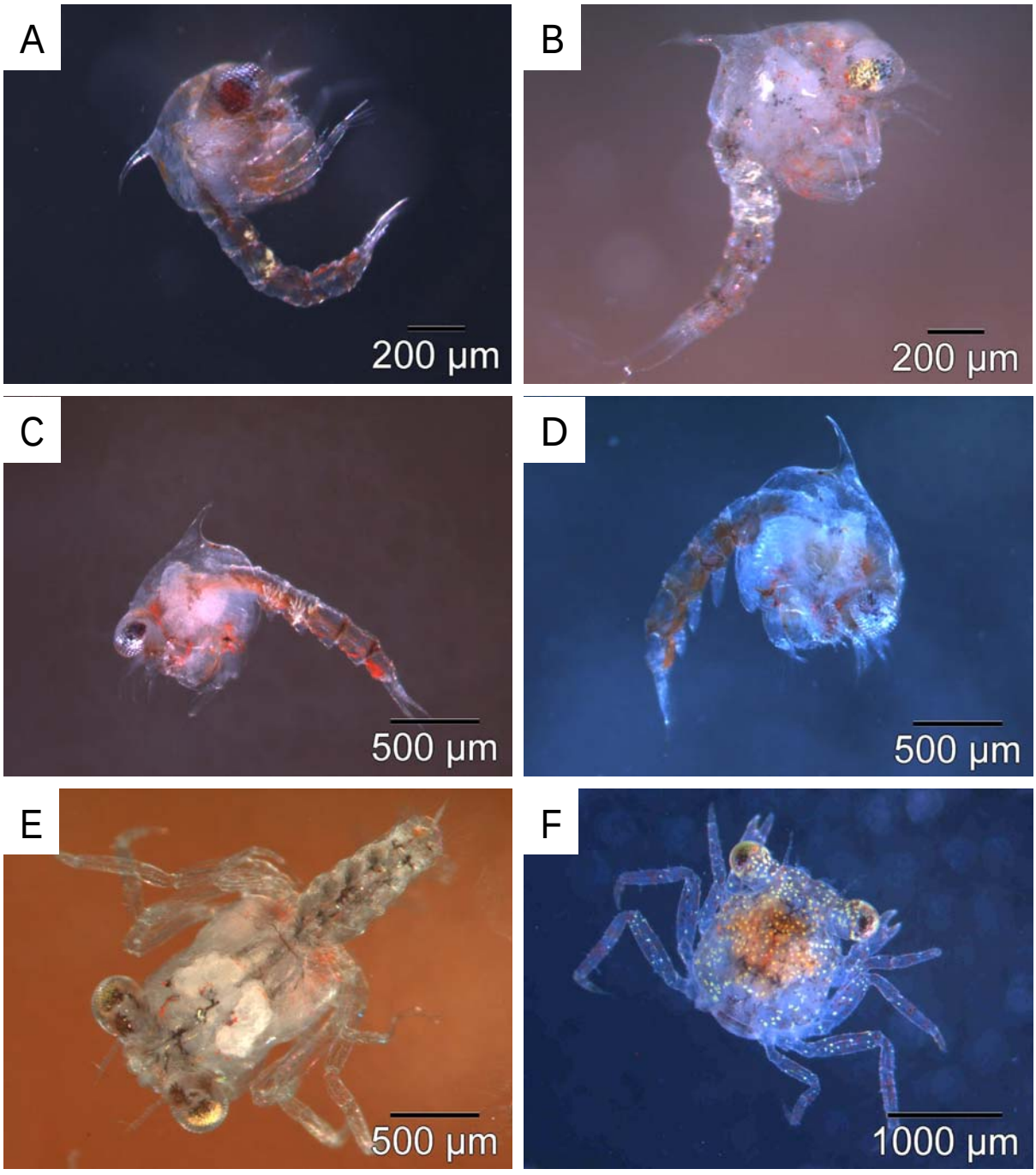

Fig. 2. *Armases roberti*, development from larval hatching through metamorphosis. A-D, zoeal stages I-IV; E, megalopa; F, first juvenile crab stage.

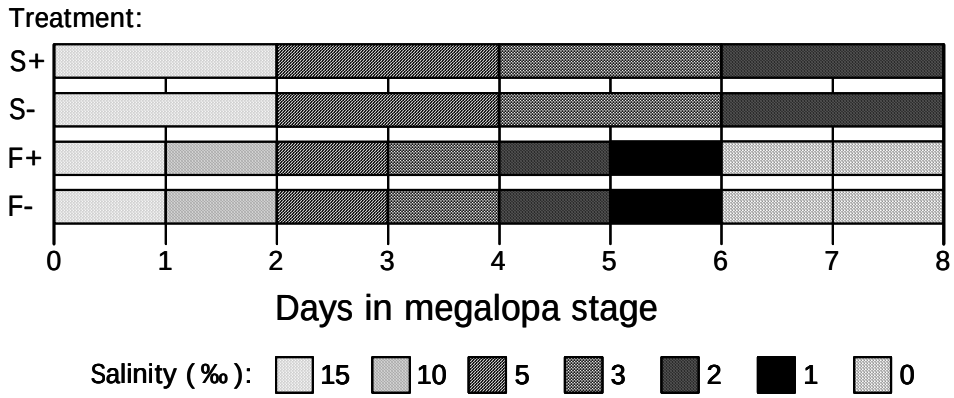

Fig. 3. Experimental design. Treatments with slow (S) or fast (F) reductions of salinity during the first 6d of megalopal development, in combination with (+) or without (-) chemical cues (odours from conspecific adult crabs).

Effects of salinity on the megalopa stage

The rate of survival through the megalopa stage was generally high (70-76%) and not significantly affected by the speed of salinity reduction (slow vs. fast; cf. Fig. 3), nor by the presence or absence of chemical cues from conspecific adults (Table 1; Fig. 4A). Our experiments proved that the megalopa stage of *A. roberti* is physiologically capable of acclimating rapidly (within 6d) to dramatically decreasing salinities. This acclimation allows not only continued survival (for another 6-8d) but also successful metamorphosis to the first juvenile crab stage under oligohaline or freshwater conditions (0.18-2.0 ‰). A recent study (Torres et al., 2006) showed that similarly low salinities (0-3 ‰) were not tolerated without previous acclimation. The limit for megalopal survival under continued exposure to low salinities was observed at 5 ‰, although this treatment caused significantly enhanced mortality and a developmental delay. The euryhalinity and the remarkable adaptability to rapidly decreasing osmotic pressures in the megalopa stage is a prerequisite for upstream migration and recruitment in brackish and limnic zones.

Table 1. Two-way ANOVA: effects of salinity reductions (fast *vs.* slow; cf. Fig. 3) and of the presence or absence of chemical cues from conspecific adults on survival and duration of development through the megalopa stage of *A. roberti*.

Factor	df	MS	F	p
Survival				
Salinity reduction (S)	1	0.000002	0.0001	0.99
Cues (C)	1	0.000066	0.0052	0.94
S x C	1	0.036317	2.8515	0.09
Error	44	0.012736		
Duration of development				
Salinity reduction	1	0.002	0.001	0.98
Cues	1	130.984	61.267	<0.0001
S x C	1	4.617	2.160	0.15
Error	44	2.138		

Since the tolerance of reduced salinities is based upon the expression of the physiological function of hyper-osmoregulation (Charmantier, 1998; Anger, 2003), our findings suggest that the megalopa of *A. roberti* enhances this function gradually during the course of its moulting cycle. This capability requires an appearance of ion-transporting cells (ionocytes) and regulating tissues, which may be located in the larval brachiostegites and/or the developing gills (see Cieluch et al., 2004, 2005; and earlier references therein). The formation of specialised ion-transporting tissues follows a developmental programme, which might be stimulated and accelerated by an increasing hypo-osmotic challenge by stepwise reductions of salinity. This hypothesis is supported by a study on another grapsoid crab, *Chasmagnathus granulata*, where a significant enhancement of early larval hyper-osmoregulation occurred after previous embryonic acclimation to a reduced salinity (Charmantier et al., 2002). Future investigations will have to identify the physiological and structural basis of the unusually great salinity tolerance of the megalopa of *A. roberti*, including the process of gradual acclimation and presumably accelerated appearance of ionocytes.

Effects of adult odours on the megalopa stage

During their migration into rivers, the megalopae of *A. roberti* are also exposed to increasing concentrations of chemical cues originating from upstream habitats, where the adults live. Our experiments showed that such cues, namely odours

released by conspecific adult crabs, had a significant metamorphosis-stimulating effect, reducing the duration of the final larval stage by about 25% (Fig. 4B; Table 1). Remarkably, this effect occurred regardless of the salinity conditions. In conclusion, our study suggests that metamorphosis of *A. roberti* is, also in the natural estuarine and riverine habitats of this species, triggered by chemical cues indicating the presence of conspecific adult populations further upstream, while rapidly decreasing salinities (from 25 ‰ to freshwater within only 6d) seem to have hardly any limiting effect on megalopal upstream migration.

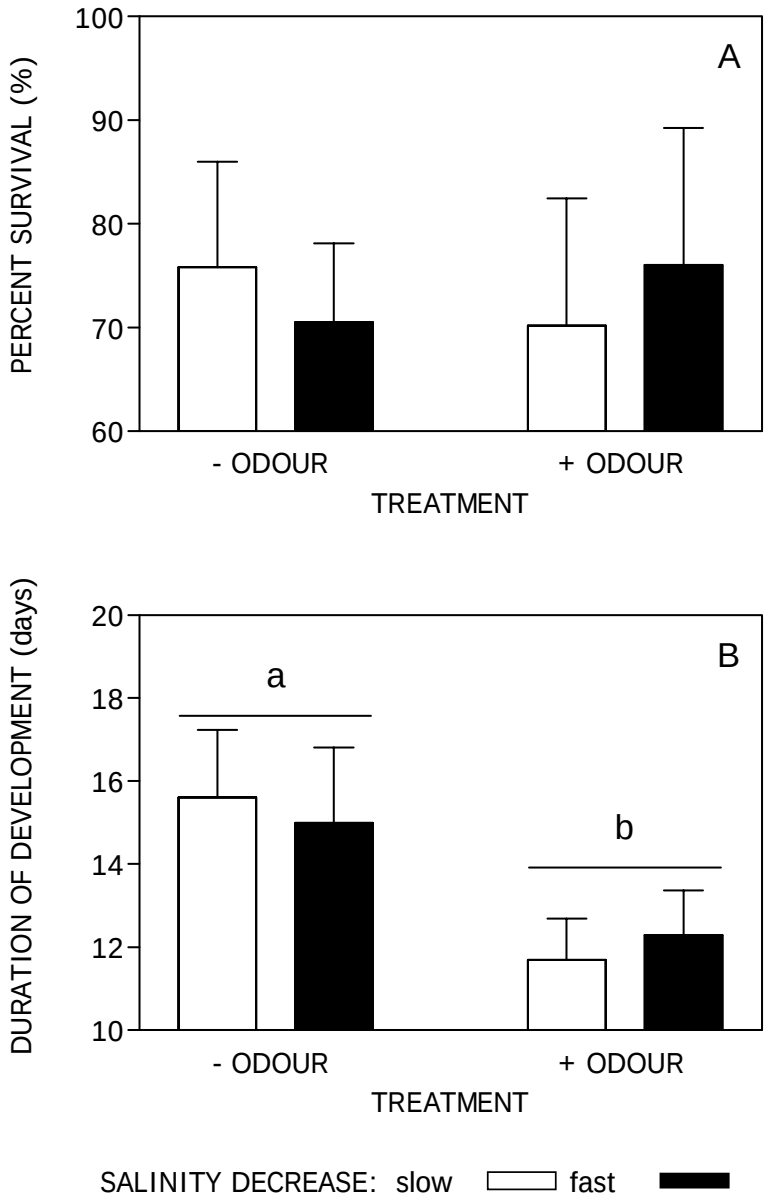

Fig. 4. *Armases roberti*. A, survival; B, duration of development through the megalopa stage to metamorphosis, during exposure to slow or fast reductions of salinity, in combination with (+) or without (-) chemical cues (odours from conspecific adult crabs; for experimental design, cf. Fig. 3).

In the two treatments without adult odours, the average durations of megalopal development were consistently longer than observed in a previous study (Torres et al., 2006). The difference (ca. 12 vs. 15d) cannot be explained by variation in salinities, chemical cues or other external factors, but reflects intraspecific variability among hatches reared at similar conditions, probably due to genetic and/or other maternal factors. Significant differences between larvae produced by different females and reared under similar conditions have been observed in most larval features including survival, duration of development, biomass, growth rate, and in some species even in the number of larval stages (for references, see Anger, 2001, Giménez and Anger, 2003). Likewise, the extent of the metamorphosis-accelerating response to adult odours has been shown to reveal significant intraspecific variability (Gebauer et al., 2005). This indicates that comparisons between results from different studies conducted with larvae from different conspecific females must be made with caution.

Chemical triggers from adult populations should not only lead megalopal re-immigration into the “right” direction. Additionally, the acceleration of megalopal development to metamorphosis should mitigate also potentially negative effects of hypo-osmotic stress, because the subsequent benthic juvenile crab stages are, compared to the megalopae, osmotically better protected by a thicker and less permeable cuticle. Moreover, young crabs have generally stronger capabilities of hyper-osmoregulation (Charmantier, 1998; Anger, 2003). As a consequence, the early juveniles may continue the upstream migration into rivers, without suffering from osmotic stress. The initial and decisive steps of recruitment, however, are in *A. roberti* most probably made already during the megalopa stage. Its euryhalinity and physiological adaptability to rapidly decreasing salinities, together with its metamorphosis-accelerating response to odours from conspecific adults, may be considered crucial life-history adaptations that permitted the invasion of freshwater environments during the evolution of this species of crab.

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IV. PHYSIOLOGICAL LEVEL

IV.1. Effects of short-term exposure to reduced salinities on biomass in species with different life histories

PUBLICATION 3

EFFECTS OF REDUCED SALINITIES ON LARVAL GROWTH AND PROXIMATE BIOCHEMICAL COMPOSITION ARE RELATED TO LIFE-HISTORIES: THE GENUS *ARMASES* AS A MODEL

Gabriela Torres, Luis Giménez, Klaus Anger (in prep.)

ABSTRACT

In the larval stages of three euryhaline species of the genus *Armases*, we tested if changes in biomass (dry mass, W; protein; lipid) under hypo-osmotic stress were related to their salinity tolerance, capabilities of osmoregulation, and migration patterns. As model species, we compared *Armases miersii*, which lives in supratidal rock pools, the riverine crab *A. roberti* (showing a larval export strategy), and *A. ricordi*, whose larvae probably develop in coastal marine waters. At each stage, larvae were exposed to different salinities (selected according to previous information on larval survival; range: 5 ‰ -32 ‰ for *A. miersii*, 10 ‰ -32 ‰ for *A. roberti*, and 15 ‰ -32 ‰ for *A. ricordi*). Biomass was measured in early postmoult and intermoult. The larvae of the strongly osmoregulating species *A. miersii*, which develop under highly variable salinity conditions, showed the smallest variations in biomass. The effect on *A. roberti* varied during its ontogeny: the Zoea I and the Megalopa, which carry out downstream and upstream migrations, respectively, showed lower biomass variations than the intermediate zoeal instars, which develop in coastal waters. The larval instars of *A. ricordi* showed the highest variations in biomass, reflecting poor adaptation to salinity variations. In addition, a common pattern was found for these estuarine species: the maximum of biomass shifted during ontogeny from 32 ‰ to 25 ‰, reflecting changes of the iso-osmotic point. The ontogeny of osmoregulation reflected ontogenetic migration patterns, which allow for avoiding detrimental effects of salinity variations.

Keywords: *Armases miersii*, *Armases ricordi*, *Armases roberti*, biochemical composition, larval development, larval growth, lipid content, osmotic stress, protein content.

INTRODUCTION

In coastal waters, changes in salinity may play a central role for larval survival and growth (Anger, 1998, 2001, 2003). In particular, biomass and chemical

composition are affected by reduced salinities (Pfaff, 1997; Anger et al., 1998, 2000; Torres et al., 2002). Variations in salinity affect the volume of water and the concentration of soluble compounds in an organism, and in consequence, alter the performance of its internal systems. The maintenance of a stable cellular composition is achieved through osmoregulatory strategies (for review see Péqueux, 1995).

Most marine crustaceans (e.g. *Cancer pagurus*, *Hyas araneus*) are osmoconformers (Péqueux, 1995; Pfaff, 1997). These species are therefore restricted to habitats with little variability in salinity (Péqueux, 1995). Many other crustaceans, however, can live in environments with variable salinity conditions. The benthic juvenile and adult stages of estuarine, semiterrestrial and terrestrial decapods, for instance, have evolved numerous physiological and behavioural adaptations to their physically variable environments (Greenaway, 1999). Nevertheless, their planktonic larvae are in most cases osmoconformers and remain therefore vulnerable to osmotic stress. Most of those species evolved larval export strategies, (Strathmann, 1982) i.e. the physiologically sensitive larvae are passively transported to waters with more stable conditions (e.g. *Armases roberti*: Diesel and Schuh, 1998; Torres et al., 2006; *Chasmagnathus granulata*: Anger et al., 1994; Charmantier et al., 2002; *Eriocheir sinensis*: Herborg et al., 2003; Cieluch et al., in press). A lesser number of species develops through euryhaline, osmoregulating larval instars, which can successfully cope with salinity variations (e.g. *Armases miersii*: Anger and Schultze, 1995; Charmantier et al., 1998). In these cases of advanced adaptation to non marine conditions, the larval stages can be retained within the parental habitats (“retention strategy”; Strathmann, 1982).

Torres et al. (2002) found that larval biomass (dry mass, protein and lipid content) of weakly osmoregulating Zoea-I larvae was less affected by low salinity than in stenohaline osmoconforming zoeae. These results suggested that effects of low salinity on larval biomass might (a) correlate with life-history strategies and ontogenetic patterns of osmoregulation and (b) be less detrimental in species with more strongly osmoregulating larvae. In the present study, we tested these predictions. We expected that a reduced salinity should produce weaker effects on larval biomass in species living in highly variable habitats and showing a retention strategy as compared to those being exported to coastal marine waters. In addition, we tested if larval instars involved in ontogenetic migrations between rivers and

coastal waters are less susceptible to salinity reductions that the intermediate instars of the same species, which normally develop in coastal waters with higher and more stable salinities.

To test our hypotheses, we compared three species of *Armases*, which live in different environments and show different reproductive strategies. The larval development of the strong osmoregulator *Armases miersii* occurs in isolated supratidal rock pools, where a wide range of salinity variation from freshwater to concentrated seawater occurs (Anger, 1995b, c; Schuh and Diesel, 1995). The larvae of *Armases ricordi* are directly released into coastal marine waters (Diesel and Schuh, 1998). *Armases roberti*, by contrast, shows a larval export strategy consisting of a rapid passive transport of the Zoea I from the riverine parental habitat to coastal waters and a later re-immigration of the Megalopa to freshwater habitats (Diesel and Schuh, 1998; Torres et al., 2006; Anger et al., in press).

MATERIALS AND METHODS

Model species

Armases miersii (Rathbun)

The distribution range of this semiterrestrial crab includes the Caribbean islands, Florida and the Bahamas (Abele, 1992). The juvenile and adult crabs live hidden in crevices between mangrove roots or on subtidal rocks as well as in coastal limestone caves with brackish or fresh water (Anger and Schultze, 1995; Anger, 1995b, c; Schuh and Diesel, 1995). On the northern shore of Jamaica, this species breeds in coastal mangrove swamps and in land-locked supratidal rock pools (Schuh and Diesel, 1995; Anger, 1995a). Its larval development consists of three zoeal stages and a Megalopa (Cuesta et al., 1999). The physico-chemical conditions in the breeding pools are highly variable, particularly regarding salinity. Larval development in such harsh environment is possible, because all larval stages are able to osmoregulate (Charmantier et al., 1998). The zoeae hyper-osmoregulate at low salinities and isoconform at salinities higher than 25 ‰. The Megalopa and the subsequent juvenile stages reveal a weakly developed but gradually increasing

capability of hyper-hypo-osmoregulation, i.e. hyper-regulation at low salinities and a slight hypo-regulation at salinities higher than 25 ‰. The adults show a strong capacity to hyper-hypo-osmoregulate in conditions ranging from freshwater to concentrated media (45 ‰) (Charmantier et al., 1998).

Armases ricordi (H Milne Edwards)

This highly terrestrial species ranges between Bermuda, Surinam and the Gulf of Mexico (Abele, 1992). It occupies typical dry coastal mangrove habitats as well as rocks and debris in the supralittoral fringe up to ca. 200 meters inland (Hartnoll, 1965; Chace and Hobbs, 1969; Abele, 1992; Diesel and Schuh, 1998). The ovigerous females migrate to the sea to release the larvae into coastal marine waters (Diesel and Schuh, 1998). All larval stages (four zoeae and a Megalopa) develop in the coastal planktonic environment (Díaz and Ewald, 1968; Alvarez and Ewald, 1990, Diesel and Schuh, 1998). They show a relatively narrow range of salinity tolerance, with high survival between 15 ‰ and 45 ‰ (G Torres, preliminary experiments). The four zoeal stages can slightly hyper-osmoregulate between 15 ‰ and 32 ‰ and isoconform at higher salinities. The Megalopa, surviving in a wider salinity range (5 ‰ -45 ‰), shows a strong hyper-osmoregulatory capacity between 5 ‰ and 25 ‰ and an incipient hypo-regulation at higher salt concentrations. In addition, the iso-osmotic point shifts during ontogeny from 32 ‰ to 25 ‰ (Charmantier et al., unpublished data).

Armases roberti (H Milne Edwards)

This little known tropical species is found in the West Indies, but not on the American mainland (Abele, 1992). The adult crabs live on the banks of rivers and streams from the mouth to several kilometres (ca. 10km) upstream (Chace and Hobbs, 1969; Abele, 1992). According to Diesel and Schuh (1998), the first zoea is released directly into freshwater near the terrestrial habitats of the adult population, from where the freshly hatched larvae are carried downstream by the river currents, so that they soon reach estuarine or coastal waters. The exported larvae develop in osmotically more stable waters through four zoeal stages to the megalopa, which is presumed to migrate back into the estuary and probably further upstream into the riverine parental habitat (Torres et al., 2006; Anger et al., in press). In agreement

with this tentative migration pattern, the Zoea I shows a wide range of salinity tolerance (0-50 ‰; Diesel and Schuh, 1998), hyper-osmoregulating between 5 ‰ and 32 ‰ and osmoconforming at higher salinities (Charmantier et al., unpublished data). The following larval stages have a narrower range of salinity tolerance (15-45 ‰; Diesel and Schuh, 1998), while their hyper-osmoregulatory capacity decreases (Charmantier et al., unpublished data). The tolerance to salinity variation increases again after metamorphosis to the Megalopa (Diesel and Schuh, 1998). This stage is able to strongly hyper-osmoregulate between 0 ‰ and 25 ‰ and slightly hypo-osmoregulate in higher salinities. The iso-osmotic point shifts during the course of larval development from 32 ‰ to 25 ‰ (Charmantier et al., unpublished data).

Handling of ovigerous females; rearing of adults and larvae

Larvae of *Armases miersii*, *A. roberti*, and *A. ricordi* were obtained from ovigerous females wich had been collected near the Discovery Bay Marine Laboratory (DBML), Jamaica. After transport to Helgoland Marine Biological Station (BAH, Helgoland, Germany), the crabs were maintained under controlled conditions of temperature (24°C), salinity (25 ‰ for *A. miersii*; 32 ‰ for *A. ricordi*; 0.2 ‰ for *A. roberti*), a 12:12h photoperiod, and feeding *ad libitum* with frozen isopods *Idotea* sp. (for further details of habitat characteristics, collection and long-term cultivation, see Anger and Moreira, 2004; Torres et al., 2006; Anger et al., in press). Ovigerous females were isolated in individual aquaria and kept under otherwise identical conditions until larvae hatched.

All larval stages were mass-reared at constant salinities (25 ‰ for *A. miersii* and *A. roberti*, 32 ‰ for *A. ricordi*) and otherwise identical conditions as the adults (see above). They were fed *ad libitum* with freshly hatched *Artemia* sp nauplii. Freshly hatched larvae were used for experiments (see below for details, Fig. 1) or mass-reared to the subsequent stages in 400-mL bowls without aeration. Larval density was initially 50 per bowl (Zoea I), thereafter decreased according to increasing size in the following zoeal stages (30, 20, 10, respectively). Filtered (pore size 1µm) seawater (32 ‰) was mixed with appropriate amounts of desalinated freshwater to obtain the experimental salinities (5 ‰, 10 ‰, 15 ‰, 20 ‰ and 25 ‰). In daily intervals, water was changed, dead larvae were removed, new food was added, and

cultures were controlled for ecdyses. Freshly moulted larvae were transferred to new bowls to ensure that all larvae used in experiments had the same moulting history and the same age within each moulting cycle.

Experimental design

Every set of experiments comprised treatments with a series of salinities (ranging from 5‰ to 32‰). Larval exposure lasted from early postmoult (<12 hs after hatching or moulting) to the intermoult stage (ca. 50 ‰ of the moulting cycle; Fig. 1). These treatments represent conditions of optimal salinity, severe, and moderate osmotic stress, respectively.

Five replicate groups of larvae were reared in 400-mL bowls. Samples of larvae were taken at hatching (Zoea I) or at early postmoult (later larval stages), and again at the end of each experiment. In all samples, larval dry mass (W), contents of lipid and protein were measured (see below).

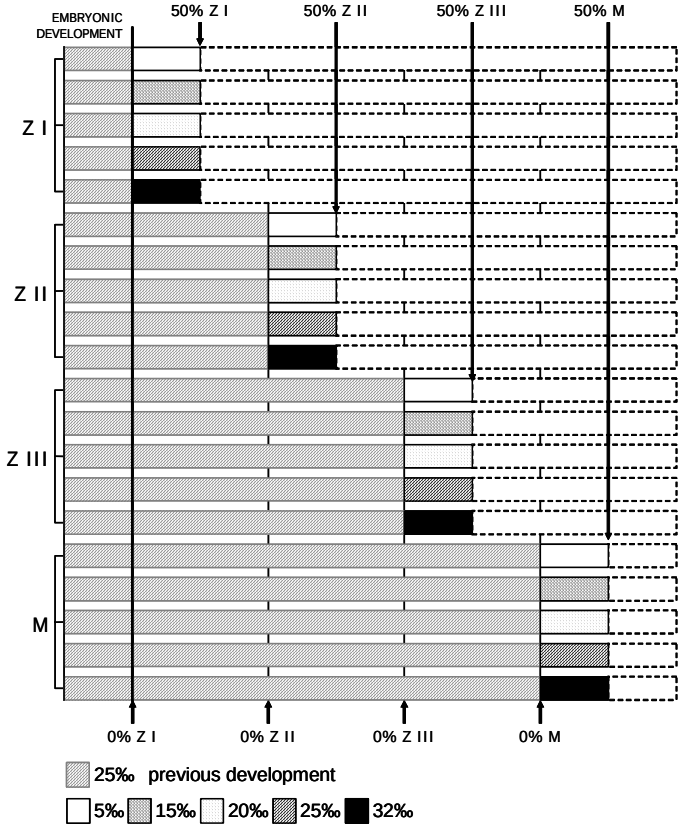
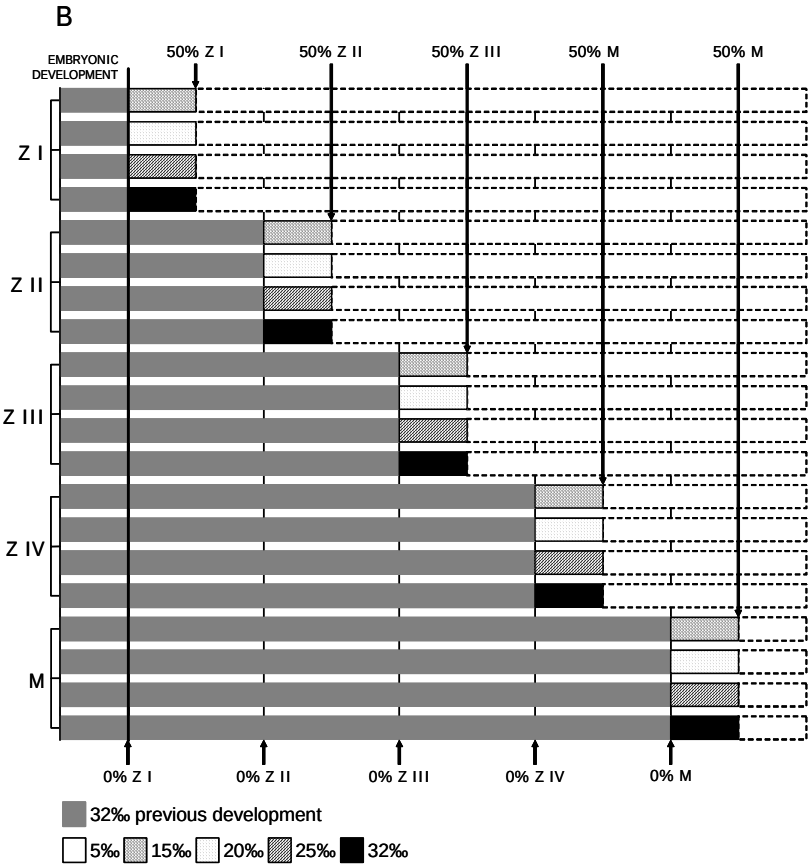


Fig. 1. Experimental design to study effects of salinity on dry mass and biochemical composition during larval development of A: *Armases miersii*, B: *A. ricordi* and C: *A. roberti*. Postmoult and intermoult samples (see arrows) were taken immediately after hatching or moulting (0%) and at 50% of the moult cycle, respectively.



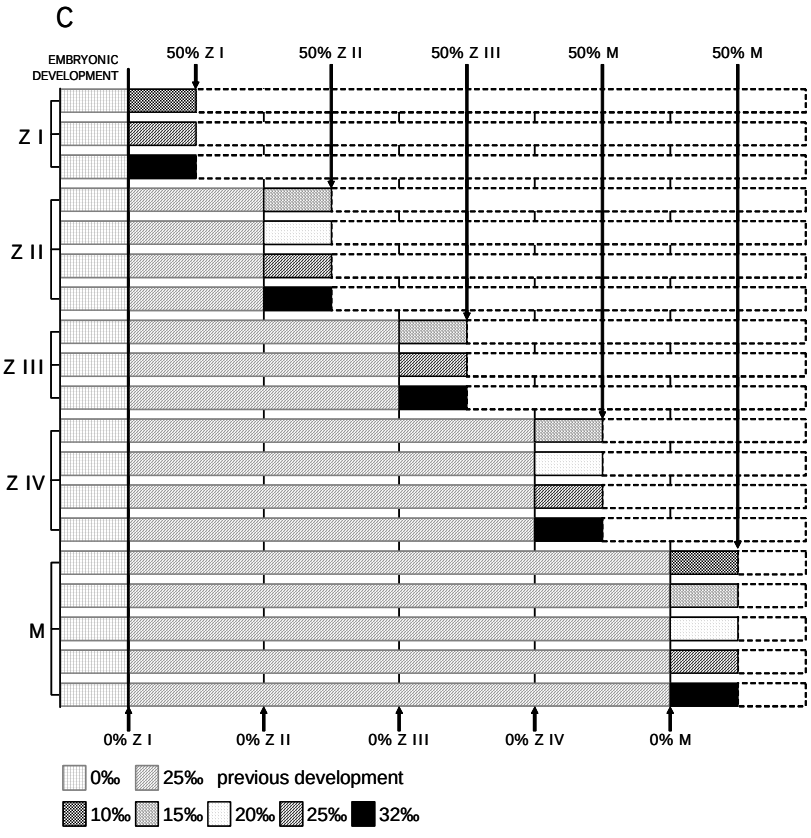


Fig. 1. continued

Biochemical analyses

Samples for biochemical analyses were gently rinsed in distilled water for 10 s and blotted on filter paper. Subsequently, they were transferred to Eppendorf vials and frozen at -80 °C. The samples were left in a vacuum drier (Finn-Aqua Lyovac GT2E) for 48 h and their dry mass was determined in a Sartorius MC1 RC 210 S balance (precision: 0, 01 mg, capacity 210 g). Afterwards, they were homogenized by sonication (Branson, Sonifier, Cell Disruptor B 15) with 5 strokes of 5 sec, on ice and each homogenate was divided in two aliquots to perform lipid and protein content determinations.

Protein determination

The total protein content of the homogenate was determined using a modified method (Anger et al., submitted) after Lowry et al., (1951; kit: BioRad D_C Protein Assay).

Lipids determination

The total lipid content of the homogenate was determined after the sulphophosphovanillin method following Zöllner and Kirsch (1962), modified for microplates (Anger et al., submitted)

Statistical analyses

Statistical analyses were performed following Zar (1996). Data-sets from *A. miersii* and *A. ricordi* were analysed by two-way ANOVA with developmental stage and salinity as fixed factors. Data from *A. roberti* were analysed for each stage separately using one-way ANOVA with salinity as factor, because different test salinities were used in different larval instars. For all stages, planned comparisons were made in order to test for significant growth from hatching or early postmoult to intermoult. The number of replicates was 4-5 for each species and stage-salinity combination.

When significant differences were detected in the ANOVAs, comparisons between different factors were performed with the Student-Newman-Keuls test (SNK). The critical level (α) to reject the null hypothesis was fixed at 0.05. Previous to performing ANOVAs, normality (normal plots) and variance homogeneity (Cochran

test) were checked. When raw data failed to meet the assumptions logarithmically transformed data showed normal errors and variance homogeneity.

RESULTS

Armases miersii

Significant increments in larval dry mass (W) were found in all larval stages and at all salinities. However, salinity affected the increase in W from postmoult to intermoult, and these salinity effects varied significantly among instars (Table. 1). In the Zoea I, W was at 5‰ significantly higher than at 15‰-32‰ (Fig. 2). The following stages, by contrast, reached lower W at 5‰ than at the other salinities, while their maximum was reached at 32‰.

In the first and second zoeal instars, the protein levels were not significantly affected by salinity. The Zoea III reached the maximum value at 15‰. The salinity of maximum protein growth shifted then to 5‰ in the Megalopa (Fig. 3).

The lipid content was generally not affected by salinity (Table 1, Fig. 4). Both the protein and lipid contents increased in all stages and salinities from postmoult to intermoult (Figs. 3, 4).

Table 1. *Armases miersii*. Two-way ANOVA to evaluate the effect of salinity and larval instar on dry mass, protein and lipid content (log-transformed data). Symbols: df: degrees of freedom, MS: means squares. Mean squares are (1 x 10-2). Significant levels are indicated as follows: ns: non significant; *: <0.05, **:<0.01, ***:<0.001.

	Dry mass			**Protein**			**Lipids**		
Factor	df	MS	F	df	MS	F	df	MS	F
Instar (I)	3	821	3747***	3	1394	7470***	3	380	355***
Salinity (S)	4	1.6	7.0***	4	1.1	5.9***	4	0.9	0.8 ns
I x S	12	1.8	8.0***	12	0.4	1.9 *	12	1.2	1.1 ns
Error	85	0.2		81	0.2		85	1.1	

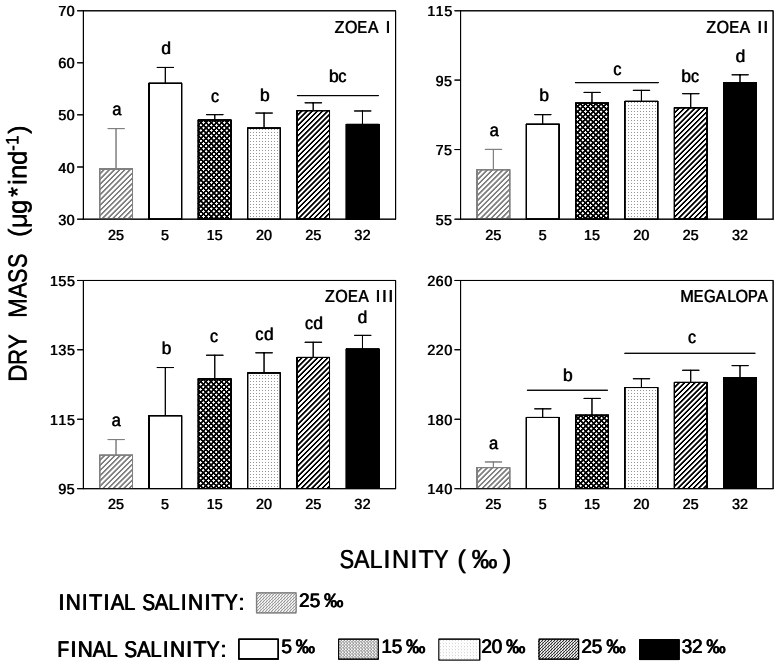


Fig. 2. *Armases miersii*. Changes in dry mass (expressed as µg*ind^{-1}) after exposure to 5‰, 15‰, 20‰, 25‰ and 32‰. Grey column: biomass at hatching or early postmoult; black columns: biomass at intermoult. Different letters show significant differences after SNK test among salinities at hatching and intermoult, n.s. not significant difference.

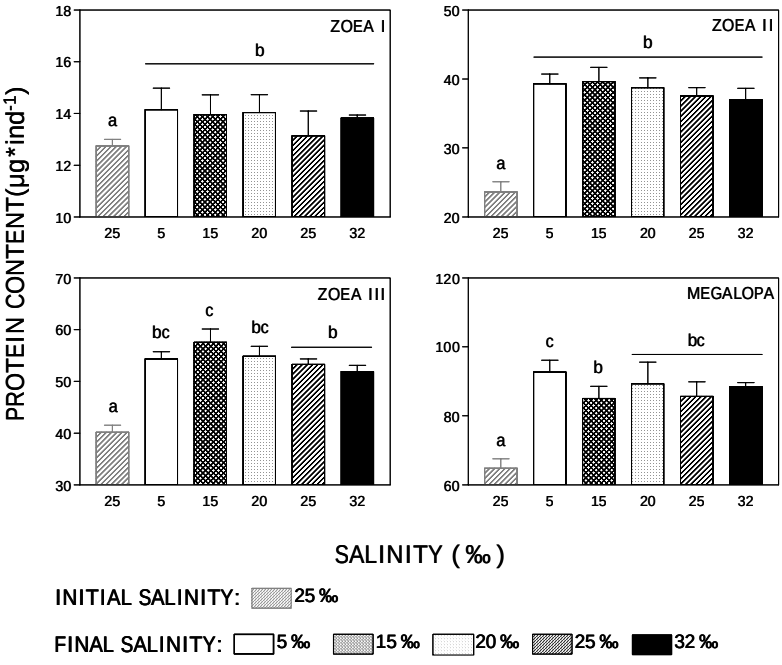


Fig. 3. *Armases miersii*. Changes in protein content (expressed as µg*ind^{-1}) after exposure to 5‰, 15‰, 20‰, 25‰ and 32‰. Grey column: biomass at hatching or early postmoult; black columns: biomass at intermoult. Different letters show significant differences after SNK test among salinities at hatching and intermoult, n.s. not significant difference.

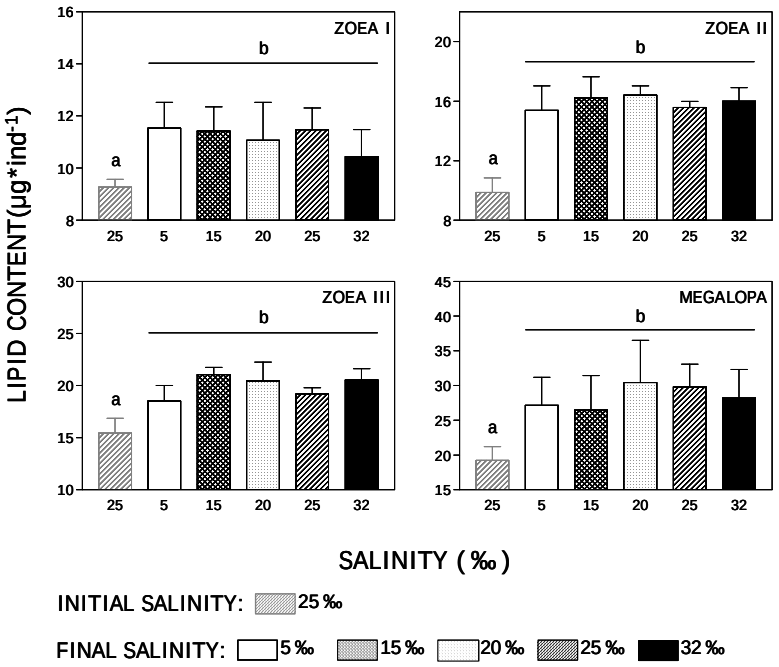


Fig. 4. *Armases miersii*. Changes in lipid content (expressed as µg*ind^{-1}) after exposure to 5‰, 15‰, 20‰, 25‰ and 32‰. Grey column: biomass at hatching or early postmoult; black columns: biomass at intermoult. Different letters show significant differences after SNK test among salinities at hatching and intermoult, n.s. not significant difference.

Armases ricordi

Effects of salinity on W, protein and lipid contents varied among larval instars (Table 2). In all stages, W was lowest at the lowest test salinity (15‰; Fig. 5). The W of the early stages (Zoea I -III) did not significantly vary in the range 20-32‰, but later instars showed a maximum at 25‰. The Zoea II and Zoea IV did not show increments in W at 15‰, while a significant increase occurred at higher salinities.

The protein content reached at the intermoult stage increased significantly with salinity in the Zoea I and Zoea II, while the Zoea III showed a peak at 20‰, and no effect was found in the Zoea IV (Fig. 6). The increments in protein measured from postmoult to intermoult were significant in all stages (Fig. 6). Interestingly, however, the megalopae showed a significantly higher protein content at 15‰ than at 32‰, reversing the pattern occurring in earlier larval instars.

In the lipid content, growth increased significantly with increasing salinity, except for the Megalopa stage (Fig. 7). In the range 20‰ - 32‰, significant effects were found in the Zoea III, i.e. the lipid content was higher at 32‰ than at 20‰. No increments in the lipid content, measured from postmoult to intermoult, were found

in the Zoea II, III and IV stages exposed to 15 ‰; otherwise, lipid growth was always significant.

Table 2. *Armases ricordi*. Two-way ANOVA to evaluate the effect of salinity and larval instar on dry mass, protein and lipid content (log-transformed data). Symbols: df: degrees of freedom, MS: means squares. Mean squares are (1 x 10^{-2}). Significant levels are indicated as follows: ns: non significant; *: <0.05, **:<0.01, ***:<0.001

	Dry mass			**Protein**			**Lipids**		
Factor	df	MS	F	df	MS	F	df	MS	F
Instar (I)	4	1344	7171***	4	2025	4101***	4	1368	3242***
Salinity (S)	3	24.7	131***	2	4.1	8.3***	3	15	37***
I x S	12	0.6	3.2***	12	1.3	2.7 **	12	1.8	4.0 **
Error	77	0.2		80	0.5		77	0.4	

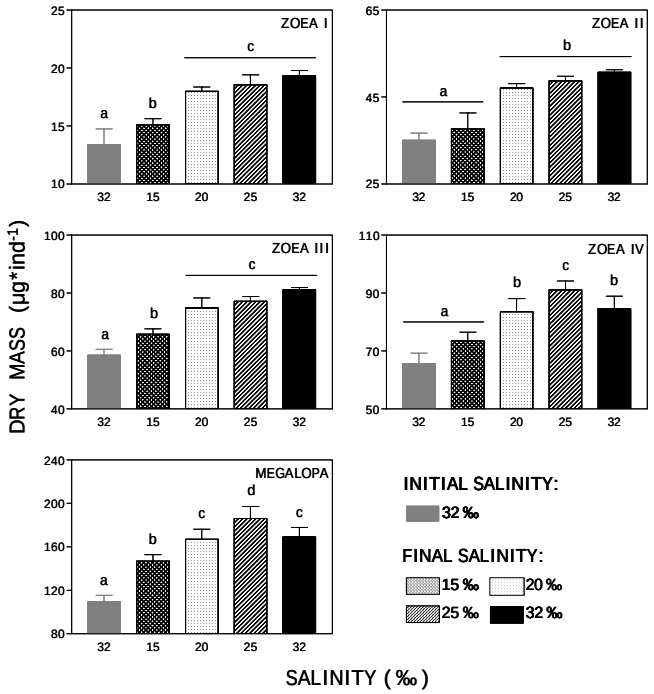


Fig. 5. *Armases ricordi*. Changes in dry mass (expressed as µg*ind^{-1}) after exposure to 15 ‰, 20 ‰, 25 ‰ and 32 ‰. Grey column: biomass at hatching or early postmoult; black columns: biomass at intermoult. Different letters show significant differences after SNK test among salinities at hatching and intermoult, n.s. not significant difference.

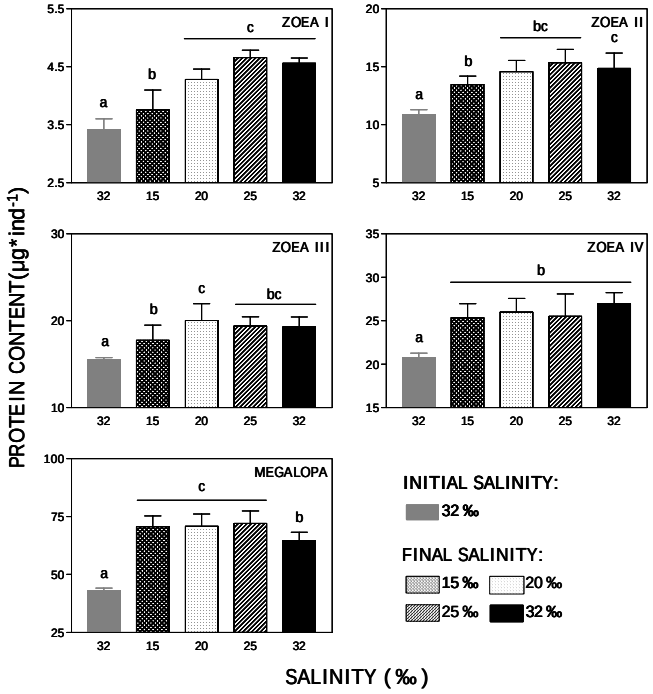


Fig. 6. *Armases ricordi*. Changes in protein content (expressed as µg*ind^{-1}) after exposure to 15 ‰, 20 ‰, 25 ‰ and 32 ‰. Grey column: biomass at hatching or early postmoult; black columns: biomass at intermoult. Different letters show significant differences after SNK test among salinities at hatching and intermoult, n.s. not significant difference.

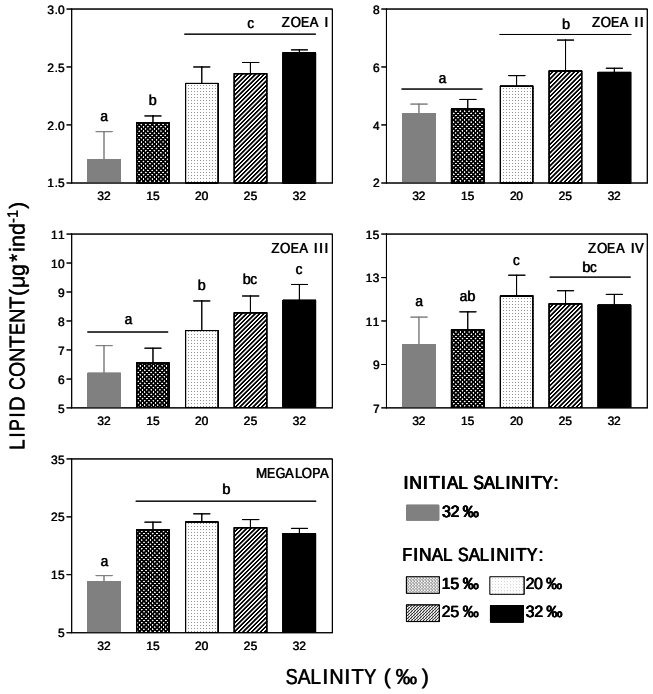


Fig. 7. *Armases ricordi*. Changes in lipid content (expressed as µg*ind^{-1}) after exposure to 15 ‰, 20 ‰, 25 ‰ and 32 ‰. Grey column: biomass at hatching or early postmoult; black columns: biomass at intermoult. Different letters show significant differences after SNK test among salinities at hatching and intermoult, n.s. not significant difference.

Armases roberti

In this species, salinity affected larval W only in the early instars (Zoea I-III, Table 3). W was lower at 10-15 ‰ than at higher salinities, reaching in the zoeal stages I and II a maximum at 25-32 ‰, and at 25 ‰ in the Zoea III (Fig. 8). Intermoult W in the Zoea IV and Megalopa tended to be higher at 10 ‰-15 ‰, but the differences were statistically not significant. In all stages, W growth from postmoult to intermoult was significant (Fig. 8).

The effect of salinity on the protein content was only in Zoea IV and Megalopa significant (Table 3). The lowest protein increments were found at 15 ‰ in the Zoea IV and at 10 ‰ in the Megalopa (Fig. 9). The Megalopa had its maximum protein content in the range 15 ‰ - 25 ‰. Increments in the protein content were significant in all stages and at all salinities.

The lipid content was in the Zoea I, III and Megalopa affected by salinity (statistically not significant in the Zoea II; Table 3). While the zoeal stages I and III showed significantly higher lipid contents at 32 ‰, the Megalopa reached its maximum at 25 ‰ (Fig. 10). The increments in the lipid content measured from postmoult to intermoult were in the Zoea I only at 32 ‰ significant, while all other instars showed significant lipid growth at all salinities.

Table 3. *Armases roberti*. One-way ANOVAs per stage to evaluate the effect of salinity and larval instar on dry mass, protein and lipid content (all data log-transformed except Lipids in Megalopa). Symbols: df: degrees of freedom, MS: means squares. Mean squares are (1×10^{-2}). Significance levels are indicated as follows: ns: non significant, *: <0.05, **:<0.01, ***:<0.001

	Dry mass			**Protein**			**Lipids**		
Factor	df	MS	F	df	MS	F	df	MS	F
Zoea I									
Salinity	2	9.72	19.8***	2	0.29	0.66 ns	2	10.3	5.85 *
Error	12	0.49		12	0.45		12	1.8	
Zoea II									
Salinity	3	2.06	7.6 **	3	0.04	0.13 ns	3	1.4	0.36 ns
Error	15	0.27		15	0.32		15	3.9	
Zoea III									
Salinity	2	3.05	16.4***	2	0.50	1.04 ns	2	4.4	6.1 *
Error	12	0.19		12	0.48		11	0.7	
Zoea IV									
Salinity	3	0.62	2.0 ns	3	2.29	9.80**	3	3.4	1.11 ns
Error	16	0.31		16	0.23		15	3.1	
Megalopa									
Salinity	4	0.40	1.7 ns	4	1.20	4.60**	4	102.2	3.70 *
Error	20	0.24		20	0.26		19	27.6	

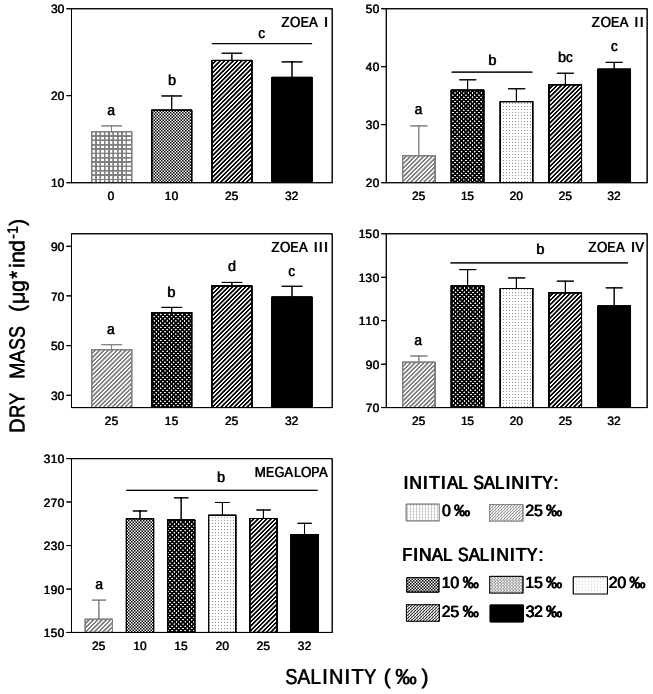


Fig. 8. *Armases roberti*. Changes in dry mass (expressed as µg*ind^{-1}) after exposure to 10-32 ‰. Grey column: biomass at hatching or early postmoult; black columns: biomass at intermoult. Different letters show significant differences after SNK test among salinities at hatching and intermoult, n.s. not significant difference.

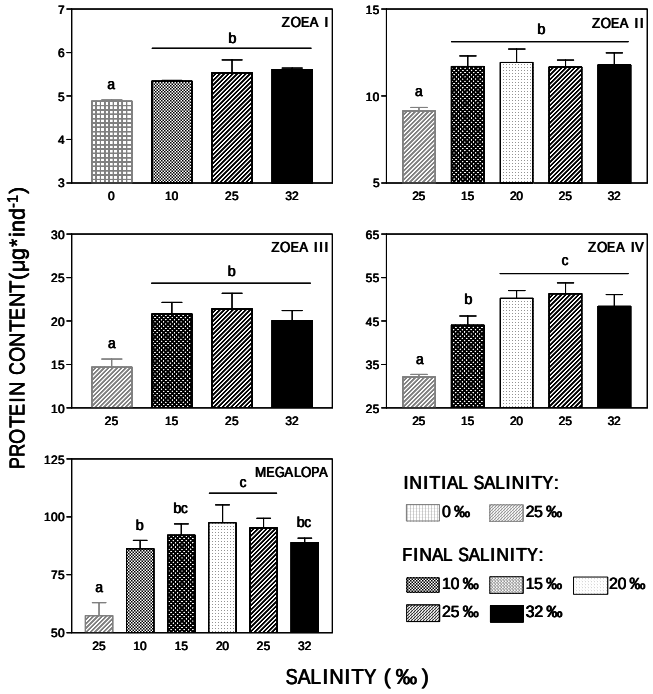


Fig. 9. *Armases roberti*. Changes in protein content (expressed as µg*ind^{-1}) after exposure to 10-32 ‰. Grey column: biomass at hatching or early postmoult; black columns: biomass at intermoult. Different letters show significant differences after SNK test among salinities at hatching and intermoult, n.s. not significant difference.

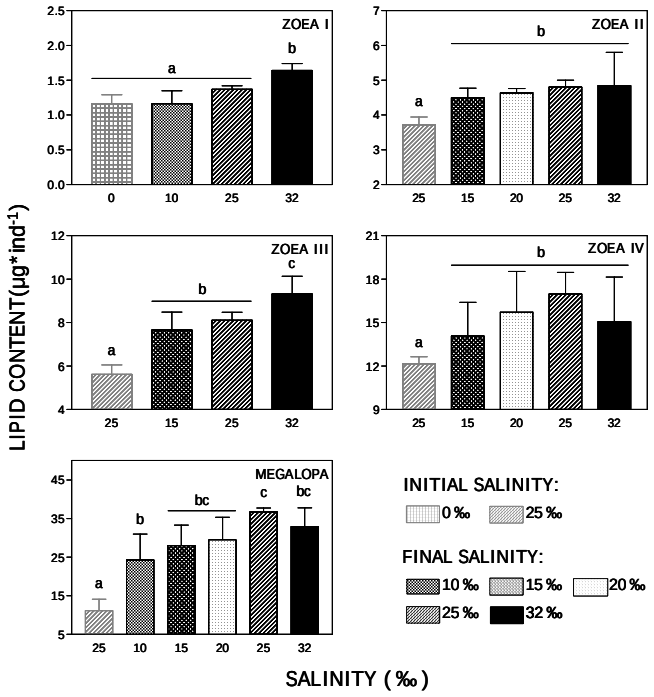


Fig. 10. *Armases roberti*. Changes in lipid content (expressed as µg*ind^{-1}) after exposure to 10-32 ‰. Grey column: biomass at hatching or early postmoult; black columns: biomass at intermoult. Different letters show significant differences after SNK test among salinities at hatching and intermoult, n.s. not significant difference.

Interspecific comparisons

Interspecific comparisons were made by means of calculating biomass-specific effects of salinity for each species and stage:

$$B'_{H\text{-}L} = (B_H - B_L) \bullet 100 \,/\, (B_{25} \bullet (S_H - S_L))$$

where B_H is individual biomass (W, protein or lipid content per larvae) measured at the end of the experiment (at intermoult); at the higher salinity (S_H) in an interval of salinities (S_H - S_L), and B_L is the biomass at the lower salinity (S_L). The dimension is the percentage of variation in biomass (in %B per ‰ of salinity change) in relation to the biomass (B_{25}) at the reference condition: 25 ‰.

Biomass-specific effects of salinity for the ranges 15-25 ‰ and 25-32 ‰ ($B'_{25\text{-}15}$ and $B'_{32\text{-}25}$) are shown in Fig. 11, for each stage and species. Positive $B'_{25\text{-}15}$ and $B'_{32\text{-}25}$ values indicate a higher biomass increment at the higher compared to the lower salinity (B_H vs. B_L). Negative B' values indicate a lower increment in biomass at the higher salinity.

Armases miersii showed very small values of B'_{25-15} and B'_{32-25} (between -1.3%/‰ and 1.1%/‰), indicating little susceptibility of biomass to variation in salinity (Fig. 11). For W, a change from positive to negative B'_{32-25}, at the early zoeal stages indicated a shift in the salinity where the increment in biomass was maximal.

Armases ricordi showed a stronger susceptibility to salinity than the other two species. The B'_{25-15} values were >2%/‰ except for the protein contents of Zoea IV and the protein and lipid contents of Megalopa (Fig. 11). Thus, the variations in the protein and lipid contents tended to decrease with development. The B'_{32-25} values for protein and lipid growth indicated that a shift of the maximum from 32‰ to 25‰.

Armases roberti showed little susceptibility to salinity, albeit a stronger response than *A. miersii* (Fig. 11). The highest variation was found in the lipid content, where the B'_{25-15} values of the Zoea IV and the Megalopa as well as the B'_{32-25} values of the Zoea I and Zoea III were ca. 2%/‰. As in *A. ricordi*, the maximum W, protein and lipid growth shifted from 32‰ to 25‰, indicated by changes in the sign of B'_{32-25}.

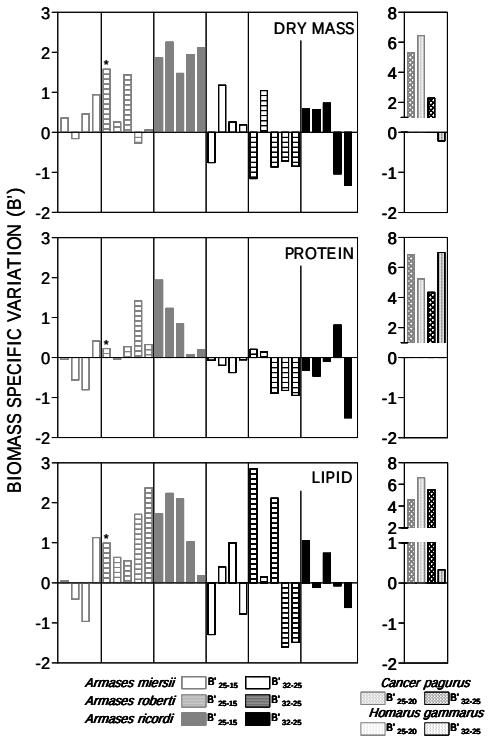


Fig. 11. Biomass-specific variation for each stage and species studied, exposed to different salinities. Variation at the interval 15‰-25‰ or 20‰-25‰: grey columns; at the interval 25‰-32‰: black columns. *Armases miersii* (ZI-M): blank columns; *A. roberti* (ZI-M): striped columns; *A. ricordi* (ZI-M): full columns; *Cancer pagurus* (ZI): crossed columns; *Homarus gammarus* (ZI): doted columns. * For Zoea I of *A. roberti*, we calculated B'_{25-10} (i.e. using 10‰), since 15‰ was not measured. Data from *C. pagurus* and *H. gammarus* After Torres et al. (2002).

DISCUSSION

Our experiments have shown that, within the genus *Armases*, effects of salinity on larval biomass vary among species and ontogenetic stages. These effects appear to be related to the osmoregulatory capacity as well as ecological characteristics of each species and larval stage.

The larval development of *Armases miersii* occurs in isolated supratidal rock pools, which are characterized by strong variations in salinity. Anger (1995 b, c) and Schuh and Diesel (1995) showed that evaporation can cause an increase in salinity up to >40 ‰, while strong rainfall leads to sudden reductions down to freshwater conditions. All larval stages already possess strong hyper-osmoregulatory capacities within the range 5-25 ‰ (Charmantier et al., 1998). This capability is most probably based on an increase in the level of mRNA encoding for the Na^+-K^+-ATPase, one of the key enzymes in the osmoregulatory mechanisms (Torres et al., submitted). These adaptive traits should have reduced the susceptibility of the larvae of *A. miersii* to salinity variation, reducing osmotic stress and physiological damage. This may explain why *A. miersii* showed the smallest changes in biomass growth, especially in that of protein and lipid. The increase in W at higher salinities may reflect an uptake of inorganic substances (Anger, 1991), while the protein content showed a different pattern of variation with a peak in the protein content of the Zoea III at 15 ‰ and no changes in the lipid content.

Compared to *Armases miersii*, *A. ricordi* and *A. roberti* showed wider variations in biomass. The larvae of *Armases ricordi*, which develop in coastal waters (Díaz and Ewald, 1968; Alvarez and Ewald, 1990, Diesel and Schuh, 1998), experience in nature a narrower range and less variation in salinity than *A. miersii*. This corresponds with a weaker osmoregulatory capacity (Charmantier et al., unpublished data). The larvae of *A. ricordi* were less successful to cope with salinity variations, showing greater changes in biomass, especially in the Zoea I. The direct release of larvae into coastal waters may prevent osmotic stress and reduced growth. In later larval instars, the maximum biomass growth shifted from 32 ‰ to 20 ‰. This might be related to the osmoregulatory pattern shown by the Megalopa (i.e. a stronger hyper-osmoregulatory capacity and a shift of the iso-osmotic point from 32 ‰ to 25 ‰; Charmantier et al., unpublished data).

The environmental conditions experienced by the larval instars of *A. roberti* vary during ontogeny. The freshly hatched Zoea I faces freshwater in the riverine habitats, the following zoeae develop in estuarine or marine waters, and finally, the Megalopa encounters brackish or freshwater conditions during its re-immigration (Torres et al., 2006; Anger et al., in press). The variations in the surrounding media experienced by successive larval stages correspond with changes in the ontogenetic pattern of osmoregulation (Charmantier et al., unpublished data). In agreement, the variations of biomass in the Zoea I exposed to salinities between 10 ‰ and 32 ‰ were low. The following zoeal stages showed higher variations in a narrower salinity range (15 ‰ -32 ‰). During development from the Zoea II to Megalopa, maximum growth shifted from 32 ‰ to lower salinities.

An index of biomass variation allowed for preliminary interspecific comparisons (see Torres et al., in prep). All euryhaline larval stages of the three species of *Armases* showed smaller changes in biomass than the Zoea I of the stenohaline species *Cancer pagurus* and *Homarus gammarus* (Fig. 11, Torres et al., 2002). Therefore, an increase in euryhalinity appears to be related to decreased sensitivity of growth to salinity variations.

In summary, our results support our predictions. Ontogenetic changes in osmoregulation are reflected in the ability of larvae to survive and grow at low salinities.

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IV.2. Relationship between effects of short-term exposure to reduced salinities on biomass and osmoregulatory capacity

PUBLICATION 4

RELATIONSHIPS BETWEEN GROWTH, EURYHALINITY AND OSMO-REGULATION IN DECAPOD CRUSTACEAN LARVAE

Gabriela Torres, Luis Giménez, Klaus Anger (in prep.)

ABSTRACT

Searching for general responses of larval growth, biomass, and chemical composition (protein and lipid content) to variations in salinity, we compared twelve species of decapod crustaceans varying in the degree of larval euryhalinity, ranging from strongly euryhaline osmoregulators to stenohaline osmoconformers. Data for seven species were obtained from previously published papers and unpublished studies. Data for five additional species are reported here for the first time. Biomass in euryhaline larval instars showed consistently a lower sensitivity to variations in salinity, even at the lower limits of salinity tolerance. Biomass in stenohaline larvae showed a higher sensitivity, high interspecific variability of the ratio Lipid:Protein, and decreasing detrimental effects of variations in salinity with increasing osmoregulatory capacity. Our data suggest that the ability to osmoregulate buffers the internal medium, so that strong euryhalinity involves a reduced sensitivity of metabolic processes, allowing for larval growth also at low salinities. The metabolic costs for osmoregulation in decapod crustacean larvae may be offset by the capacity of maintaining high growth in a variable environment.

Keywords: *Hyas araneus*, *Liocarcinus pusillus*, *Nephrops norvegicus*, *Perisesarma fasciatum*, *Sesarma curacaoense*, salinity, osmoregulation, biomass, growth.

INTRODUCTION

Salinity is a key factor to explain patterns of growth and distribution of coastal marine invertebrates (Anger, 2003). For benthic stages, important variations in salinity may be frequently experienced in species occurring in estuarine areas. By contrast, unusual low salinities should be experienced only sporadically by benthic stages occupying fully marine habitats, perhaps as a consequence of long-term variations in oceanographic conditions. However, the planktonic larvae of semiterrestrial, estuarine and fully marine species might experience low salinities. For instance, the larvae of estuarine crabs retained in estuarine areas or in land locked

habitats experience the low and variable salinities characterizing these habitats (*Armases miersii*: Anger, 1995a, b; *Rhithropanopeus harrisii*: Chen et al., 1997; *Crangon crangon*: Paula, 1998). For species with larval exporting strategy, low salinity is experienced during the reinvasion of estuarine habitats by the last larval stage (e.g. *Callinectes sapidus*: Tankersley et al., 1995; *Eriocheir sinensis*: Anger, 1991), or in some species also by the first larval stage (e.g. *Chasmagnathus granulata*: Anger et al., 1994; *Armases roberti*: Torres et al., 2006).

Besides, fully marine species might experience low salinities, at least for short times. Planktonic larvae are transported by currents and therefore may disperse at larger scales than benthic stages, increasing the risk of experiencing osmotic stress. In addition, storm and heavy rainfall events, occurring at a seasonal scale may lead to high levels of freshwater discharge reducing the salinity in wide portions of coastal waters. Decapod larvae exhibit a certain capacity for movement associated partly to behavioural responses to salinity changes (Queiroga and Blanton, 2005). However, these responses are restricted to a change of the vertical position in the water column and thus in the local oceanographic conditions.

In consequence, the evaluation of the physiological responses to salinity in larval stages has greater ecological importance than in benthic stages. Short-term fluctuations in salinity affect planktonic invertebrate larvae in general (Richmond and Woodin, 1996; Anger, 2003). Studies on the effect of low salinity on larval survival and developmental rates of marine and estuarine decapod crustaceans are abundant in the literature (reviewed in Anger, 2003). In the recent years, a considerable progress has been made in the study of osmoregulatory patterns during larval development of decapod crabs (reviewed in Charmantier, 1998). There are diverse patterns of ontogeny of osmoregulation. In several species, both osmoconforming and osmoregulating larval instars occur during larval development, (Charmantier, 1998; Charmantier et al., 2002; Cieluch et al., in press). Less information is available about general patterns of biomass and chemical composition in response to exposure to low salinity or its relationship to the osmoregulation pattern. Evaluations of the effect of salinity in larval growth and metabolism of a few species, have found reductions in feeding, assimilation and growth, when larvae experience osmotic stress (Johns, 1982; Anger et al., 1998). Torres et al. (2002) found that the negative effects of low salinity on growth of Zoea I were larger in two stenohaline species

(*Cancer pagurus* and *Homarus gammarus*) than in the weakly euryhaline *Carcinus maenas* and *Chasmagnathus granulata*. In addition, osmoregulating Megalopa of *C. granulata* show cumulative effects of long-term exposure to low salinity (Torres et al., in prep a). An evaluation on the ontogeny of salinity effects on biomass and growth was made in three strong euryhaline species of the genus *Armases* with different life-history strategies (Torres et al. in prep b). In this paper, we review the available information and add data of five species in order to determine whether there are general responses to salinity in the larval growth, biomass and chemical composition (protein and lipid content) in decapod crustacean larvae.

In particular, the available data allowed us to explore the relationships between effects of salinity on biomass vs. (a) degree of euryhalinity, (b) minimum salinity for which larval survival was enough (>80%) for biomass determinations and (c) the osmoregulatory capacity. According to previous findings (Torres et al. 2002, Torres et al., in prep b) we expected that the detrimental effects of low salinity on larval growth declined from stenohaline to euryhaline species and as osmoregulatory capacity increased.

MATERIALS AND METHODS

Experiments: handling of animals and larval rearing

Five sets of experiments with Zoea I of three stenohaline species (*Hyas araneus*, *Nephrops norvegicus* and *Liocarcinus pusillus*) and two euryhaline (*Perisesarma fasciatum* and *Sesarma curacaoense*) were performed to obtain additional data on biomass (Table 1.) The experiments were run following the methodology described in Torres et al. (2002, in prep a, in prep b). Briefly, ovigerous females of each species were kept under optimal controlled conditions of temperature, photoperiod, salinity and food (frozen isopod *Idotea* sp.). Replicate groups of freshly hatched larvae were assigned to 3-5 salinity treatments according to the species salinity tolerance. Thus, test salinities varied among species (see Results). Otherwise, larval rearing was made under identical conditions to those of ovigerous females, with *Artemia* sp. nauplii as *ad libitum* food. Water and food were changed every day. Experimental salinities were obtained mixing filtered seawater (32‰, Orion, pore

size 1µm) with appropriate amounts of desalinated tap water. Samples were taken shortly after hatching (<24 hs.), and at 50% of the moulting cycle (postmoult stage). Dry mass, protein and lipid contents were determined following Torres et al, in prep. a, Torres et al., in prep b).

Table 1. Species and larval stages for which data on variations in biomass (dry mass, protein and lipid content) and osmoregulatory capacity are available. Salinity range refers to the range for which biomass data were obtained.

SPECIES	SALINITY TOLERANCE	SALINITY RANGE	VARIABLE	SOURCE
Hyas araneus Zoea I	Stenohaline	20-32	OC Biomass	Pfaff, 1997 Present paper
Nephrops norvegicus Zoea I	Stenohaline	20-32	Biomass	Present paper
Liocarcinus pusillus Zoea I	Stenohaline	20-32	Biomass	Present paper
Homarus gammarus Zoea I	Stenohaline	20-32	Biomass	Torres et al., 2002
Cancer pagurus Zoea I	Stenohaline	20-32	Biomass	Torres et al., 2002
Carcinus maenas Zoea I	Euryhaline	20-32	OC Biomass	Cieluch et al., 2004 Torres et al., 2002
Chasmagnathus granulata Zoea I	Euryhaline	15-32	OC Biomass	Charmantier et al., 2002 Torres et al., 2002
Perisesarma fasciatum Zoea I	Euryhaline	10-32	Biomass	Present paper
Sesarma curacaoense Zoea I	Euryhaline	10-32	OC Biomass	Anger and Charmantier 2000 Present paper
Armases miersii all larval stages	Euryhaline	5-32	OC Biomass	Charmantier et al., 1998 Torres et al. in prep b
Armases ricordi all larval stages	Euryhaline	15-32	OC Biomass	Charmantier et al., unpublished Torres et al. in prep b
Armases roberti all larval stages	Euryhaline	10-32	OC Biomass	Charmantier et al, unpublished Torres et al. in prep b

Experiments: statistical analysis

Statistical analyses were performed following Zar (1996). Data were analysed using one-way ANOVA with salinity as factor. For all species, planned comparisons were made in order to test significant growth from hatching to intermoult. The number of replicates was 4-5 for each species and salinity combination.

Comparisons between different factors, after finding significant differences in the ANOVAs, were performed with the Student-Newman-Keuls test (SNK). The critical level (α) to reject the null hypothesis was fixed at 0.05. Previous to performing

ANOVAs, normality (normal plots) and variance homogeneity (Cochran test) were checked.

Data analysis

Data on biomass (dry mass, protein and lipid content) and osmoregulatory capacity of twelve species were obtained from the literature or from the above-mentioned experiments (Table 1). These data were gained in laboratory experiments under controlled conditions of temperature, salinity and photoperiod. The data from the stenohaline species were obtained after exposure to a narrow salinity range (20-32 ‰). The same range was used for the weak hyper-osmoregulator Zoea I of *Carcinus maenas* (Cieluch et al., 2004). By contrast, the salinity range used for the other euryhaline species varied according to each species tolerance from 5 ‰-32 ‰ to 15 ‰-32 ‰.

For the analysis of the relationship between osmoregulatory capacity and biomass-specific effects of salinity, we selected data obtained from individuals coming from the same population, i.e. from ovigerous females and larvae reared in the same conditions, in order to avoid confounding by genetic or acclimation effects on salinity tolerance. The salinities at which osmoregulatory capacity (OC) has been determined (5.3 ‰, 10.3 ‰, 17.0 ‰, 26.7 ‰, and 32.7 ‰) fall quite near the values used for the evaluation of the biomass-specific effects of salinity (5 ‰, 10 ‰, 15 ‰, 20 ‰, 25 ‰, and 32 ‰). For calculations, the salinity values that varied <1 ‰ between determinations of OC and biomass were approximated to the nearest neighbouring value. Otherwise, in order to attain a better adjustment at 15 ‰, 20 ‰ and 25 ‰, we used linear interpolation to estimate OC.

Interspecific comparisons were made by means of calculating biomass-specific effects of salinity for each species and stage:

$$B'_{H\text{-}L} = (B_H - B_L) \bullet 100 / (B_H \bullet (S_H - S_L))$$

where B_H is individual biomass (W, protein or lipid content per larvae) measured at the end of the experiment (at intermoult); at the higher salinity (S_H) in an interval of salinities (S_H - S_L), and B_L is the biomass at the lower salinity (S_L). The dimension

is the percentage of variation in biomass (in %B per ‰ of salinity change) in relation to the biomass (B_H) at the higher salinity.

Biomass-specific effects of salinity for the ranges 15-25 ‰ and 25-32 ‰ (B'_{25-15} and B'_{32-25}) are shown in Fig. 11, for each stage and species. Positive B'_{25-15} and B'_{32-25} values indicate a higher biomass increment at the higher compared to the lower salinity (B_H vs. B_L). Negative B' values indicate a lower increment in biomass at the higher salinity. We have previously used this index (Torres et al., 2002) but with the opposite sign. In this paper, we have changed the sign in order to facilitate the presentation of data in the graphs. Therefore, here positive values indicate increased biomass growth at the higher salinity; negative values decreased biomass growth at that salinity.

RESULTS

Experiments

The effect of salinity on dry mass (W), protein or lipid content depended on species. Low salinity caused lower W in all species studied (Fig. 1, Table 2). An increment in W, from postmoult to intermoult, was found in *H. araneus* and *N. norvegicus*, while a decrease occurred in *S. curacaoense* and *P. fasciatum* larvae exposed to 10 ‰. In addition, *L. pusillus* larvae only accumulated dry mass at 32 ‰.

Table 2. Summary of one-way ANOVA to evaluate the effect of salinity on dry mass, lipid and protein content of zoea I larvae of five decapod crustacean species. Abbreviations: MSf, MSe: mean squares of factors and error; dff, dfe: degrees of freedom of factors and errors. Significant differences are in bold.

	dff	MSf	dfe	MSe	F	p
DRY MASS						
S. curacaoense	2	117.74	12	9.86	11.9	**<0.005**
P. fasciatum	3	1.85	15	0.11	16.1	**<0.000**
H. araneus	2	445	12	17.20	25.9	**<0.000**
N. norvegicus	2	504781	40	3391	148.8	**<0.000**
L. pusillus	1	13.17	8	1.48	8.9	**<0.025**
PROTEINS						
S. curacaoense	2	2.81	12	0.80	3.5	0.064
P. fasciatum	3	0.03	15	0.001	27.6	**<0.000**
H. araneus	2	6.76	12	2.98	2.27	0.14
N. norvegicus	2	4070	40	374	10.9	**<0.000**
L. pusillus	1	0.89	8	0.10	8.7	**<0.025**
LIPIDS						
S. curacaoense	2	0.73	12	0.15	4.9	**<0.05**
P. fasciatum	3	0.003	16	0.002	1.4	0.27
H. araneus	2	22.73	12	4.06	5.6	**<0.025**
N. norvegicus	2	2636	39	140	18.8	**<0.000**
L. pusillus	1	0.21	7	0.015	13.7	**<0.01**

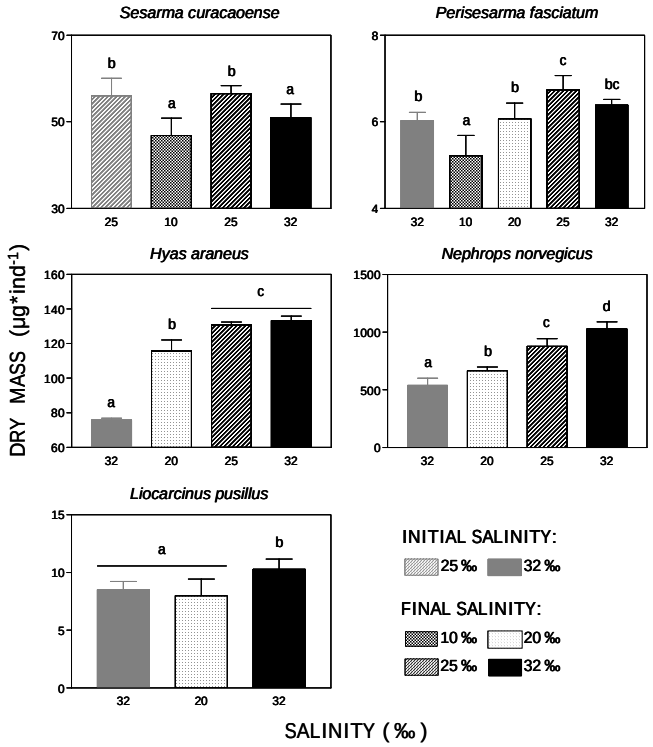

Fig. 1. Changes in dry mass (expressed as µg*ind^{-1}) after exposure to test salinities (10 ‰-32 ‰). Grey column: hatching; black columns: intermoult biomass (50% of the moult cycle). Different letters show significant differences after SNK test among salinities at hatching and intermoult, n.s. not significant difference.

Exposure to reduced salinity led to decreased protein content in *N. norvegicus*, *L. pusillus*, and *P. fasciatum* (Fig. 2, Table 2). Significant growth from hatching to intermoult was found in all species, but in *L. pusillus* only at 32 ‰. In *P. fasciatum* biomass decreased at all tested salinities but at 25 ‰.

The lowest lipid content was found at the lowest salinity in all species, except in *P. fasciatum* (Fig. 3, Table 2). In this species there was no significant growth and salinity did not affect the lipid content. By contrast, *H. araneus*, *N. norvegicus* and *L. pusillus* showed an increment of lipid content at 20 ‰; *S. curacaoense* lost lipids when exposed to 10 ‰; at higher salinities increment in lipid was not significant.

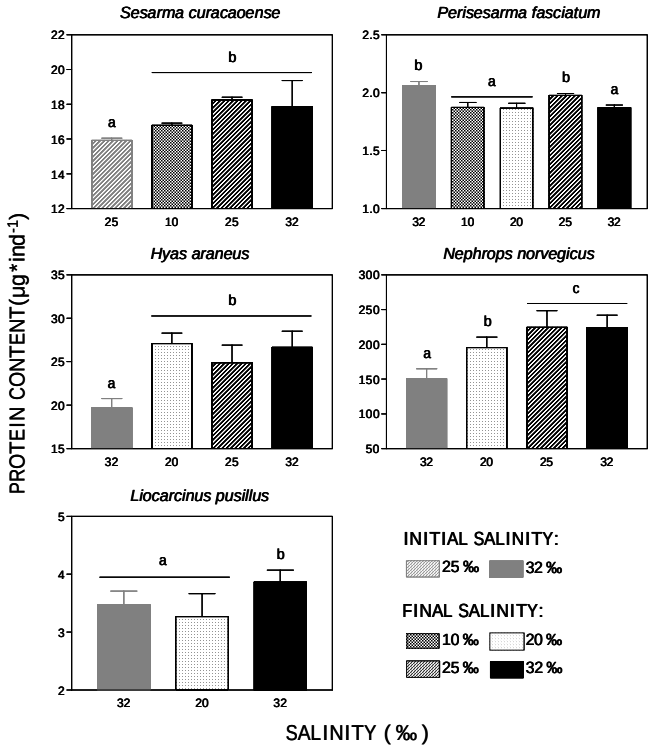

Fig. 2. Changes in protein content (expressed as µg*ind^{-1}) after exposure to test salinities (10 ‰ - 32 ‰). Grey column: hatching; black columns: intermoult biomass (50% of the moult cycle). Different letters show significant differences after SNK test among salinities at hatching and intermoult, n.s. not significant difference.

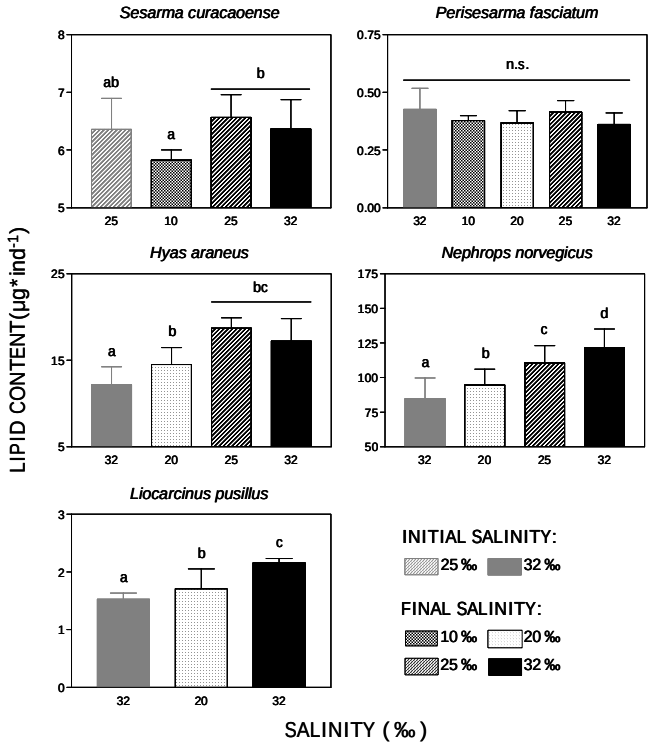

Fig. 3. Changes in lipid content (expressed as µg*ind^{-1}) after exposure to test salinities (10 ‰ -32 ‰). Grey column: hatching; black columns: intermoult biomass (50% of the moult cycle). Different letters show significant differences after SNK test among salinities at hatching and intermoult, n.s. not significant difference.

Biomass-specific effects of salinity (B') and euryhalinity

The ranges of salinity 20‰-25‰ and 25‰-32‰ allowed for comparisons of biomass variations (B'_{25-20} and B'_{32-25} respectively). In general, stenohaline species showed high and positive values of both B'_{25-20} and B'_{32-25}. By contrast, euryhaline species had small and/or negative values indicating increased biomass at the lower salinity. When both indices were plotted together (Fig. 4) euryhaline and stenohaline species segregated. The segregation occurred even considering only Zoea-I (Fig. 4, compare the encircled white dots with the black dots). The B'_{25-20} values corresponding to dry mass were, in all stenohaline species, in the range 2 – 7%/‰, while B'_{32-25} values were larger than 2%/‰ in *C. pagurus* and *N. norvegicus*. Euryhaline species showed values of B'_{25-20} and B'_{32-25} within the range: -2%/‰ – 1%/‰.

The B'_{25-20} values calculated for proteins and lipids of all stenohaline species (exception, proteins *H. araneus*) were >2%/‰, reaching maximum values of 7%/‰ and 12%/‰, respectively. By contrast, the euryhaline species had always values < 2%/‰ for proteins and < 4%/‰ for lipids (Fig. 4). In addition, all stenohaline species had B'_{32-25} values in the range 0%/‰ – 6%/‰, except in *H. araneus* for the lipid content. The euryhaline species, with the exception of *C. maenas* had B'_{32-25} values within the range -2%/‰ – 2%/‰ (Fig. 4, Table 3).

Table 3. Values of B'_{32-25} in euryhaline species not plotted in Fig. 4 since data on B'_{25-20} were not available.

SPECIES	DRY MASS	PROTEIN	LIPIDS
Sesarma curacaoense (zoea I)	-1.56	-0.32	-0.46
Chasmagnathus granulata (zoea I)	-0.42	-0.94	-1.24
Armases roberti (zoea I)	-1.25	0.20	2.37
Armases roberti (zoea III)	-0.92	-0.94	1.85

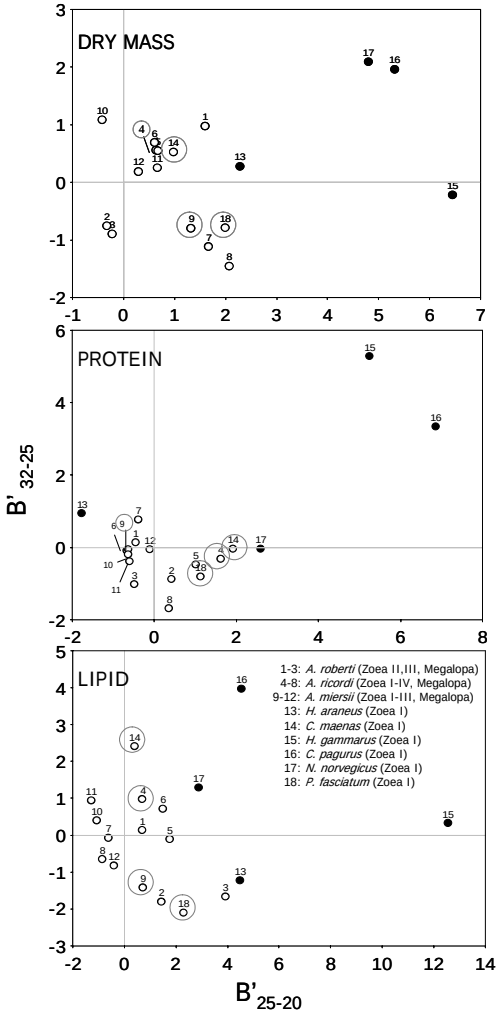

Fig. 4. Patterns of biomass-specific effects of salinity within two different salinity ranges, in larval instars of nine species of decapod crustaceans.

Minimum salinity vs. biomass-specific variation

Further comparisons were made using the range defined by the minimal (S_m) and the maximal salinity (32 ‰) used for each set of experiments. The biomass-specific variation was calculated as:

$$B'_{32\text{-}m} = (B_{32} - B_m) \bullet 100 \,/\, (B_{32} \bullet (32 - S_m))$$

The species with lower S_m showed also lower $B'_{32\text{-}m}$ values (Fig. 5). In addition, there was a considerable interspecific variability in $B'_{32\text{-}m}$ values at the highest S_m (20 ‰). For protein and lipid contents, *H. gammarus* and *C. pagurus* showed the highest values and the lowest belonged to *H. araneus*.

One-way ANOVA and the following posthoc tests showed that instars able to cope only with a S_m of 20 ‰, had significantly higher $B'_{32\text{-}m}$ than other instars (dry mass: $F_{3,19} = 7.39$, $p < 0.01$; lipids: log-transformed data: $F_{3,19} = 7.86$, $p < 0.01$; protein: t-test with separate variance estimates, 20 ‰ vs. all other salinities pooled: $t = 4.02$, $p < 0.05$). Comparisons using only Zoea I gave results alike only for W ($p < 0.02$). For lipid and protein contents, however, differences were marginally significant (lipids: $F_{3, 8} = 3.71$, $p = 0.061$; proteins: $t = 2.56$, $p = 0.054$).

The species that were reared at 20 ‰ as S_m (Table 4) were previously categorized as stenohaline, with the exception of the weak euryhaline *C. maenas* (survival <80 ‰ at 15 ‰); those whose S_m was lower were classified as euryhaline. Thus, most euryhaline species showed consistently smaller reduction in biomass, while in stenohaline the decrease was larger and varied considerably among species.

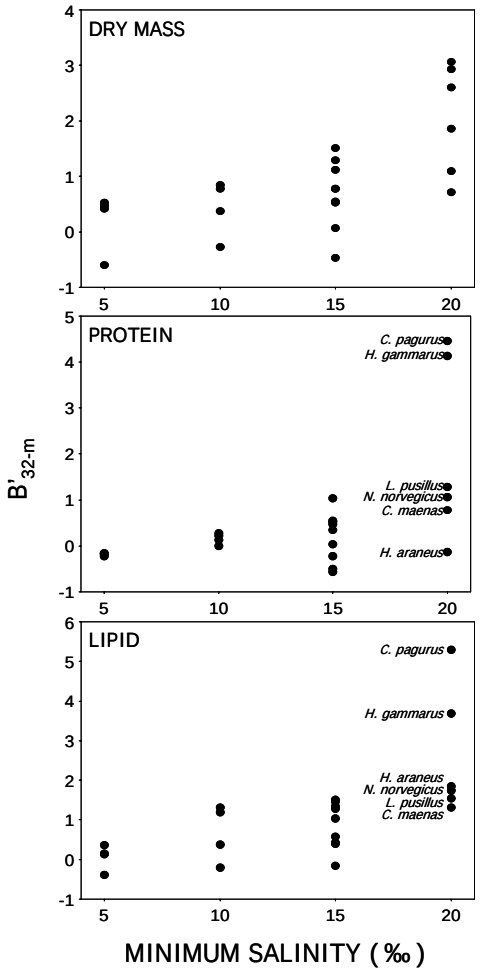

Fig. 5. Relationship between minimal salinity (S_m) for biomass determination (set to ensure larval >80% survival) and biomass specific effects of salinity in larval instars of twelve species of decapod crustaceans.

Table 4. Minimum salinity used for biomass determinations, discriminated by species and larval instar. Abbreviations: Z = zoea, M = megalopa, roman numbers refer to zoeal instars.

5 ‰		10 ‰		15 ‰		20 ‰	
SPECIES	INSTAR	SPECIES	INSTAR	SPECIES	INSTAR	SPECIES	INSTAR
A. miersii	all	*A. roberti*	ZI, M	*A. ricordi*	all	*C. maenas*	ZI
		S. curacaoense	ZI	*A. roberti*	ZII-IV	*H. gammarus*	ZI
		P. fasciatum	ZI	*C. granulata*	ZI	*C. pagurus*	ZI
						H. araneus	ZI
						L. pusilus	ZI
						N. norvegicus	ZI

Osmoregulatory capacity and biomass-specific effects

The relationship between osmoregulatory capacity (OC) and biomass-specific effects of salinity was evaluated using the biomass variation at the range 20-32 ‰, with OC at 20 ‰ (OC_{20}), and the maximal OC (OC_M) reached by each instar. We used this salinity range in order to include in our analyses the data corresponding to the stenohaline species.

In general, there were significant negative correlations between OC_{20} and $B'_{32\text{-}20}$, and between OC_M and $B'_{32\text{-}20}$ (Fig. 6). These correlations suggested that an increase in the osmoregulatory capacity led to lesser detrimental biomass-specific effects of salinity during larval growth. The only exception was found in *H. araneus*, where the correlation between OC_{20} and protein-specific effects of salinity were marginally significant, probably due to an excessively low $B'_{32\text{-}20}$ value (see Fig. 6, dot within circle).

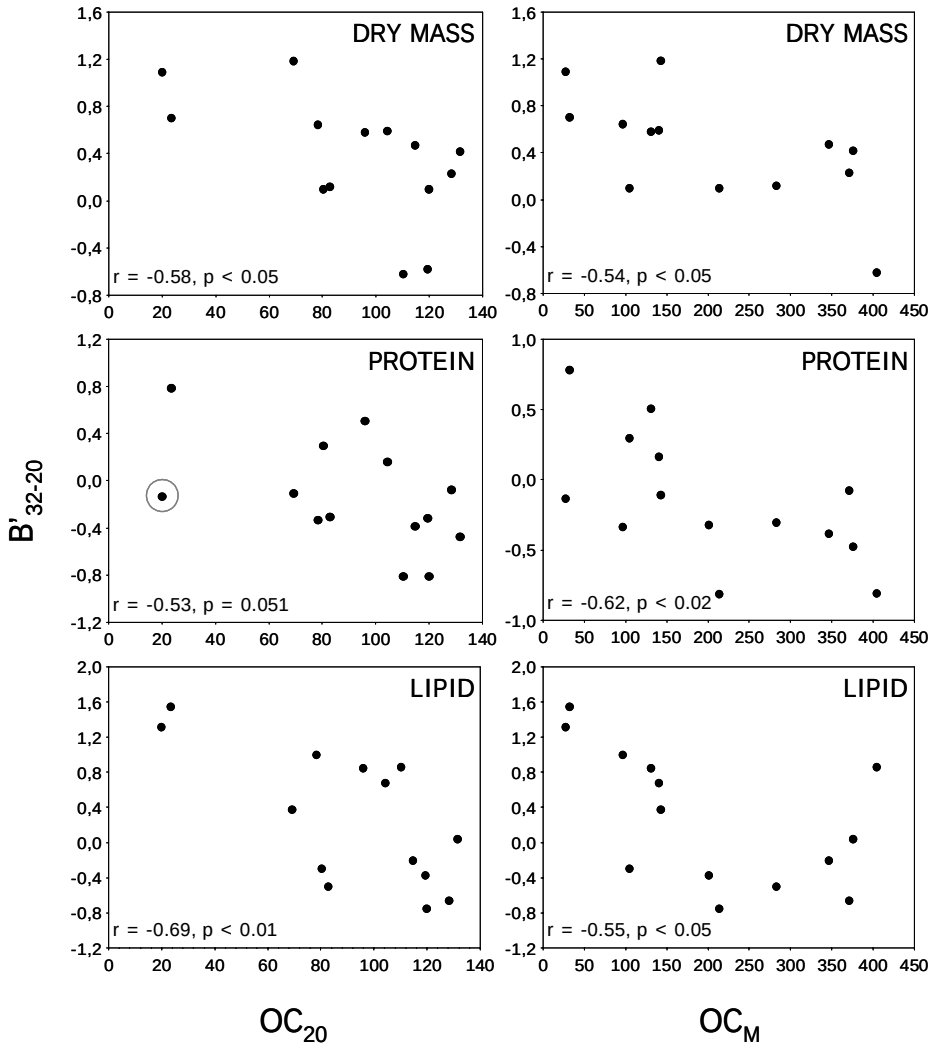

Fig. 6. Relationship between osmoregulatory capacity (OC_{20} and OC_M) and biomass-specific effects of salinity (B'_{32-20}), in larval instars of six species of decapod crustaceans.

DISCUSSION

According to Anger (2001, 2003), few comparative data for larval decapods are available to allow generalizations about the effects of salinity on larval growth. We present a data set that includes the effect of variations in salinity on larval growth for species and larval instars varying in their degree of euryhalinity and osmoregulatory capacity. We found the following patterns: (a) biomass in euryhaline instars shows consistently low sensitivity to variations in salinity, even at the lowest extreme of salinity tolerance. By contrast, in stenohaline instars the sensitivity to salinity varies from medium to high levels. (b) The higher the osmoregulatory capacity, the lower the biomass-specific effects of low salinities.

The small sensitivity to salinity during larval growth exhibited by euryhaline species, even at extremely low salinities, is clearly due to a higher osmoregulatory capacity. Thus, osmoregulation plays a key role in larval growth, as it ensures that metabolic and developmental processes take place with high efficiency even at extreme conditions. In adult stages of brachyuran crustaceans, osmoregulatory capacity is positively correlated with the activity of Na^+-K^+-ATPase (Lucu and Towle, 2003), and such pattern might be also found in larval stages. The strong osmoregulating juvenile of *Eriocheir sinensis*, showed increased activity of the ion-transport Na^+-K^+-ATPase after long-term exposure to low salinity (Torres et al., submitted a). Furthermore, the posterior gills of these juvenile exposed to 5‰ showed a typical differentiation in their lamellae: i.e. increased number of Na^+-K^+-ATPase molecules widely distributed along the gill lamellae. By contrast, juvenile exposed to 25‰ showed only scarcely scattered Na^+-K^+-ATPase molecules at the base of the gill lamellae (Torres et al., submitted a). In addition, an investigation of the molecular aspects of the response of Na^+-K^+-ATPase to reduced salinities on larval and first juvenile stages of the strong osmoregulator *Armases miersii*, showed that *de novo* synthesis of the enzyme is involved (Torres et al., submitted b). Therefore, we might conclude that even if osmoregulation requires an energetic cost; this is successfully traded for sustained growth.

On the contrary, low salinity led to reduced biomass growth in larval instars with low osmoregulatory capacity. The reduction in W growth may reflect effects on inorganic as well as organic substances at low salinities (Anger, 2003). Variability in the lipid and protein fraction should reflect, either negative effects on feeding and assimilation, or increased costs for the maintenance of growth and developmental processes. For instance, zoeal stages of the stenohaline crab *Cancer irroratus* exposed to low salinities showed reduced feeding and respiration rates, as well as increased nitrogen excretion rate and reduced energy content (Johns, 1982); *C. maenas* Zoea I exposed to 15‰ showed low respiration rate, carbon and nitrogen content (Anger et al., 1998). In these species, low salinity caused a decrease of the assimilation capacity and efficiency of conversion of food into tissue (Anger, 2003). In the stenohaline species studied here we found two patterns: (a) reduction of both lipid and protein growth leading to rather constant L:P ratios in *L. pusillus* (0.52-0.56), *C. pagurus* (0.10-0.11) and *N. norvegicus* (0.48-0.54), and (b) reduction of

lipid but not protein growth, leading to reduced L:P ratios at low salinity: *H. araneus* (0.54 to 0.65). The lobster *H. gammarus* may fall in an intermediate category because, even if the L:P ratio varied as in *H. araneus* (0.19 to 0.30), there was a significant decrease in protein growth. In the first group, low salinity must have led to less assimilation capacity and efficiency of conversion of food into tissue as in *C. irroratus*. This was most clearly seen as a negative growth between postmoult and intermoult. However, the unaffected protein content in *H. araneus* suggests that low salinity affected the stored reserves of lipids rather than feeding or assimilation efficiency. Differences in the way salinity affects metabolism must explain the high interspecific variability in the effects of salinity on larval growth and ultimately survival. These differences may be associated to small differences in the degree of euryhalinity. For instance, the zoeal development of *C. pagurus* cannot be completed when reared at 20 ‰ (G.T. unpublished data), while this is possible for *H. araneus* (Anger, 1985; Pfaff, 1997). The latter species may be able to develop populations in the western Baltic Sea (Anger, 1985). It is interesting to note that within the range 20-32 ‰, the OC_{20} of the osmoconformer Zoea I of *H. araneus* is as high as that of the weak hyper-osmoregulator Zoea I of *C. maenas*. Unfortunately, no data of OC are available of other stenohaline species such as *C. pagurus* or *H. gammarus*. The existing OC data of *H. gammarus* correspond to a different population as the one we used for our biomass determination, thus we did not use these data for comparisons.

It remains to be elucidated why different effects were found for stenohaline species. Is there a different response to osmotic stress involved? Or does osmotic stress negatively affect different physiological processes? What is the role of osmoconforming strategies for larval growth and survival?

ACKNOWLEDGEMENTS

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grant. The experiments comply with the current animal and manipulation laws in Germany.

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IV.3. Effects of long-term exposure to reduced salinities on species with larval export strategy

PUBLICATION 5

CUMULATIVE EFFECTS OF LOW SALINITY ON LARVAL GROWTH AND PROXIMATE BIOCHEMICAL COMPOSITION IN AN ESTUARINE CRAB, *CHASMAGNATHUS GRANULATA*

Gabriela Torres, Luis Giménez, Klaus Anger (in prep.)

ABSTRACT

Cumulative effects of salinity on dry mass (W) and biochemical composition (total lipid and protein contents) were evaluated throughout the larval development of the euryhaline crab *Chasmagnathus granulata*. The larvae were reared at three salinities (15‰, 25‰ and 32‰). Samples for biochemical measurements were taken <24h after hatching or moulting to successive larval stages (early postmoult), and at 50% of each larval moulting cycle (intermoult). Effects of salinity were generally insignificant in the early zoeal instars (Zoea I, II). Only the lipid content of the Zoea I was slightly lower at 15‰. Conspicuous effects appeared in later stages (Zoea IV, Megalopa). In the Zoea IV, for instance, W, lipid and protein contents were notably lower in the 15‰ treatment. Biomass accumulation rates (daily increments) showed increasingly negative effects of low salinity. In the early larval stages, reduced salinity caused a delay in the time of moulting, while W and biochemical composition at moulting remained unaffected. This suggests that the ability of Zoea-I larvae to osmoregulate mitigated effects of hypo-osmotic stress on biochemical composition. In later stages, we found reduced biomass after continuous osmotic stress, probably due to the inability of the stages Zoea III-IV to osmoregulate. These effects persisted throughout the osmoregulating Megalopa stage, indicating cumulative effects of continued osmotic stress since hatching. This has implications for population dynamics, as cumulative effects may influence the number and quality of larvae reaching metamorphosis.

Keywords: *Chasmagnathus granulata*, biochemical composition, cumulative osmotic stress, growth, lipid content, protein content.

INTRODUCTION

Decapod crustaceans such as crabs and lobsters develop through complex life cycles characterized by a series of planktonic larval instars. The larval development of these species involves dramatic changes in growth, which are affected by

environmental conditions. In the case of estuarine and semiterrestrial crabs, for instance, an exposure to low salinities may affect larval metabolism and growth (Anger, 1998, 2001, 2003).

The effects of salinity on larval biomass and biochemical composition should vary with physiological adaptations such as the ability to osmoregulate. Torres et al. (2002) showed that Zoea-I larvae of stenohaline osmoconformers like *Cancer pagurus* and *Homarus gammarus* showed in their dry mass (W), lipid and protein content a stronger response to variations in salinity than those of the euryhaline osmoregulators *Carcinus maenas* and *Chasmagnathus granulata*. According to these observations, we may expect that stenohaline and osmoconforming larval instars should exhibit a stronger response of their lipid and protein contents when exposed to reduced salinity than euryhaline and osmoregulating instars.

The ability to osmoregulate changes during ontogeny (Charmantier, 1998). There are species whose larvae are osmoconformers from hatching until metamorphosis (e.g. *Libinia marginata*), while some osmoregulating species have larvae that show the adult type of osmoregulation throughout their development (e.g. *Armases miersii*; Charmantier et al., 1998). In estuarine species with a larval export strategy, e.g. the South American salt-marsh crab *Chasmagnathus granulata* (Anger et al., 1994), there is a typical ontogenetic pattern of change in osmoregulation: the Zoea I, which hatches in estuaries, shows a slight hyper-osmoregulatory ability. The following zoeal stages, which develop in an osmotically more stable coastal environment, are osmoconformers. The ability of hyper-osmoregulation re-appears after metamorphosis, so that the Megalopa is physiologically prepared to re-immigrate into the physically variable parental habitat. This ability increases during the juvenile and adult phase, allowing for successful development in harsh estuarine environments (Charmantier et al., 2002).

Changes in the ontogeny of osmoregulation should lead to instar-dependent effects of salinity on larval growth in advanced stages. Changes in the biochemical composition of advanced stages may also be affected by conditions experienced during previous stages. For instance, exposure to low salinity during osmoconforming zoeal stages may lead to cumulative stress effects in subsequent osmoregulatory stages. Information about this topic is available for a few species. In the euryhaline larvae of *Armases miersii*, which are capable of osmoregulation early in development,

a significant accumulation of biomass was observed in a wide range of salinities (Charmantier et al., 1998, Anger et al. 1998), although also cumulative stress effects are observed (Anger, 1996). In *Hyas araneus*, whose larvae are stenohaline and osmoconforming, Pfaff (1997) found at a reduced salinity (20 ‰) already at the end of the moulting cycle of the Zoea I a weaker lipid accumulation as compared with higher salinities.

Here, we focused on the importance of cumulative effects of osmotic stress on larval biomass in a species with both osmoconforming and osmoregulating larval instars, namely the estuarine crab *Chasmagnathus granulata*. It is an ideal model to evaluate such potential stress effects, as the Zoea II-IV instars are osmoconformers, while the Zoea I and the Megalopa stages are capable of hyper-osmoregulation (Charmantier et al., 2002). If cumulative effects play an important role in the larval development, we expect that: (a) stress will not be strongly manifested in the Zoea II due to the osmoregulatory ability of the preceding larval instar, and (b) stress effects experienced during later zoeal instars (III-IV) should be visible in the Megalopa, although this stage is capable of osmoregulation. In a previous investigation, Giménez and Anger (2003) found high mortality rates at the megalopal stage of *C. granulata* when the zoeal development took place at 15 ‰. Cumulative stress effects on larval biochemical composition may play a key role to explain this pattern of larval survival (Giménez et al., 2004).

MATERIALS AND METHODS

Handling of ovigerous females and obtaining of larvae

Larvae of *Chasmagnathus granulata* were obtained from ovigerous females maintained in the Helgoland Marine Biological Station (BAH, Helgoland, Germany) since 1990 under controlled conditions of temperature (21°C), salinity (32 ‰), and photoperiod (12:12h), fed *ad libitum* with isopods (*Idotea* sp.). This laboratory population was established with animals captured in the Mar Chiquita lagoon, Argentina (1990), and later complemented with specimens from Mar Chiquita (1997) and from the Solís Chico River, Uruguay (2006). When females laid eggs, they were isolated in individual aquaria and kept under otherwise identical conditions.

Laboratory experiments

Freshly hatched larvae were assigned to three groups and reared at 15 ‰ , 25 ‰ and 32 ‰ (Fig. 1), 18°C, and photoperiod of 12:12hs, with freshly hatched *Artemia* sp. nauplii provided *ad libitum* as food. Salinities of 15 ‰ and 25 ‰ were obtained by mixing filtered seawater (Orion, pore size: 1µm), with appropriate amounts of desalinated tap water. These treatments represent conditions of severe, moderate and no hypo-osmotic stress. Water and food were changed daily. Cultures were controlled daily for moulted or dead larvae.

Seventy Zoea I were group-reared in replicate 400-mL bowls without aeration, until 50% of the moulting cycle (intermoult) was reached. For the production of subsequent stages, larvae were mass-reared with slight aeration in 5-L bottles (approximate density: 200 ind*L^{-1}; Table 1). Early postmoult larvae were separated from the cultures and reared until intermoult in 400-mL bowls, under identical conditions of the Zoea I. Thus, each set of experiments was performed with individuals with the same moulting history (Table 1). The stocking density decreased in successive larval instars (50, 40, 30, 20 per bowl, respectively).

Samples of larvae were taken for biochemical analyses at early postmoult (<24 after hatching for Zoea I or moulting for later stages) and again at intermoult (after ca. 50% of the moulting cycle).

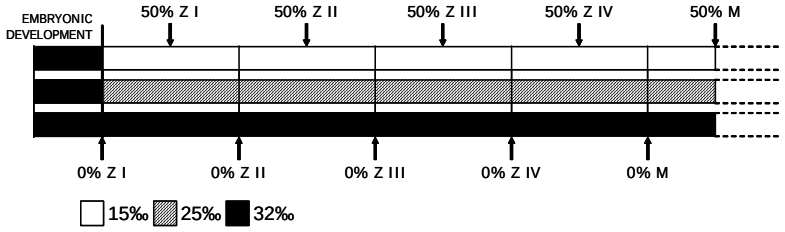

Fig. 1. *Chasmagnathus granulata*. Experimental design to study the cumulative effects of salinity on dry mass and biochemical composition during larval development. Postmoult and intermoult samples (see arrows) were taken immediately after moulting (0%) or at 50% of the moult cycle, respectively.

Table 1. *Chasmagnathus granulata.* Cumulative duration of development, in days, from hatching to early postmoult (post) and intermoult (int) from Zoea-II to Megalopa.

	15 ‰		**25 ‰**		**32 ‰**	
	post	**int**	**post**	**int**	**post**	**int**
Zoea II	8	11	6	9	6	9
Zoea III	12	15	10	13	10	13
Zoea IV	19	23	17	21	17	21
Megalopa	27	31	23	27	23	27

Biochemical analyses

Samples for biochemical analyses were gently rinsed in distilled water for 10 s and blotted on filter paper. Then, they were transferred to an Eppendorf vial and frozen at -80 °C. These samples were left in a vacuum drier (Finn-Aqua Lyovac GT2E) for 48 h and their dry mass was determined in a Sartorius MC1 RC 210 S balance (precision: 0, 01 mg, capacity 210 g). Afterwards, they were homogenized by sonication (Branson, Sonifier, Cell Disruptor B 15) with 5 strokes of 5 sec, in ice and each homogenate was divided in two aliquots to perform lipid and protein content determinations.

Total lipids determination

The lipid content of the homogenate was determined after the sulphophosphovanillin method following Zöllner and Kirsch (1962), modified for microplates. 40 µL of homogenate were mixed with 300 µL of ice-cold $CHCl_3/CH_3OH$ (2:1). After 15 min incubation at room temperature, the samples were centrifuged at 10.000g for 20 min at 4°C. 180 µL of the lower phase were transferred to new tubes. These were left open to dry in a Thermomixer Eppendorf for 90 min at 56°C with shaking at 700 rpm. The dried pellet was dissolved in 200 µL of H_2SO_4 conc. and incubated for 10 min at 95°C with shaking at 1400 rpm (in a Thermomixer Eppendorf, with the tubes closed). After cooling for 20 min at room temperature, 8 replicates of 20 µL from each sample were distributed in two 96-well microplates. In the first plate (Blank: Plate 0) 300 µL of H_3PO_4 conc. were added; in the second (Plate 1), 300 µL of vanillin solution (8 mM H_3PO_4 conc.). The microplates were incubated for colour development, 40 min at room temperature and subsequently measured (Thermoelectron Multiskan® Spectrum spectralphotometer, wavelength: 530 nm). The final values were obtained as the difference between Plate 1 and Plate 0. The calibration curve was obtained by dilutions of a standard solution of cholesterol.

Protein determination

The protein content of the homogenate was determined using a modified method after Lowry et al. (1951; kit: BioRad D_C Protein Assay). 25 µL of homogenate were mixed with 100 µL of ice-cold 20% trichloroacetic acid (TCA). After 10 min incubation

at 4°C, the samples were centrifuged at 10.000g for 10 min at 4°C; the supernatant was discarded. The remaining pellet was dissolved in 300 µL NaOH (1 M) and incubated with shaking at 1400 rpm for 30 min at 56°C in a Thermomixer Eppendorf. Afterwards, 30 µL of the dissolved sample (4 replicates) were mixed with 20 µL of Reagent A and 300 µL of Reagent B (kit: BioRad D_C Protein Assay) in a 96-well microplate. The microplates were incubated for 15 min at room temperature in the dark and absorbance was measured (Thermoelectron Multiskan® Spectrum spectralphotometer, wavelength: 750 nm). The calibration curve was obtained by dilutions of bovine serum albumin (BSA, kit: BioRad D_C Protein Assay).

Data analyses

We expected two types of effects of salinity: (a) a stage-dependent change, i.e. the effect on biomass was observed at a particular stage (e.g. Zoea II, intermoult); (b) an effect on the accumulation rate, due to effects on the developmental rate. The stage-dependent change was evaluated using biomass (W, protein and lipid content) per individual as response variable. The effect of salinity on the accumulation rates was evaluated using the biomass per individual divided by the number of days required by freshly hatched larvae to reach the desired moulting stage (see Table 1).

Statistical analyses were performed following Zar (1996). All data-sets were analysed with ANOVA separately for each larval instar, because data from each instar originated from different females. We were not able to conduct all experiments with larvae from one hatch since a high number of larvae were required for the biochemical methods. Zoea I data were analysed with a one-way ANOVA with salinity (15‰, 25‰ and 32‰) as factor; planned comparisons were made in order to test significant growth from hatching to intermoult. Data from Zoea II-Megalopa were analysed with a two-way ANOVA with salinity and moult-stage (postmoult and intermoult) as factors. The number of replicates was 4-5 for each stage-salinity combination.

Comparisons between different factors, after finding significant differences in the ANOVAs, were performed with the Student-Newman-Keuls test (SNK). The critical level (α) to reject the null hypothesis was fixed at 0.05. Previous to performing ANOVAs, normality (normal plots) and variance homogeneity (Cochran test) were checked. In case of failing to meet the assumptions (i.e. variance homogeneity), data

were logarithmically transformed. In a few occasions, and even after data transformation, variances were not homogeneous. In these cases differences were significant even after we reduced the critical level below the level of significance of variance heterogeneity.

RESULTS

In all larval instars, there was always a significant increase in dry mass (W), lipid and protein content per individual between early postmoult and intermoult (Table 2). Biomass accumulation rates increased significantly in most instars also from postmoult to intermoult (Table 3).

Table 2. *Chasmagnathus granulata.* Summary of ANOVA analysis for dry mass, lipid and protein content per individual (log transformed data). Symbols: MS: mean squares; significant effects are in bold.

	Dry mass			Lipid			Protein		
	MS	**F**	**p**	**MS**	**F**	**p**	**MS**	**F**	**p**
Zoea I									
Salinity (S)	0.012	3.65	0.058	0.053	15.13	**<0.0006**	0.012	2.44	0.13
Error	0.003			0.003			0.005		
Zoea II									
Salinity (S)	0.010	5.9	**0.0087**	0.019	2.60	0.096	0.009	8.3	**<0.002**
Moult-stage (M)	0.122	68.7	**<0.0001**	0.044	5.84	**0.024**	0.115	101.3	**<0.0001**
S*M	0.005	2.7	0.085	0.002	0.34	0.717	0.002	2.0	0.16
Error	0.002			0.007			0.001		
Zoea III									
Salinity (S)	0.0027	2.6	0.10	0.016	14.8	**<0.0001**	0.0134	27.1	**<0.0001**
Moult-stage (M)	0.1165	112.8	**0.0001**	0.216	202.9	**<0.0001**	0.1791	363.3	**<0.0001**
S*M	0.0005	0.5	0.59	0.002	2.2	0.13	0.0174	35.2	**<0.0001**
Error	0.0010			0.001			0.0005		
Zoea IV									
Salinity (S)	0.0479	129	**<0.0001**	0.032	31.8	**<0.0001**	0.0124	9.0	**<0.002**
Moult-stage (M)	0.2011	541	**<0.0001**	0.095	94.6	**<0.0001**	0.4758	345.9	**<0.0001**
S*M	0.0001	<0.1	0.83	0.004	4.0	**0.032**	0.0237	17.2	**<0.0001**
Error	0.0004			0.001			0.0014		
Megalopa									
Salinity (S)	749.7493	977445	**<0.0001**	0.0209	15.4	**<0.0001**	0.0274	17.7	**<0.0001**
Moult-stage (M)	0.0258	33	**<0.0001**	1.3208	969.4	**<0.0001**	0.1288	83.2	**<0.0001**
S*M	0.2220	289	**<0.0001**	0.0613	45.0	**<0.0001**	0.0076	4.9	**<0.02**
Error	0.0056			0.0014			0.0015		

Table 3. *Chasmagnathus granulata*. Summary of ANOVA analysis for dry mass, lipid and protein content accumulation rates. Symbols as in Table 2; significant effects are in bold.

	Dry mass			Lipid			Protein		
	MS	**F**	**p**	**MS**	**F**	**p**	**MS**	**F**	**p**
Zoea II									
Salinity (S)	2.43	42.48	**<0.0001**	0.0082	11.87	**<0.0003**	0.054	12.62	**0.0002**
Moult-stage (M)	0.26	4.61	**0.042**	0.0058	8.42	**0.008**	0.044	10.42	**<0.004**
S*M	0.19	3.37	0.052	0.0012	1.68	0.21	0.029	6.61	**<0.005**
Error	0.06			0.0007			0.004		
Zoea III									
Salinity (S)	2.09	30.24	**<0.0001**	0.016	56.30	**<0.0001**	0.37	86.16	**<0.0001**
Moult-stage (M)	0.15	2.30	0.14	0.008	29.50	**<0.0001**	0.17	40.40	**<0.0001**
S*M	0.03	0.39	0.68	0.0006	2.16	0.14	0.09	23.37	**<0.0001**
Error	0.07			0.0003			0.004		
Zoea IV									
Salinity (S)	9.91	217.97	**<0.0001**	0.048	45.25	**<0.0001**	0.58	25.23	**<0.0001**
Moult-stage (M)	5.55	122.03	**<0.0001**	0.003	3.04	0.09	3.52	152.25	**<0.0001**
S*M	0.04	0.96	0.391	0.003	3.29	0.06	0.41	17.88	**<0.0001**
Error	0.04			0.001			0.02		
Megalopa									
Salinity (S)	17.90	64.47	**<0.0001**	0.069	35.40	**<0.0001**	1.14	17.31	**<0.0001**
Moult-stage (M)	26.79	96.49	**<0.0001**	0.712	365.22	**<0.0001**	1.36	20.68	**<0.0002**
S*M	2.19	7.91	**0.0025**	0.066	33.86	**<0.0001**	0.22	3.31	0.054
Error	0.28			0.002			0.06		

Zoea I

W and protein content per individual were not affected by salinity. However, larvae exposed to 15 ‰ showed a significantly lower lipid content than those at 25 ‰ and 32 ‰ (Table 2, Fig. 2B).

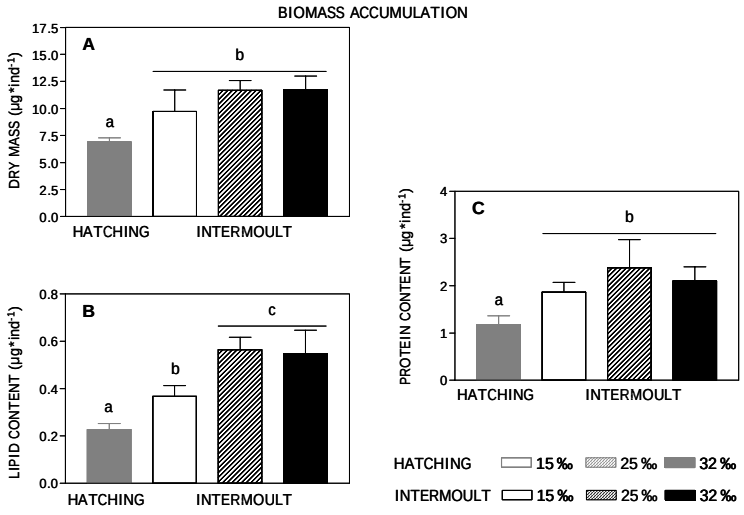

Fig. 2. *Chasmagnathus granulata*. Changes in Zoea I biomass: dry mass, lipid, protein content per individual (expressed as µg*ind^{-1}) after exposure to 15 ‰, 25 ‰ and 32 ‰. Grey column: biomass at hatching; black columns: biomass at intermoult (50% of the moult cycle). Different letters show significant differences between salinities (SNK tests) in biomass at hatching and at intermoult.

Zoea II

In the Zoea II, W per individual was significantly lower at 15 ‰ than at 32 ‰ both at postmoult and at intermoult (Table 2; Fig. 3A). The effect of salinity on dry mass was stronger at intermoult than at postmoult. No significant effects were detected in the lipid content (Fig. 3B). The protein content was significantly higher at 15 ‰ than at the other test salinities (Fig. 3C). Although this pattern was clearer at intermoult than at early postmoult, the interaction term (Salinity * Moult-stage) in the ANOVA (Table 2) was not significant.

Biomass accumulation rates per day were significantly affected by salinity, with lower levels in W and in the lipid content, and this effect was observed both at postmoult and intermoult (Fig. 3D-E, Table 3). For the protein content, this pattern was found only at postmoult (Table 2, Fig. 3F).

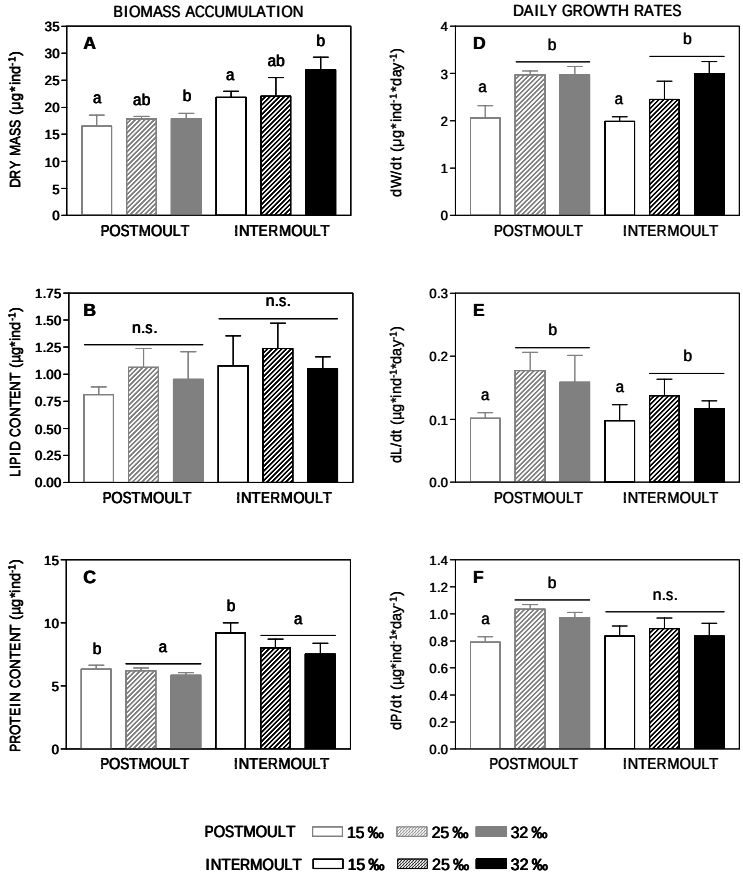

Fig. 3. *Chasmagnathus granulata*. A-C: Changes in Zoea II biomass: dry mass, lipid, protein content per individual (expressed as µg*ind^{-1}) after continuous exposure to 15 ‰, 25 ‰ and 32 ‰. D-F: Changes in accumulation rates: dry mass, lipid, protein content per individual (expressed as µg*ind^{-1}*days^{-1}). Grey columns: biomass at postmoult (0% of the moult cycle); black columns: biomass at intermoult (50% of the moult cycle). Different letters show significant differences between salinities (SNK tests), separately for postmoult and intermoult.

Zoea III

No effects of salinity were found in W (Table 2, Fig. 4A). For the lipid fraction, the effect of salinity was similar in early postmoult and intermoult, with lower values in larvae exposed to 15 ‰ and 25 ‰ as compared to those at 32 ‰. The effect of salinity on the protein content depended on the moult-stage, with no effects at early postmoult, but reduced levels observed at intermoult in larvae exposed to 15 ‰ (Fig. 4C).

Significant effects of salinity were found in the accumulation rates of W, lipid and protein at both early postmoult and intermoult, with lower levels in larvae maintained at 15 ‰ (Table 3, Fig. 4D-F). In addition, larvae exposed to 25 ‰ showed also a significantly lower lipid accumulation rate than those at 32 ‰. The effect on the accumulation rate of proteins was stronger at intermoult than at early postmoult.

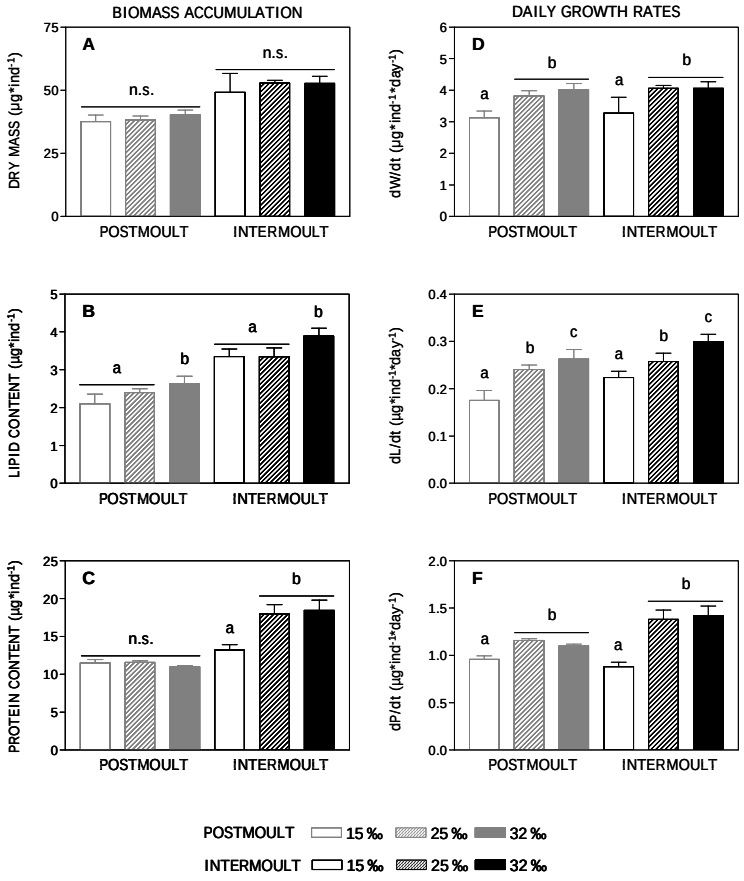

Fig. 4. *Chasmagnathus granulata*. A-C: Changes in Zoea III biomass: dry mass, lipid, protein content per individual (expressed as µg*ind^{-1}) after continuous exposure to 15 ‰, 25 ‰ and 32 ‰. D-F: Changes in accumulation rates: dry mass, lipid, protein content per individual (expressed as µg*ind^{-1}*days^{-1}). Symbols as given in Fig. 3.

Zoea IV

Continuous exposure to reduced salinities affected W and the lipid and protein contents depending on the moulting stage (Fig. 5A-C, Table 2). At early postmoult, W and the lipid content were lower at 15 ‰ compared to the other test salinities. By contrast, the protein content increased significantly at 15 ‰ and 25 ‰ compared to 32 ‰. At intermoult, larvae exposed to 15 ‰ showed significantly lower W, lipid and protein contents (Fig. 5A-C). At 25 ‰, the larvae showed a significantly lower lipid content than those at 32 ‰.

The Zoea IV showed reduced accumulation rates of W and lipids at 15 ‰, both at postmoult and intermoult (Fig. 5D-F, Table 3). The lipid accumulation rate showed a marginally insignificant interaction ($p = 0.06$) suggesting a reduced accumulation at 25 ‰ as compared to 32 ‰ at intermoult. The accumulation rate of proteins was reduced only at intermoult in larvae exposed to 15 ‰. At early postmoult, accumulation rates differed only slightly, with a maximum at 25 ‰.

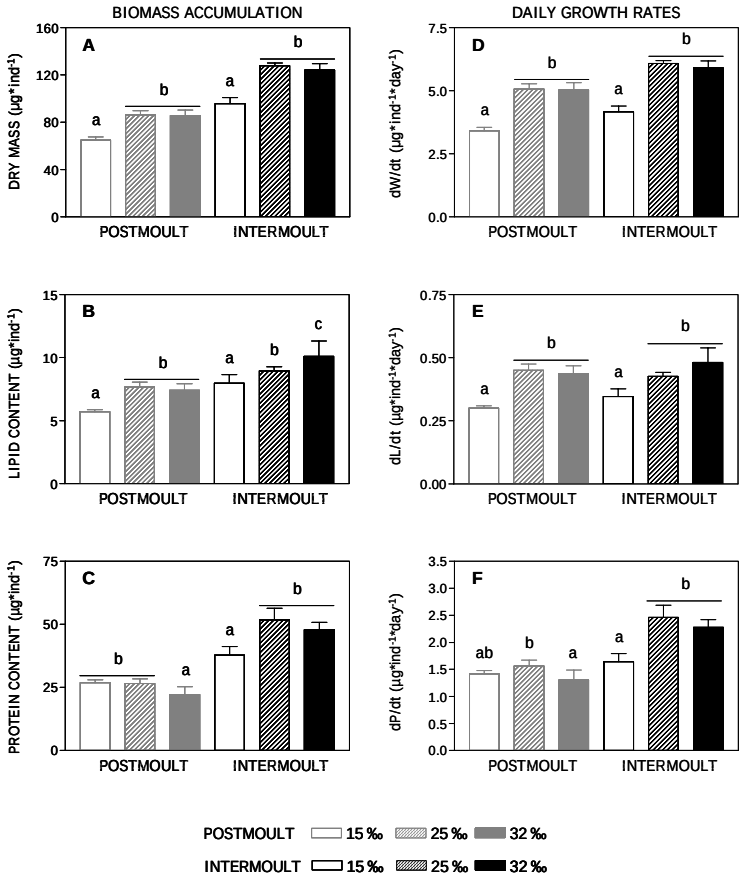

Fig. 5. *Chasmagnathus granulata*. A-C: Changes in Zoea IV biomass: dry mass, lipid, protein content per individual (expressed as µg*ind^{-1}) after continuous exposure to 15 ‰, 25 ‰ and 32 ‰. D-F: Changes in accumulation rates: dry mass, lipid, protein content per individual (expressed as µg*ind^{-1}*days^{-1}). Symbols as given in Fig. 3.

Megalopa

Effects of salinity on W, lipid and protein contents depended on the moulting stage (Fig. 6A-C, Table 2). Continuous exposure to 15 ‰ caused a decreased W at early postmoult and intermoult compared to larvae maintained at 32 ‰. W at postmoult was at 25 ‰ also lower than at 32 ‰. At early postmoult, the lipid and protein contents at 32 ‰ were slightly lower than at the other test salinities. At intermoult, reduced salinity (15 ‰) caused a very low lipid content, but it did not significantly affect the protein level.

The effects of salinity on accumulation rates of W and the lipid content depended on the moulting stage considered (Table 3, Fig. 6D-F). For W, the growth rates were lower at 15 ‰ than at any other salinity, both at postmoult and intermoult. Accumulation rates of lipids were at early postmoult, slightly affected by salinity with a maximum at 25 ‰, and significantly reduced rates at intermoult at 15 ‰. The protein accumulation rate was significantly higher at 25 ‰ than at the other salinities, regardless of the stage in the moulting cycle.

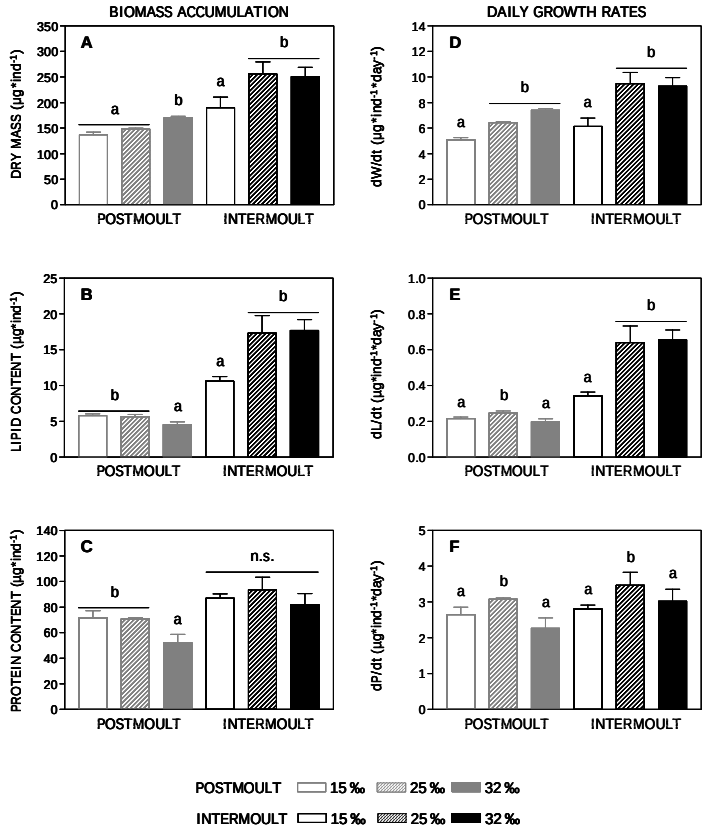

Fig. 6. *Chasmagnathus granulata*. A-C: Changes in Megalopa biomass: dry mass, lipid, protein content per individual (expressed as µg*ind^{-1}) after continuous exposure to 15 ‰, 25 ‰ and 32 ‰. D-F: Changes in accumulation rates: dry mass, lipid, protein content per individual (expressed as µg*ind^{-1}*days^{-1}). Symbols as in Fig. 3.

Lipid-protein relationships

Overall patterns in the lipid and protein data are shown in Fig. 7 as a lipid-protein plot. Cumulative effects of low salinity on lipids and proteins are clearly shown at intermoult in the Megalopa and the Zoea IV stage (Fig. 7A). The lipid-protein ratios (L:P) at intermoult of the Megalopa were lower at 15 ‰ (0.12) compared to 25 ‰ and 32 ‰ (0.18-0.21). The same pattern was found in the Zoea IV at postmoult (15 ‰ : 0.21, 32 ‰ : 0.41). However, at intermoult the Zoea IV showed higher L:P at 15 ‰ (0.18) than at 25 ‰ and 32 ‰ (0.10-0.11).

Effects of salinity were also visible at intermoult in the Zoea II and III (Fig. 7B). In the Zoea II, there was a consistent reduction of the L:P ratio at 15 ‰ compared to the other salinities both at early postmoult (0.13 vs. 0.16-0.17) and intermoult (0.11 vs. 0.14-0.15). The L:P ratio showed in the Zoea III no consistent response to salinity. In early postmoult, it was lower at 15 ‰ than at 32 ‰ (0.19 vs. 0.32) but the opposite pattern occurred at intermoult (0.25 vs. 0.18).

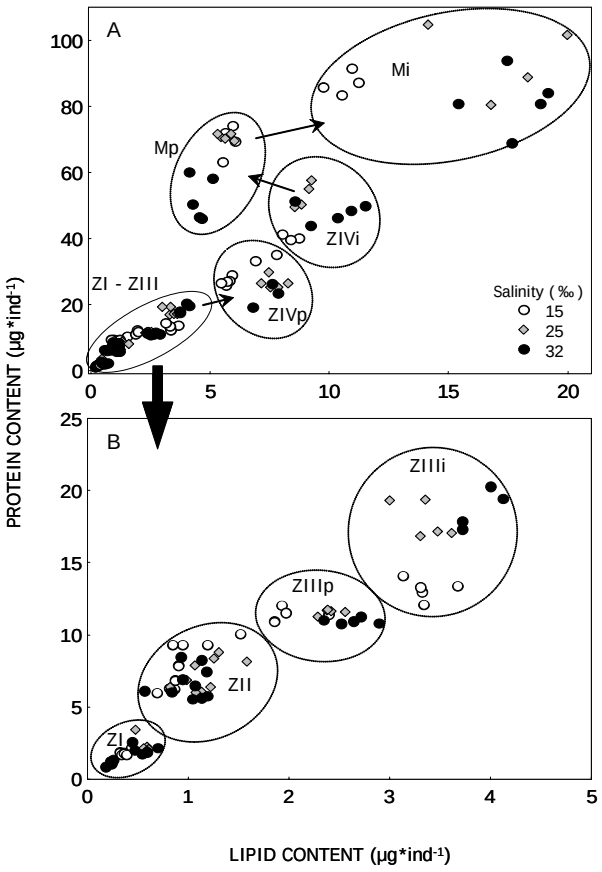

Fig. 7. *Chasmagnathus granulata*. Lipid content versus protein content plot (µg*ind^{-1}) showing overall salinity effects throughout the larval development. A: all data, B: amplification of A, to show the early larval stages clearer. Symbols: ZI for Zoea I; ZII for Zoea II; ZIIIp for Zoea III postmoult, ZIIIi for Zoea III intermoult, ZIVp for Zoea IV postmoult; ZIVi for Zoea IV intermoult, Mp for Megalopa postmoult and Mi for Megalopa intermoult.

DISCUSSION

Our data show that salinity affected biomass (measured as W, protein and lipid content per individual) and daily growth rates. The latter indicate effects on both the biomass reached at a particular instar or moult stage, as well as the duration of a developmental period. Corresponding with observations by Giménez and Anger (2003), our data show that larvae kept at 15 ‰ revealed a longer duration of development and a reduced average of daily accumulation rate of biomass. At this salinity, the larvae showed reduced W and lipid contents compared with larvae reared at an optimal salinity (32 ‰). These differences were small in the early zoeal instars (6-7% in W, 10-20% in lipids), but they increased in the Zoea IV and the Megalopa (20-25% in W, 20-40% in lipids). Reduced W and lipid contents at low salinities are consistent with previous investigations on decapod larvae (Pfaff, 1997; Torres et al., 2002). Lower W is caused by reduced accumulation of both organic and inorganic substances. Osmotic stress affects in particular metabolic processes involved in the assimilation of lipids. For proteins the effect, if significant, consisted on higher levels (8-37%) at 15 ‰ than at 32 ‰.

The effects of salinity on larval biomass were small or absent in the early zoeal instars, but strong in late instars. By contrast, clear effects of salinity on average daily growth rates were already conspicuous in early postmoult of the Zoea II, with reduced accumulation rates of W, lipids and proteins at 15 ‰ compared to the other salinities. In this stage, moulting was delayed by low salinity, while the larvae showed little change in their biochemical composition. Weak salinity effects on the biomass of the Zoea II may be due to hyper-osmoregulation during the Zoea I of *C. granulata* (Charmantier et al., 2002). Torres et al (2002) compared the Zoea I of various species differing in their osmoregulatory capacities, and suggested that hyper-osmoregulating larvae should suffer smaller effects on biomass at low salinities.

After moulting to the Zoea III, cumulative stress effects led to an increased delay in moulting, but also to reduced biomass. Effects on the biochemical composition could clearly be seen in a protein-lipid plot (Fig. 7). Osmotic stress was probably enhanced, as the Zoea II and III instars are osmoconformers (Charmantier et al., 2002). The present data are consistent with the findings of Torres et al (2002),

showing that an exposure of osmoconforming instars to low salinity causes reduced biomass. The ability to osmoregulate in the Megalopa (Charmantier et al., 2002), did not suffice to compensate previous effects of salinity on biochemical composition, so that low biomass values at low salinity were found also in this stage. As a consequence, megalopal mortality at 15 ‰ was high after previous zoeal exposure to this salinity (Giménez and Anger, 2003). At least in *C. granulata*, cumulative effects of osmotic stress may therefore be a physiological key process impeding a retention strategy, with larval development in estuaries or coastal lagoons where the conspecific adults live (Giménez, 2003).

In conclusion, cumulative osmotic stress may be a potentially important phenomenon along the larval development of estuarine decapod crabs. It has also been detected in the crab *Armases miersii*, although this is characterized by strongly osmoregulating larvae (Anger, 1996; Charmantier et al., 1998; Anger et al., 2000). In this case, stress due to long-term exposure to reduced salinities overcharged the capacity of acclimation, leading to increased mortality and delayed development. In *C. granulata* cumulative effects of salinity stress may lead to prolonged and alternative developmental pathways (Giménez and Torres, 2002), reduced survival (Giménez and Anger, 2003), and reduced larval quality in terms of biochemical composition (present paper), and these effects may transcend the larval phase, leading to reduced juvenile survival and growth (Giménez et al. 2004; Giménez, 2006).

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V. BIOCHEMICAL LEVEL

V.1. Effects of variation in salinity on the localization and activity of Na^+-K^+-ATPase

PUBLICATION 6

EFFECTS OF LONG-TERM EXPOSURE TO DIFFERENT SALINITIES ON THE LOCATION AND ACTIVITY OF NA^+-K^+-ATPASE IN THE GILLS OF JUVENILE MITTEN CRAB, *ERIOCHEIR SINENSIS*

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Comparative Biochemistry and Physiology A

ABSTRACT

The euryhalinity of mitten crab, *Eriocheir sinensis*, is based on osmoregulation, and thus on the activity of Na^+-K^+-ATPase. We studied location and activity of this enzyme in gills of juvenile crabs exposed to 5‰, 25‰, and 40‰ salinity. The posterior gills showed always a high number of immunopositive cells (IPC), staining with fluorescent antibody against Na^+-K^+-ATPase, covering at 5‰ the entire lamellae. At 25‰, they showed fewer IPC which occurred only at the bases of the lamellae. Enzyme activity was consistently higher in posterior than in anterior gills. Low salinity stimulated the activity only in posterior gills. Both histochemical and enzymatic results are consistent with previous ultrastructural observations showing that the epithelial cells of the posterior, but not the anterior gills exhibit typical traits of ionocytes. While an increase in Na^+-K^+-ATPase activity at a reduced salinity is consistent with a strong hyper-osmoregulatory capacity in juvenile crabs, a low activity at an enhanced salinity suggests a physiological response, directed towards a reduction of Na^+ uptake. The activity increase of ion-transporting enzymes is directly related to spatial changes in their distribution along the osmoregulatory tissue, i.e. an enhanced number of IPC scattered along the entire lamellae. In juveniles, this allows for successful development and growth at reduced salinities.

Keywords: *Eriocheir sinensis*, gills, Na^+-K^+-ATPase activity, Na^+-K^+-ATPase immunolocalisation, osmotic stress.

INTRODUCTION

The Chinese mitten crab, *Eriocheir sinensis* H. Milne-Edwards 1854 is a strongly euryhaline brachyuran. During its complex life cycle, the adults migrate downstream towards estuarine waters (Herborg et al., 2003), where they reach maturity, mate and release the planktonic larvae. Their following larval development, consisting of 5 zoeal stages and a megalopa (Kim and Hwang, 1995; Montú et al., 1996) takes place in estuarine and marine coastal waters. After metamorphosis, the juveniles start the upstream migration towards the limnic parental habitat (Herborg et al., 2003). This

upstream migration involves an acclimation of the migrating juveniles to decreasing salinity, reaching eventually fresh water. The ability of *E. sinensis* to cope with the salinity variations occurring during its life cycle involves ontogenetic changes in the osmoregulatory capacity, i.e. from a moderately hyper-iso-regulating zoeal phase to a strongly euryhaline hyper-hypo-regulating first juvenile crab stage (Cieluch et al., in press).

The increasing osmoregulatory capacity is indispensable to allow for upstream migration. It is achieved by maintaining an osmotic gradient through an active uptake of ions such as Na^+ and Cl^- across the posterior gills (Péqueux and Gilles, 1988). The absorption of Na^+ occurs through a coordinated action of apical Na^+ channels and basolateral Na^+-K^+-ATPase, while the uptake of Cl^- proceeds via an apical Cl^-/HCO_3^- antiporter and basolateral Cl^- channels (Onken et al., 1991) driven by the apical V-type H^+ pump (Putzenlechner et al., 1992; for review see Towle, 1997). The study of developmental changes in histological characteristics and physiological capabilities showed a strong correlation between the ontogeny of osmoregulation and the expression of Na^+-K^+-ATPase in transport epithelia (Cieluch et al., in press).

Adult *E. sinensis* possess eight lateral pairs of gills. The five anterior pairs are predominantly formed by thin epithelial cells, which are typical of gas exchange tissues (Péqueux, 1995). By contrast, ionocytes predominate in the three posterior gill pairs involved in the ion transport. They typically reveal abundant apical infoldings and basolateral invaginations with numerous mitochondria (Péqueux, 1995; Cieluch et al., in press). The presence of numerous Na^+-K^+-ATPase molecules in the posterior gills was shown by immunofluorescence light microscopy (ILM), while immunopositive cells were undetectable in the anterior gills of the juvenile I and adult crabs (Cieluch et al, in press).

The present study focuses on the involvement of Na^+-K^+-ATPase in the osmoregulation of juvenile *E. sinensis* during long-term exposure to different salinities, by localizing and measuring the activity of this enzyme in the gill tissues. The combination of histochemical and biochemical techniques should allow to explain how the regulation of Na^+ concentration occurs during hyper-osmoregulation. We expected (a) an increase in Na^+-K^+-ATPase activity during exposure to reduced salinities, as already documented for other crustacean species, and (b) that this

effect is due to a modification in the abundance and distribution of Na^+-K^+-ATPase molecules along the posterior gills.

MATERIALS AND METHODS

Animals

Ovigerous females were collected near the island of Scharhörn in the mouth of River Elbe, northern Germany. They were transported to the Marine Biological Station Helgoland (Helgoland, Germany) and maintained in a circulating water system (25 ‰, 15°C, and photoperiod 12:12 h). Freshly hatched larvae were mass-reared in 400-mL beakers (150 larvae*L^{-1}) at 25‰ (optimal salinity; Anger, 1991), 18°C, and a 12h:12h photoperiod. Water and food (*Artemia* sp. nauplii *ad libitum*) were changed in daily intervals. After metamorphosis to the juvenile-I stage, the young crabs were divided in three groups and cultured for ca. one year at a reduced (5‰), an intermediate (25‰) and an enhanced (40‰) salinity under the same conditions of temperature, photoperiod, and food. Feeding and water change, however, were performed only every second day. Two months before the sampling of gills, the crabs were isolated to allow for control of the moulting cycle. Sampling took place during the intermoult stage.

Localisation of Na^+-K^+-ATPase: immunofluorescence light microscopy

Crabs (ca. 15 mm carapace width) previously exposed to 5‰ and 25‰, respectively, were fixed for 48 h in Bouin's fixative. After rinsing in 70% ethanol and removal of the carapace, the posterior gills were dissected from the inner body wall. Subsequently, the gills were fully dehydrated in a graded ethanol series and embedded in Paraplast X-tra (Sigma). Sections (4 µm) were cut on a Leitz Wetzlar microtome, collected on poly-L-lysine-coated slides, and stored overnight at 38 °C. Sections were then pre-incubated for 10 min in 0.01% Tween20, 150 mM NaCl in 10 mM phosphate-buffered saline (PBS, pH 7.3) to permeabilize the tissues. In order to remove the fixatives' free-aldehyde groups, samples were treated for 5 min with 50 mM NH_4Cl in PBS. Sections were washed in PBS and incubated for 10 min with a blocking solution (BS) containing 1% bovine serum albumin (BSA) and 0.1% gelatine

in 20 mM PBS to block non-specific binding sites. The primary antibody (monoclonal antibody IgGα5, raised against the avian α-subunit of the Na^+-K^+-ATPase; DSHB, University of Iowa, USA) was diluted in PBS to 20 µg*ml^{-1}, placed in small droplets of 100 µl on the sections, and then incubated for 2 h at room temperature in a moist chamber. Control sections were incubated in BS without primary antibody. To remove unbound antibodies the sections were then washed (3x5 min) in PBS, and subsequently incubated for 1 h with small droplets (100 µl) of the secondary antibody: fluoresceinisothiocyanate-labeled (FITC) goat anti-mouse IgG (Jackson Immunoresearch, West Baltimore, USA). After extensive washes in PBS (4x5 min), the sections were covered with a mounting medium and examined with a fluorescent microscope (Leitz Diaplan coupled to a Ploemopak 1-Lambda lamp) with an appropriate filter set (450 nm to 490 nm band-pass excitation filter).

Gill sampling for enzymatic determinations

Fifteen (five per each salinity) one-year old crabs (carapace width 13.0 - 27.5mm) were killed by short exposure to -20°C. The gills were dissected, washed 2 sec in pre-homogenisation buffer (see Table 1), blotted dry, and frozen at -80°C for later measurements of Na^+-K^+-ATPase activity and protein content.

Na^+-K^+-ATPase activity in gills tissues

The gills were homogenised in 300 µL of ice-cold homogenisation buffer (see Table 1) by sonication (Branson Sonifier, Cell Disruptor B 15) with 5 strokes of 5 sec, on ice. Na^+-K^+-ATPase activity was obtained as the difference between the inorganic phosphate (P_i) produced from the reactions in absence (total ATPase-activity) and presence of the Na^+-K^+-ATPase specific inhibitor ouabain (activity of "other" ATPase). The P_i concentration was determined using a modification of the method described by Chifflet et al. (1988). Reactions were initiated by the addition of 25 µL of the homogenate to 225 µL of incubation buffer I (IB-I; see Table 1) and to 225 µL of incubation buffer II (IB-II; see Table 1). After 15 min at 37°C, the incubation was stopped by an addition of 250 µL solution A (see Table 1). To determine the P_i photometrically, 4 replicates of 75 µL from each of the above mentioned incubation mixtures (IB-I + solution A and IB-II + solution A) were placed in a 96-well microplate. Immediately thereafter, they were mixed with 75 µL of solution BD (1:1;

see Table 1) and incubated for 5 min at room temperature. Subsequently, 115 µL of solution E (see Table 1) were added, and after 15 min at room temperature, absorbance was determined (Thermoelectron Multiskan® Spectrum spectralphotometer, wavelength: 850nm). The calibration curve was obtained with appropriate dilutions of a K_2HPO_4 standard solution.

Table 1. *Eriocheir sinensis*. Reagents for homogenisation and incubation buffers, and solutions to determination of inorganic Phosphate (modified from Chifflet et al., 1988)

SOLUTION	REAGENTS
Pre-homogenisation buffer (PHB)	250.0 mM saccharose 10.0 mM imidazol 150.0 mM NaCl
Homogenisation buffer (HB)	250.0 mM saccharose 10.0 mM imidazol
Incubation buffer I (IB-I)	144.0 mM NaCl 22.0 mM KCl 3.3 mM $MgCl_2$ 4.4 mM NaN_3 56.0 mM HEPES 26.0 mM TRIS 3.3 mM ATP 1.1 mM ouabain
Incubation buffer II (IB-II)	144.0 mM NaCl 22.0 mM KCl 3.3 mM $MgCl_2$ 4.4 mM NaN_3 56.0 mM HEPES 26.0 mM TRIS 3.3 mM ATP
Solution A	12 % SDS
Solution B	6 % ascorbic acid (in HCl 1N)
Solution D	1 % ammonium molybdate (in 12% SDS)
Solution E	2 % sodium citrate 2 % sodium meta-arsenite 2 % acetic acid

Protein determination

The protein content of the homogenate was determined using a modified method after Lowry et al. (1951) (kit: BioRad D_C Protein Assay). 25 µL of homogenate were mixed with 100 µL of ice cold 20% trichloroacetic acid (TCA). After 10 min incubation at 4°C, the samples were centrifuged at 10.000 g for 10 min at 4°C; the supernatant was discarded. The remaining pellet was dissolved in 300 µL NaOH (1 M) and incubated with shaking at 1400 rpm for 30 min at 56°C in a Thermomixer Eppendorf. After incubation, 4 replicates of 30 µL each of the dissolved sample were mixed with 20 µL of Reagent A and 300 µL of Reagent B (kit: BioRad D_C Protein Assay) in a 96-well microplate. The microplates were incubated for 15 min at room temperature in the dark and absorbance was measured (Thermoelectron Multiskan® Spectrum spectralphotometer, wavelength: 750 nm). The calibration curve was obtained by dilutions of bovine serum albumin (BSA, kit: BioRad D_C Protein Assay).

Statistical analysis

The data are expressed as mean values ± SD. The effect of exposure to different salinities on the activity of total ATPases and Na^+-K^+-ATPase in the different gills was tested with ANOVA following Zar (1996). Position (right vs. left), type (anterior vs. posterior) and gill number (3 - 8) were treated as repeated measures within-subject factors, while salinity (5, 25, 40 ‰) was a between-subject factor. When the ANOVA showed a significant effect, post hoc comparisons between mean values were performed using the Student-Newman-Keuls (SNK) test. Homogeneity of variance (Cochran test) and normality (normality plots) were checked. Statistical significance was accepted at $\alpha = 0.05$.

RESULTS

Localisation of Na^+-K^+-ATPase

Long-term exposure of *Eriocheir sinensis* to two different salinities (5 ‰, 25 ‰) induced changes in the location and number of ionocytes in the ion-transporting epithelia of the posterior gills. When animals were exposed to 5 ‰, these epithelia showed numerous immunopositive cells (IPC) scattered throughout the gill lamellae,

indicating abundant Na^+-K^+-ATPase molecules (Fig. 1A). In gills of animals maintained at 25 ‰, by contrast, IPC were present in smaller amounts and located only at the base of the lamellae (Fig. 1B). Thus, a comparison between animals maintained at 5 ‰ and 25 ‰ showed not only a change in abundance of Na^+-K^+-ATPase molecules, but also in its distribution.

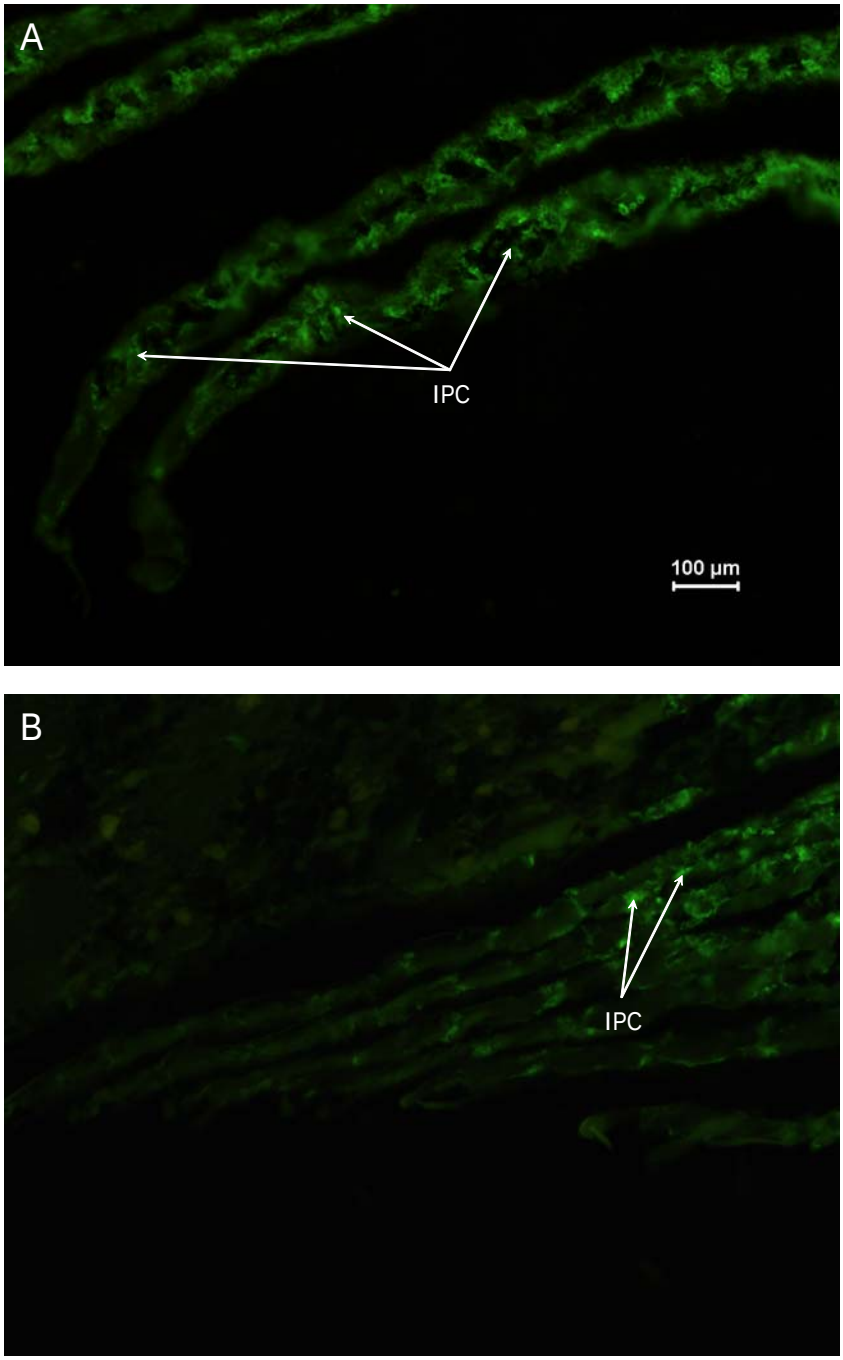

Fig. 1. *Eriocheir sinensis*. Immunolocalisation of Na^+-K^+-ATPase in ionocytes of juvenile crabs (ca. 15mm carapace width) after long-term exposure (one year) to 5 ‰ (A) or 25 ‰ (B). Equivalent sections of posterior gills show the apical side of the gill lamellae: more lamellae are shown in B, because they are ca. 1.5 times thinner than in A. IPC: immunopositive cells.

Na^+-K^+-ATPase activity in gill tissues

We show here only biochemical determinations from gills 3-8, because, the smallest anterior gills (1-2) did not reveal detectable enzymatic activities (neither total-ATPase nor Na^+-K^+-ATPase).

ATPase-activity (measured as P_i; given in nmoles*min^{-1}*mg^{-1} protein) was significantly affected by salinity and gill type (anterior vs. posterior), but not by the lateral gill position (left vs. right; Table 2). The anterior gills (3-5) were not affected by salinity (Fig. 2A). In the posterior gills (6-8), by contrast, increasing salinities caused a significant decrease in enzyme activity (Fig. 2A). The anterior gills showed consistently low activities (245-367 nmoles*min^{-1}*mg^{-1} protein), while the posterior gills revealed values of 485 - 933.

Table 2. *Eriocheir sinensis*. Four-way between-within subject ANOVA analysis to evaluate the effect of salinity, gill position, type and number of total ATPase activity. Significant differences are in bold. Symbols: MSf: mean square of factors; Dff: degrees of freedom of factors; MSe: mean square of error; Dfe: degrees of freedom of error.

	MSf	**Dff**	**MSe**	**Dfe**	**F**	**p**
Salinity (S)	523075	2	54000	12	9,69	**0,003**
Position (P)	15860	1	5609	12	2,83	0,118
P*S	11725	2	5609	12	2,09	0,166
Type (T)	6022160	1	32251	12	186,73	**<0,001**
T*S	393202	2	32251	12	12,19	**0,001**
Gill N° (N)	22389	2	8605	24	2,60	0,095
N*S	9318	4	8605	24	1,08	0,387
P*T	3074	1	3967	12	0,77	0,396
P*T*S	1328	2	3967	12	0,33	0,722
P*N	7660	2	2459	24	3,11	0,063
P*N*S	1797	4	2459	24	0,73	0,580
T*N	118203	2	9001	24	13,13	**<0,001**
T*N*S	21266	4	9001	24	2,36	0,082
P*T*N	16214	2	5013	24	3,23	0,057
P*T*N*S	5159	4	5013	24	1,03	0,412

In the enzyme essay, the P_i produced through the activity of ATPase can be divided in two different fractions: (1) P_i produced by the activity of Na^+-K^+-ATPase

alone and (2) P_i produced by "other" ATPases. In the anterior gills Na^+-K^+-ATPase activity represented only 30-60% of total ATPase activity, in the posterior gills, by contrast, this activity amounted to 70-73% of total ATPase activity.

Na^+-K^+-ATPase activity showed the same pattern of activity modification in response to different salinities as the total ATPase activity (Table 3, Fig. 2B). Thus, the effect on total ATPase activity, i.e. an increase at decreasing salinity in the posterior but not in the anterior gills, could be attributed to an increase in Na^+-K^+-ATPase activity. In the former gill type, it increased on average from 329 to 723 nmoles P_i*min^{-1}*mg^{-1} protein, while it remained low (122-166 nmoles P_i produced*min^{-1}*mg^{-1} protein) in the latter.

Table 3. *Eriocheir sinensis*. Four-way between-within subject ANOVA analysis to evaluate the effect of salinity, gill position, type and number of Na^+-K^+-ATPase activity. Symbols as in Table 2.

	MSf	**Dff**	**MSe**	**Dfe**	**F**	**p**
Salinity (S)	416595	2	35579	12	11.71	**0.002**
Position (P)	1088	1	3072	12	0.35	0.563
P*S	6424	2	3072	12	2.09	0.166
Type (T)	5141084	1	26602	12	193.26	**<0.001**
T*S	310537	2	26602	12	11.67	**0.002**
Gill number (N)	17813	2	6295	24	2.83	0.079
N*S	7788	4	6295	24	1.24	0.322
P*T	25	1	2676	12	0.01	0.925
P*T*S	5047	2	2676	12	1.89	0.194
P*N	5030	2	1380	24	3.65	**0.041**
P*N*S	1648	4	1380	24	1.19	0.339
T*N	9390	2	6842	24	1.37	0.273
T*N*S	15834	4	6842	24	2.31	0.087
P*T*N	8829	2	3162	24	2.79	0.081
P*T*N*S	2423	4	3162	24	0.77	0.558

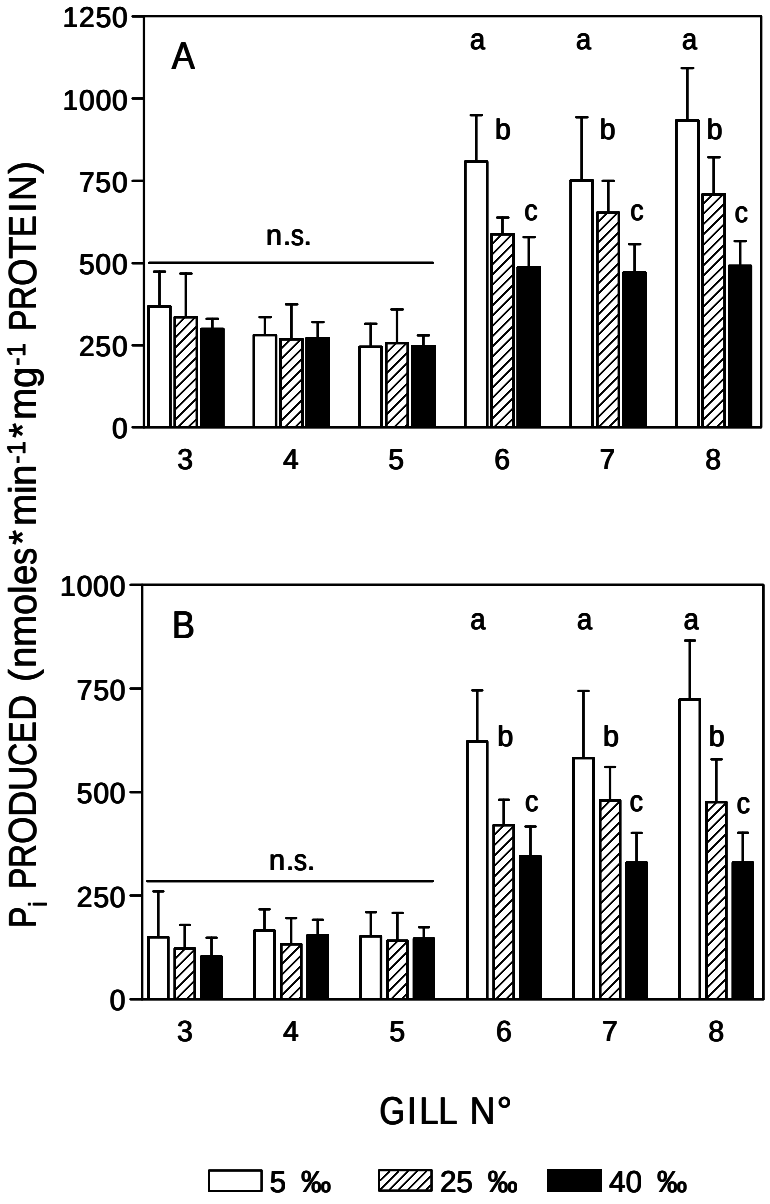

Fig. 2. *Eriocheir sinensis*. Activity of total ATPases (A) and Na^+-K^+-ATPase (B), expressed as amounts (in nmoles) of inorganic phosphorous (P_i) produced per minute per milligram of protein. Different letters indicate significant differences between means; n.s., no significant difference.

DISCUSSION

In previous studies, long-term acclimation to different salinities (through one year) has not been tested, but only exposures for several weeks (e.g. Li et al., 2006).

Our study shows for the first time effects of salinity on the location of Na^+-K^+-ATPase molecules within ion-transporting gill tissues of juvenile crabs. Also, the differential significance of individual gills, of the lateral position (left vs. right), and the type of gill (anterior vs. posterior) for Na^+-K^+-ATPase activity in relation to

salinity is demonstrated. In *Eriocheir sinensis*, the early juvenile stages have previously been studied only with immunofluorescence light microscopy using specimens maintained at optimal salinities (Cieluch et al., in press). Na^+-K^+-ATPase activities were studied in the posterior gills only (e.g. Cooper and Morris, 1997), with in pooled samples of anterior or posterior gills (e.g. Castilho et al., 2001), without testing for variability among the various gills. We found that, within either the posterior or anterior gills, Na^+-K^+-ATPase activity was independent of the body side or the individual gill number; while it differed substantially between anterior and posterior gills.

Our immunocytochemical study corroborates the importance of Na^+-K^+-ATPase in the posterior gills of brachyurans to cope with hypo-osmotic stress. In juveniles maintained at a low salinity (5‰), the gills showed an enhanced number of ionocytes, indicated by immunopositive cells (IPC) scattered throughout the entire lamellae. In specimens exposed to 25‰, by contrast, a lower number of IPC was detected, concentrated near the base of the lamellae. Besides, lamellae were thicker after exposure to 5‰ than to 25‰. In a previous study on early juvenile *Eriocheir sinensis* exposed to 25‰, the IPC were also located mainly on the base of the lamellae, but not on their apical side (Cieluch et al., in press). This indicates that the number of ionocytes within the ion-transporting gill tissues increases in diluted media. The proliferation of ionocytes may provide the suitable machinery to face hypo-osmotic stress, allowing an enhanced expression of Na^+-K^+-ATPase, and thus an increase of the Na^+-K^+-ATPase activity.

The localisation of ionocytes in our study is consistent with previous investigations on the European lobster, *Homarus gammarus* (Lignot et al., 1999; Lignot and Charmantier, 2001). When juveniles of this species were exposed to brackish seawater, they showed an increased number of widely scattered ionocytes in the epithelia of the epipodites and branchiostegites, which are in this species the ion-transporting epithelia involved in osmoregulation in this species. In contrast, lobsters maintained in seawater possessed only low amounts of immunostained cells (Lignot et al., 1999; Lignot and Charmantier, 2001). The increased thickness of the lamellae in juvenile *E. sinensis* exposed to low salinity was consistent with previous studies of the posterior gills of *Carcinus maenas* and *Pachygrapus marmoratus* (Compère et al., 1989; Pierrot, 1994) and of the epithelium of epipodites and

branchiostegites of *Homarus gammarus* (Haond et al., 1998). In conclusion, our study points to a common pattern in the relationship between variations in salinity and the distribution and abundance of ionocytes in ion-transporting tissues of decapod crustaceans.

The patterns of Na^+-K^+-ATPase activity found with biochemical techniques (present study) are also consistent with previous histological evidence (Péqueux, 1995). In crabs, all posterior gills, showed both typical characteristics of ion-transport tissues and higher enzyme activities than the anterior gills. The latter, by contrast, possessed typical features of respiratory tissues and changes in salinity did not influence their structure or abundance of IPC.

The increase in Na^+-K^+-ATPase activity in the posterior gills at 5 ‰ appears to be an adaptive physiological response, which enhances the function of hyper-osmoregulation. Lower salinities may either stimulate enzyme production, or uncover hidden active sites. Both mechanisms should cause an increase in the active transport of Na^+ ions. For *Eriocheir sinensis*, the immunocytochemical evidence, suggests that *de novo* synthesis is the likely explanation for the increase in Na^+-K^+-ATPase activity. The same pattern was found not only in *Eriocheir sinensis* (Péqueux et al., 1984), but also in other hyper-regulating decapods (for review see Lucu and Towle, 2003; e.g. *Carcinus maenas* (Siebers et al., 1982, 1983), *Homarus gammarus* (Flick and Haond, 2000), *Chasmagnathus granulata* (Castilho et al., 2001). Inversely, the enzyme activity decreases during a transfer from freshwater to brackish water, e.g. in *Procambarus clarkii* (Sarver et al., 1994) or *Macrobrachium rosenbergii* (Wildera et al., 2000). In addition, osmoconformers, which are incapable of regulating their hemolymph concentration, do not show changes in the Na^+-K^+-ATPase activity during salinity variation, e.g. *Palinurus elephas* (Lucu et al., 2000).

The reduction of Na^+-K^+-ATPase activity after an extended exposure to 40 ‰ might be explained (a) as an adaptive response to reduce active Na^+ uptake, or (b) as a consequence of physiological disturbance due to high internal osmolality. *E. sinensis* is a weak hypo-osmoregulator at high salinities (Cieluch et al., in press). In our experiments, the activity of ATPases other than Na^+-K^+-ATPase (data not shown) did not significantly decrease at 40 ‰. This suggests that a decrease occurred only in Na^+-K^+-ATPase activity. We may therefore conclude that this effect is an adaptive physiological response, which reduced the uptake of Na^+ ions.

The response of hypo-osmoregulators to changes in salinity varies among species. For instance, *Chasmagnathus granulata* shows in the posterior gills similar changes in Na^+-K^+-ATPase activity as *E. sinensis* (Castilho et al., 2001). However, it shows also a decreasing activity in the anterior gills. This suggests that, in this case, the hypo-osmoregulatory capacity may not be strong enough to prevent physiological stress. Other species do not show any significant changes of enzyme activity when exposed to concentrated media (e.g. *Carcinus maenas*: Hake and Teller, 1983; *Uca pugnax*: Holliday, 1985; *Uca pugilator*: Hake and Teller, 1983; D'Orazio and Holliday, 1985; *Sesarma reticulatum*: D'Orazio and Holliday, 1985). Others, by contrast, show under such conditions an increase in Na^+-K^+-ATPase activity (e.g. *Uca tangeri*: Drews, 1983; *Artemia salina*: Holliday et al., 1990). These inconsistent findings indicate that the mechanisms of hypo-osmoregulation in concentrated media require further comparative studies to enhance our understanding of responses to hyper-osmotic stress in decapod crustaceans.

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VI. MOLECULAR LEVEL

VI.1. Effects of variations in salinity on expression of an ion-transporting enzyme

PUBLICATION 7

EFFECTS OF SALINITY ON GENE EXPRESSION OF NA^+-K^+-ATPASE IN THE EARLY LIFE-HISTORY STAGES OF A EURYHALINE CRAB

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ABSTRACT

The ion-transporting enzyme Na^+-K^+-ATPase plays a major role in hyper-osmoregulating crustaceans. Its activity increases in the posterior gills of brachyuran crabs, when these are exposed to diluted media. Studies conducted on adult crabs have investigated whether this response is achieved by regulation of pre-existing enzyme or through an increased gene transcription and mRNA translation. In the present investigation, we addressed this question in the early postembryonic developmental stages of a euryhaline and strongly osmoregulating crab, *Armases miersii*. We studied the effects of salinity on the expression of the Na^+-K^+-ATPase α-subunit gene in the Zoea I, Zoea III, Megalopa, and Juvenile I, exposed to 5‰, 25‰, and 40‰. A partial cDNA sequence (1429bp) corresponding to the Na^+-K^+-ATPase α-subunit was obtained through RT-PCR from total RNA extracted from larvae and juveniles. This α-subunit is known to be highly conserved among species. In *A. miersii*, the partial nucleotide sequence showed 82-92% homology with those of other decapod crustaceans. The expression of the Na^+-K^+-ATPase α-subunit gene was analysed through quantitative real-time PCR. Elongation Factor 1α was used as an internal control or house-keeping gene. After acclimation to 5‰, all studied stages showed higher levels of α-subunit transcripts than those acclimated to 25‰ or 40‰. The Zoea I stage showed the lowest transcript levels, followed by the Zoea III. The Megalopa and Juvenile I had slightly higher transcript levels. We conclude that, as in adult crabs, the response to low salinity involves *de novo* synthesis of Na^+-K^+-ATPase in the early life-cycle stages.

Keywords: *Armases miersii*, crab larvae, Na^+-K^+-ATPase, osmoregulation, real-time PCR, salinity.

INTRODUCTION

The enzyme Na^+-K^+-ATPase was first identified in homogenates of nerve tissue from the shore crab *Carcinus maenas* (Skou, 1957). The ATP-driven Na^+-K^+-ATPase

is an ubiquitous transport enzyme in membranes of animal cells, where it extrudes Na^+ from the cytosol, simultaneously importing K^+ into the cell (reviewed by Therien and Blostein, 2000). Localised in the basolateral membrane of epithelial cells, it consists of a catalytic α-subunit and an accompanying β-subunit (Broude et al., 1989). The highly conserved catalytic α-subunit (Skou and Esmann, 1992) contains the ATP-binding domain and the cation-binding sites, as well as a binding site for its specific inhibitor ouabain (Jørgensen and Andersen, 1988).

In many aquatic animals including crustaceans, the Na^+-K^+-ATPase plays a major role in the regulation of Na^+ ions in the transporting epithelia (for reviews see Péqueux, 1995; Lucu and Towle, 2003). The effect of salinity on the activity of Na^+-K^+-ATPase has been extensively studied in a wide range of species. In brachyuran crustaceans, such studies were mainly conducted in the posterior gills of adults. An enhanced activity in homogenates of ion-transporting gills was found when euryhaline crustaceans were exposed to diluted media (Péqueux, 1995; Lucu and Towle, 2003).

A few studies have tried to elucidate the molecular basis of the response to salinity variations. Investigations on the expression levels of the gene coding for the Na^+-K^+-ATPase α-subunit in gills of the hyper-hypo-osmoregulating crab *Pachygrapsus marmoratus* have shown an increase upon exposure to reduced salinity (Lucu and Towle, 2003). In addition, the expression of the gene encoding the Na^+-K^+-ATPase α-subunit increased when adults of the hyper-hypo-osmoregulating crabs *Chasmagnathus granulata* and *Scylla paramamosain* were exposed to diluted seawater (Luquet et al., 2005; Chung and Lin, 2006). However, no information has been available concerning the early life-history stages (larvae, early juveniles), so that it has remained uncertain if the mechanisms known from adult crabs appear early in ontogeny or only later.

Armases miersii (Rathbun 1897) breeds in coastal mangrove swamps and in land-locked supratidal rock pools (Anger, 1995a; Schuh and Diesel, 1995). The larval development consists of three zoeal stages and a megalopa, which develop in isolated supratidal rock pools until metamorphosis to the first juvenile stage occurs. In such pools, the physico-chemical conditions are highly variable, particularly regarding salinity. The semiterrestrial juvenile and adult crabs hide in crevices between mangrove roots or inhabit subtidal rocks as well as coastal limestone caves

with brackish or fresh water (Anger, 1995b, c; Anger and Schultze, 1995; Schuh and Diesel, 1995). The utilization of this harsh environment as a breeding habitat was possible due to the ability of early larval stages to osmoregulate (Charmantier et al., 1998). Larvae can hyper-osmoregulate at low salinities and are osmoconformer at salinities higher than 25 ‰. The megalopa and the subsequent juvenile stages reveal a weakly developed but increasing capability of hyper-hypo-osmoregulation, i.e. strong hyper-regulation at low salinities and slight hypo-regulation at salinities higher than 25 ‰. The adults show strong hyper-hypo-osmoregulatory capacities in conditions ranging from freshwater to concentrated media (45 ‰) (Charmantier et al., 1998).

To further elucidate the molecular aspects of hyper-hypo-osmoregulation in early stages of *Armases miersii*, we identified and characterised the mRNA expression of the Na^+-K^+-ATPase α-subunit during the ontogeny. In addition, variations in this expression following exposure to different salinities were investigated.

MATERIALS AND METHODS

Animals

Larvae were produced by females maintained at the Helgoland Marine Biological Station (BAH, Helgoland, Germany) under controlled conditions of temperature (24°C), salinity (25 ‰) and photoperiod (12:12h), and fed with isopods (*Idotea* sp.) *ad libitum*. The first juvenile specimens were reared in 1993 at the Discovery Bay Marine Laboratory (Jamaica) and subsequently transported to the BAH, where they reached adulthood and reproduced. New crabs were collected in Jamaica in 2003 and 2004. Ovigerous females were isolated in individual aquaria and kept under otherwise identical conditions.

Freshly hatched larvae were either used for experiments with the Zoea I (see below for details, Fig. 1) or mass-reared to the subsequent stages. All larval stages were reared under controlled conditions (24°C, 25 ‰, photoperiod 12:12h), and fed *ad libitum* with freshly hatched *Artemia* sp. nauplii. For rearing, filtered (Orion, mesh size 1µm) seawater (32 ‰) was mixed with appropriate amounts of desalinated freshwater to obtain a salinity of 25 ‰. Mass-rearing took place in 3L-beakers

(approximate density 250 ind*L^{-1}) with aeration. Water was changed daily, dead larvae were removed, and new food was added. Cultures were controlled daily for moulting and freshly moulted larvae were transferred to new beakers to ensure that all larvae used in experiments had the same moulting history.

Experimental design

For all experiments, the larvae were group-reared in replicate (Table 1) 400-mL bowls without aeration, under the same conditions of temperature, light and feeding as in mass-rearing. The number of larvae per bowl was adjusted according to the size of each instar (see Table 1). Each set of experiments consisted of an exposure to three experimental salinities (5 ‰, 25 ‰, 40 ‰) from early postmoult (0% of the moult cycle) to the intermoult stage (ca. the 50% of the moult cycle; Fig. 1). These treatments represent conditions of reduced, optimal and enhanced salinity conditions, respectively. Low salinity (5 ‰) was obtained by mixing filtered seawater (32 ‰) with appropriate amounts of desalinated freshwater, concentrated seawater (40 ‰) by desiccation.

Table 1. *Armases miersii*. Rearing density, number (N) of larvae or stage-I juveniles pooled for each replicate extraction of total RNA.

	Rearing density per 400-mL bowl ind*ml^{-1}	N individuals per replicate	N replicate		
			5 ‰	25 ‰	40 ‰
Zoea I	0.125	70	4	3	4
Zoea III	0.075	30	4	3	4
Megalopa	0.050	20	4	4	4
Juvenile I	0.050	15	3	3	3

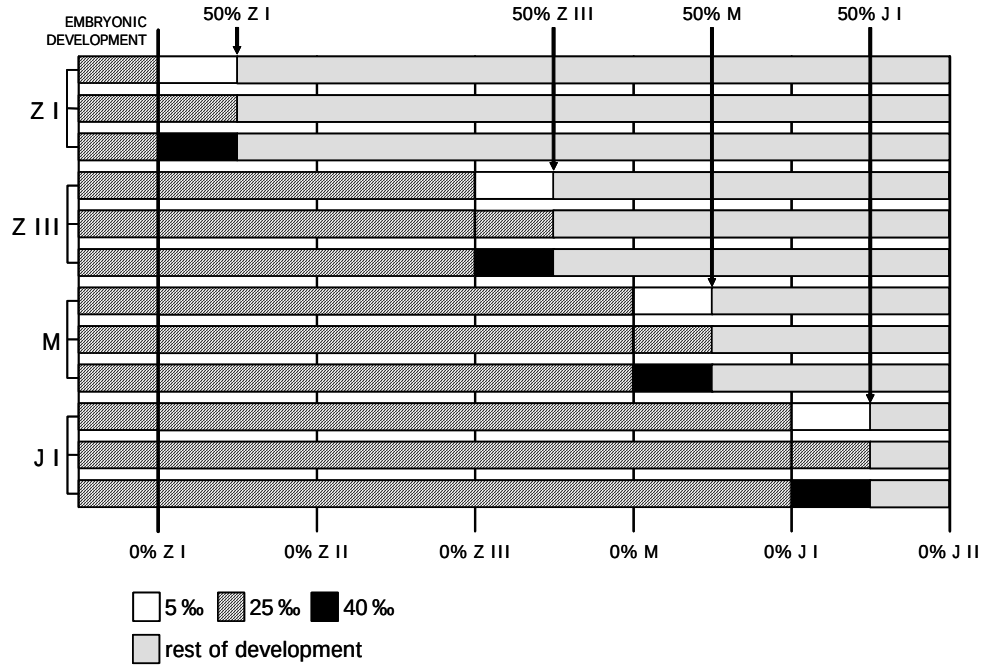

Fig. 1. *Armases miersii*. Experimental design used to study the effects of salinity on Na^+-K^+-ATPase α-subunit gene expression. Intermoult samples were taken after 50% of the moult cycle occurred (early postmoult: 0%, intermoult: 50% of the moult cycle).

Larval sampling, total RNA extraction and reverse transcription

The larvae and juveniles were briefly washed in RNAse-free distilled-water and placed in RNAse-free 1.5-mL vials containing a large excess of RNA*later*® (Ambion, Cambridgeshire, UK) to preserve samples against endogenous RNAse. These samples were stored at -20°C until air transport to Montpellier (France), where they were stored again at -20°C.

Total RNA was extracted from a pool of individuals (Table 1) following an adaptation of the method of Chomczynski and Sacchi (1987) using the kit RNAgents® Total RNA Isolation System (Promega, Mannheim, Germany). Total RNA quality was checked electrophoretically on 1% agarose gels and visualised by ethidium bromide staining. Total RNA concentration of each sample was determined by measuring the absorbance at 260nm (Eppendorf Biophotometer, Le Pecq, France). Afterwards, identical amounts of total RNA (2 µg) for each sample were reverse-transcribed using the kit SuperScript™ II First-Strand Synthesis System (Invitrogen, Karlsruhe, Germany) with oligo-dT as primers.

Sequencing and primer design

Target cDNAs were those encoding the Na^+-K^+-ATPase α-subunit (NaK), and the Elongation Factor 1α (EF1α), a putative housekeeping gene. Degenerate and non-degenerate primers (Table 2) were based on conserved regions identified by multiple alignments of target amino acid sequences from other species, and synthesised by Sigma Genosys, U.K. (NaK10F & NaK16R: Towle et al., 2001; NaK-Pam184-F1, EF1-F2, EF1-R1), and by Eurogentec S.A., Seraing, Belgium (GABI-R1). Conventional PCR was performed in an Eppendorf Mastercycler® (Le Pecq, France) using Taq DNA polymerase (storage buffer A, Sigma, Lyon, France). The protocol included 40 cycles consisting of an initial denaturation step (92°C, 1 min), followed by an annealing step (50°C, 1 min), and an elongation step (72°C, 2 min), using the primers mentioned above (Table 2). Subsequently, a final extension step (72°C, 5 min) was carried out and the resulting products were stored at 4°C. The PCR products were isolated electrophoretically on 1% agarose gels and visualised by ethidium bromide staining. Selected fragments were extracted (700bp and >1000bp for the NaK and 300bp for EF1α) using the MinElute Gel Extraction Kit protocol (Qiagen, Courtaboeuf, France).

Following gel extraction, these fragments were sequenced using the BigDye® Terminator Sequencing kit (Applied Biosystems, Foster City, California, USA) in an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Raw sequences were analysed and trimmed using the BioEdit sequence Alignment Editor (Hall, 1999). To obtain the consensus sequence for NaK, the Contig Assembly Program (CAP) from BioEdit (Huang, 1992) was used. A BLASTX analysis was performed to obtain functional identification (Altschul et al., 1997). Additionally, these nucleotide sequences were translated to open reading frames, and aligned using ClustalW (http://antheprot-pbil.ibcp.fr/ie_sommaire.html) and GeneDoc software (http://www.psc.edu/biomed/genedoc/) to find possible conserved regions.

Species-specific primers (Table 2) based on the consensus sequences were designed with Primer Premier Software (Premier Biosoft) and VectorNTI Primer (Invitrogen) and synthesised by Eurogentec. S.A. (Belgium). They were checked using conventional PCR. The size of the obtained products was checked electrophoretically on 1% agarose gels and visualised by ethidium bromide staining.

Table 2. *Armases miersii*. Nucleotide sequences of degenerated and non-degenerated primers used for conventional and quantitative PCR to amplify the cDNAs of the Na^+-K^+-ATPase α-subunit and the elongation factor 1α.

TARGET cDNA	NAME	SEQUENCE (5'-3')	Tm (°C)
Primers used for initial amplification in conventional PCR			
Na^+-K^+-ATPase α-subunit	NaK-10F	ATG ACI GTI GCI CAY ATG TGG	54.8
	NaK-16R	GGR TGR TCI CCI GTI ACC AT	55.2
	NaK-Pam184-F1	AAC CAG TCT CAC CCT TTT CCC TT	62.4
	GABI-R1	ATG AGT CCC ACA AAC CTG AGA CCA	54.0
Elongation factor 1α	EF1-F2	GHT TCG TGG CYT CTG ACT C	60.0
	EF1-R1	TTH ACG ATG CAD GAG TCV CC	60.2
Species-specific primers used in quantitative real-time PCR			
Na^+-K^+-ATPase α-subunit	ARMI-NaK-F1	GTT ACC TTC TTC GTC ATT GC	58.0
	ARMI-NaK-R1	ACA GTA GCC AGC AGA CCT T	58.0
Elongation factor 1α	ARMI-EF1-F1	AGG TGA TCG TGC TGA ACC	56.0
	ARMI-EF1-R1	GTA CGC CTG TCA ATC TTC TG	60.0

D=A/G/T; H=A/C/T; I=A/C/G/T; R=A/G; V=A/C/G; Y=C/T

Quantification of gene expression

The relative abundance of mRNA encoding Na^+-K^+-ATPase α-subunit in total RNA extracts was estimated by means of real-time quantitative PCR (Q-PCR) using a LightCycler (Roche Molecular Biochemicals, Meylan, France). Q-PCR was performed using the RT-PCR products (0.5 µL-aliquot), the PCR Mix (SYBR Green and Taq DNA polymerase, purchased from Roche, France) and the species-specific primers (Table 2). When binded to double-stranded DNA, the SYBR Green produces a fluorescent signal which is monitored at each cycle of the Q-PCR to establish the threshold cycle, or Ct value, at which the fluorescence (proportional to the product amplification) starts to be detected. The thermal profile used for Q-PCR consisted of 40 cycles of denaturing at 95°C for 10 sec, annealing at 60°C for 5 sec and elongation at 72°C for 10 sec. After the last cycle, temperature increased from 65°C to 95°C with a temperature transition rate of 0.5°C*sec^{-1} to generate the melting curves. A dilution

series of a selected reference cDNA (extract of Megalopa acclimated to 25 ‰) was performed to obtain a calibration curve representing the relationship between the Ct values and the $\log_{10}$ of template availability. Absence of unspecific PCR products and primer dimers was checked by the melting curves.

Data and statistical analyses

The data (Ct) are expressed as mean values ± SD. The linearity of the relationship between Ct values and the $\log_{10}$ of the dilution factors was tested with the Runs test (GraphPad Prism) following Motulsky (1999). The regression lines of NaK and EF1α dilution curves were compared with an F-test (Motulsky, 1999). Since slopes of regression analyses did not differ significantly (see Results), we used the ($2^{-\Delta\Delta Ct}$) method for calculating relative expression (Livak and Schmittgen, 2001; Pfaffl, 2001; Kubista et al., 2006). The values corresponding to salinity 25 ‰ were used as calibrator for the ΔΔCt calculations according to methods described by Livak and Schmittgen (2001).

To validate the EF1α as a reference gene we tested the effect of salinity on the 2^{-Ct} values, following Hauton et al. (2005). We performed two-way factorial ANOVA using salinity (5 ‰, 25 ‰ and 40 ‰) and developmental stages (ZI, ZIII, M and JI) as fixed factors (Zar, 1998).

The effect of salinity on Na^+-K^+-ATPase expression was evaluated using $2^{-\Delta Ct}$ values ($\Delta Ct = Ct_{NaK} - Ct_{EF1\alpha}$) with a two-way factorial ANOVA, with the fixed factors mentioned above.

Before performing the ANOVA analyses, we checked for homogeneity of variance (Cochran test) and normality (plot of residuals). The critical level (α) was 0.05. Comparisons between different factors, after finding significant differences in the ANOVAs, were performed with the Student-Newman-Keuls test (SNK).

RESULTS

Sequence of Na^+-K^+-ATPase α-subunit and Elongation Factor 1α genes

The partial sequence encoding the EF1α (Fig. 2) and the Na^+-K^+-ATPase α-subunit (Fig. 3) were translated to amino acid sequences that yielded high-scoring

BLASTX matches to known sequences in the GenBank database. The translated amino acid sequence encoding the Na^+-K^+-ATPase α-subunit of *Armases miersii* (this study, NCBI Accession n°: DQ983378) showed a very high percentage of homology with various crab species, e.g. *Pachygrapsus marmoratus* (NCBI Accession n°: AAK54644): 98%, *Callinectes sapidus* (AAG47843): 97%, and *Carcinus maenas* (AAK62046): 95%. In addition, the homology with different groups was also high, reaching 84-85% with the insects *Drosophila melanogaster* (AAC05260) and *Aedes aegypti* (EAT35802), and 75-76% with the vertebrates *Gallus gallus* (AAA48982) and *Homo sapiens* (AAH09394). The alignment of the amino acid sequence of *Armases miersii* with sequences of these species revealed conserved regions, likely to be essential for protein function. In fact, some of the transmembrane domains and the likely ATP-binding site could be identified (Fig. 4).

The translated amino acid sequence of EF1α (this study, NCBI Accession n° DQ983379) presented a high homology with the crustaceans *Libinia marginata* (AAC03149), *Heteromysis formosa* (AAD21849) and *Armadillidium vulgare* (AAC03145; 96%, 89% and 88%, respectively), and the fungus *Physoderma maydis* (ABB90944; 93%), among others.

The species-specific primers were designed based on the obtained sequences (Figs 2, 3). Both pairs of species-specific primers yielded products of ca. 100-150 bp, suitable for Q-PCR.

```
  1  CTCCAAGAACGACCCCGCCAGGGTAAAGCTGGAGACTTCACAGCCCAGGTGATCGTGCTG  60
  1   L  Q  E  R  P  R  Q  G  K  A  G  D  F  T  A  Q  V  I  V  L   20

 61  AACCACCCCGGCCAGATCCAGGCTGGCTACTCGCCTGTGCTGGATTGCCACACCGCTCAC 120
 21   N  H  P  G  Q  I  Q  A  G  Y  S  P  V  L  D  C  H  T  A  H   40

121  ATTGCTTGCAAGTTCGCTGAGCTCATCCAGAAGATTGACAGGCGTACTGGTAAGGAGATC 180
 41   I  A  C  K  F  A  E  L  I  Q  K  I  D  R  R  T  G  K  E  I   60

181  GAGGCCAACCCCAAGCACATCAAGTCAGGCGACTCCTGGCATCGTAAAA           229
 61   E  A  N  P  K  H  I  K  S  G  D  S  W  H  R  K             76
```

Fig. 2. *Armases miersii*. Partial nucleotide sequence of Elongation factor 1α (in bold). The corresponding predicted amino acid sequence (NCBI Accession n°: DQ983379) is also shown. Locations of species-specific primers used in quantitative PCR are indicated in dark green.

```
   1 ATTGGCATTGTACTCACTGCTGTCGTGATCATCACAGGCATCTTCTCATACTACCAAGAG   60
   1  I  G  I  V  L  T  A  V  V  I  I  T  G  I  F  S  Y  Y  Q  E   20

  61 AGCAAAAGTTCACGCATCATGGAGTCCTTCAAAAACCTTGTTCCTCAGTATGCCATCGTC  120
  21  S  K  S  S  R  I  M  E  S  F  K  N  L  V  P  Q  Y  A  I  V   40

 121 ATCCGTGAAGGTGAGAAACAAAACGTCCAGGCTGAGGAGCTCTGCATAGGTGACATTATT  180
  41  I  R  E  G  E  K  Q  N  V  Q  A  E  E  L  C  I  G  D  I  I   60

 181 GACGTCAAGTTTGGTGATCGTATCCCAGCTGACGTGCGCGTCATTGAGGCCCGAGGCTTC  240
  61  D  V  K  F  G  D  R  I  P  A  D  V  R  V  I  E  A  R  G  F   80

 241 AAGGTTGACAATTCTTCTCTCACTGGTGAATCTGAGCCTCAGAGCCGCTCTCCAGAATTC  300
  81  K  V  D  N  S  S  L  T  G  E  S  E  P  Q  S  R  S  P  E  F  100

 301 ACCTCAGAGAACCCTCTTGAGACGAAGAATCTCGCCTTCTTCTCTACCAATGCTGTGGAG  360
 101  T  S  E  N  P  L  E  T  K  N  L  A  F  F  S  T  N  A  V  E  120

 361 GGTACTGCAAAGGGTATTGTAATCAACATTGGTGACAACACTGTGATGGGCCGCATTGCT  420
 121  G  T  A  K  G  I  V  I  N  I  G  D  N  T  V  M  G  R  I  A  140

 421 GGTTTGGCTTCTGGCTTGGAGACTGGAGAGACCCCCATTGCTAAGGAAATCAGTCACTTC  480
 141  G  L  A  S  G  L  E  T  G  E  T  P  I  A  K  E  I  S  H  F  160

 481 ATTCACATCATCACCGGTGTGGCAGTCTTCCTGGGTGTTACCTTCTTCGTCATTGCCTTC  540
 161  I  H  I  I  T  G  V  A  V  F  L  G  V  T  F  F  V  I  A  F  180

 541 ATCTTGGGCTACCACTGGCTGGATGCTGTTGTGTTCCTCATCGGTATCATTGTGGCCAAT  600
 181  I  L  G  Y  H  W  L  D  A  V  V  F  L  I  G  I  I  V  A  N  200

 601 GTGCCGGAAGGTCTGCTGGCTACTGTGACTGTGTGTCTCACCCTCACTGCCAAGCGTATG  660
 201  V  P  E  G  L  L  A  T  V  T  V  C  L  T  L  T  A  K  R  M  220

 661 GCTGCCAAGAACTGCCTGGTCAAGAACTTGGAAGCTGTGGAGACCCTGGGTTCGACCTCG  720
 221  A  A  K  N  C  L  V  K  N  L  E  A  V  E  T  L  G  S  T  S  240

 721 ACCATCTGCTCCGACAAGACTGGCACCCTCACCCAGAACCGTATGACCGTTGCCCACATG  780
 241  T  I  C  S  D  K  T  G  T  L  T  Q  N  R  M  T  V  A  H  M  260

 781 TGGTTTGACAATACCATCATCGAGGCCGACACCTCCGAGGACCAGTCTGGCTGCCAGTAC  840
 261  W  F  D  N  T  I  I  E  A  D  T  S  E  D  Q  S  G  C  Q  Y  280

 841 GACAAGAGCTCTGAGGGGTGGAAGACTCTCTCCAGGATCGCTGCTCTCTGCAACCGTGCT  900
 281  D  K  S  S  E  G  W  K  T  L  S  R  I  A  A  L  C  N  R  A  300

 901 GAGTTTAAGACTGGCCAGGAGGACGTCCCCATCCTGAAGCGAGAGGTGAACGGTGATGCC  960
 301  E  F  K  T  G  Q  E  D  V  P  I  L  K  R  E  V  N  G  D  A  320

 961 TCTGAGGCAGCTCTCCTGAAGTGCGTGGAACTGGCTGTCGGAGACGTCAGGGGCTGGCGC 1020
 321  S  E  A  A  L  L  K  C  V  E  L  A  V  G  D  V  R  G  W  R  340

1021 TCGCGCAATAAGAAGGTTTGTGAGATCCCCTTCAACTCCACCAACAAGTACCAAGTGTCC 1080
 341  S  R  N  K  K  V  C  E  I  P  F  N  S  T  N  K  Y  Q  V  S  360

1081 ATCCACGAGACCCAGGACAAGAACGACCCTCGTTACCTCCTTGTGATGAAGGGTGCCCCT 1140
 361  I  H  E  T  Q  D  K  N  D  P  R  Y  L  L  V  M  K  G  A  P  380

1141 GAGAGAATCCTTGAGCGGTGCTCTACCATCTTCATGAATGGCGAGGAGAAGGCCCTGGAC 1200
 381  E  R  I  L  E  R  C  S  T  I  F  M  N  G  E  E  K  A  L  D  400

1201 GAGGAGATGAAGGAAGCCTTCAACAATGCCTACCTGGAGCTCGGAGGTCTTGGAGAGCGT 1260
 401  E  E  M  K  E  A  F  N  N  A  Y  L  E  L  G  G  L  G  E  R  420

1261 GTGCTGGGCTTCTGCGACTACATGCTTCCTTCAGACAAGTATCCCCTAGGTTACCCCTTT 1320
 421  V  L  G  F  C  D  Y  M  L  P  S  D  K  Y  P  L  G  Y  P  F  440

1321 GATGCCGATTCCGTCAACTTCCCCGTACATGGTCTCAGGTTTGTGGGACTCATGTCCATG 1380
 441  D  A  D  S  V  N  F  P  V  H  G  L  R  F  V  G  L  M  S  M  460

1381 ATTGACCCTCCCCGCGCTGCTGTGCCTGATGCCGGGCCAAGTGCCGCTT           1429
 461  I  D  P  P  R  A  A  V  P  D  A  G  P  S  A  A             476
```

Fig. 3. *Armases miersii*. Consensus partial nucleotide sequence of Na^+-K^+-ATPase α-subunit (in bold). The corresponding predicted amino acid sequence (NCBI Accession n°: DQ983378) is also shown. Locations of species-specific primers used in quantitative PCR are indicated in dark green.

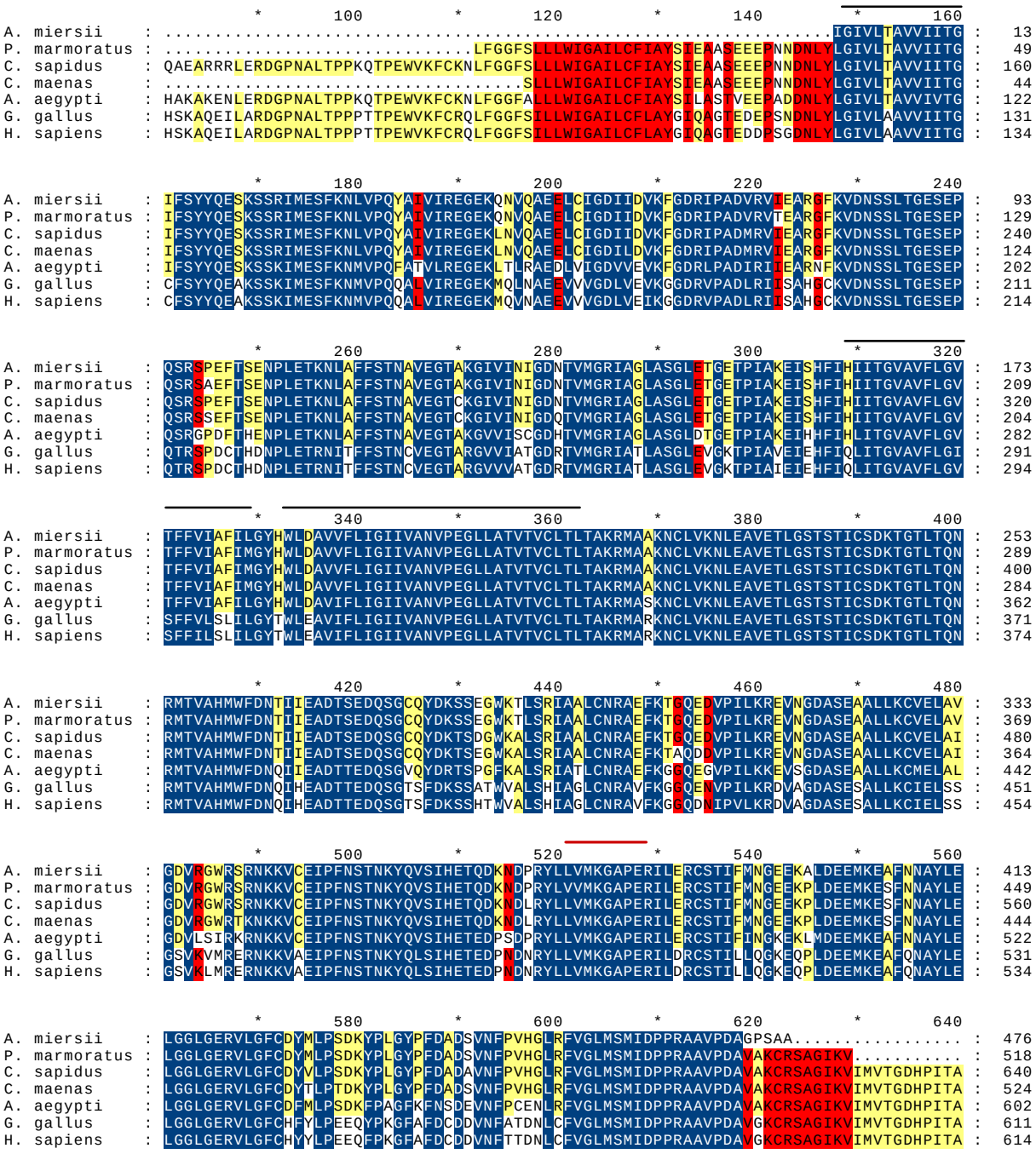

Fig. 4. *Armases miersii*. Multiple alignment of the predicted amino acid sequence of the Na^+-K^+-ATPase α-subunit with examples of other crustaceans *Pachygrapsus marmoratus* (AAK54644), *Callinectes sapidus* (AAG47843) and *Carcinus maenas* (AAK62046), insect *Aedes aegypti* (EAT35802), and vertebrates *Gallus gallus* (AAA48982) and *Homo sapiens* (AAH09394). Alignment was performed with ClustalW and GeneDoc software. Blue shading: 100% agreement, red shading: 80% and yellow shading: 60%. The likely ATP-binding site is shown with a red line; putative transmembrane domains are pointed out by black lines (after Horisberger et al., 1991; Towle et al., 2001).

Determination of real-time PCR efficiency

The amplification efficiency (E) of each gene was calculated as E = 10(-1/b). The slope values (b) were obtained from the regression line Ct = a + b (log_{10} dilution

factor) generated by plotting the Ct values against the $\log_{10}$ of the dilution factors (Fig. 5). The relationship between the Ct values and $\log_{10}$ dilution factor was linear for the dilution range used ($p>0.05$). In addition, the amplification efficiencies of NaK and EF1α were not significantly different ($F_{1,19} = 1.13$, $p>>0.05$), which allowed the analysis of relative changes using the "comparative Ct method" ($2^{-\Delta\Delta Ct}$) according to Livak and Schmittgen (2001).

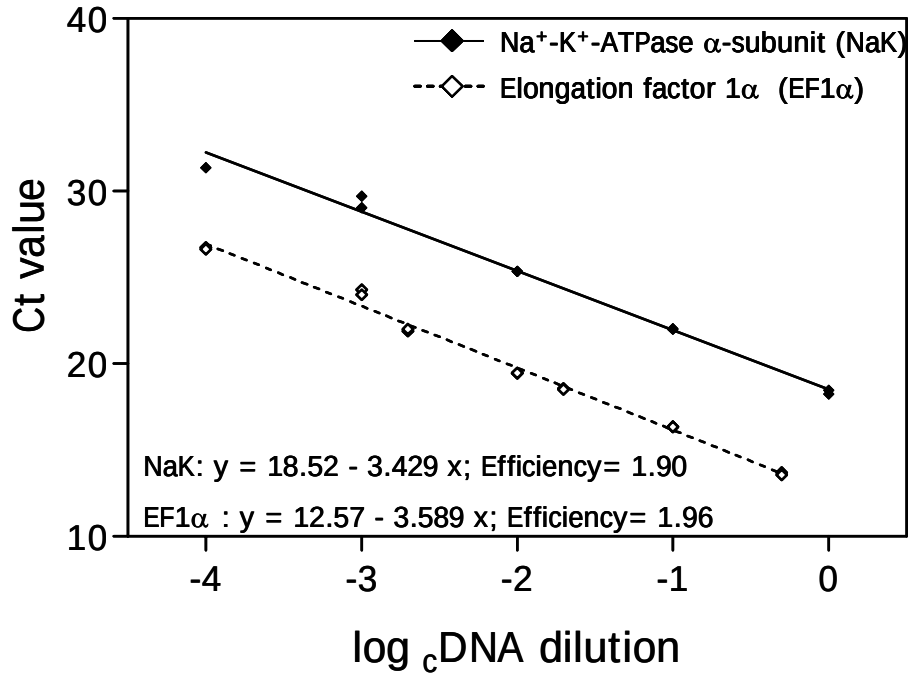

Fig. 5. *Armases miersii*. Comparison of amplification efficiencies of the Na^+-K^+-ATPase α-subunit and the elongation factor 1α expression.

Validation of the Elongation Factor 1α as reference gene

The levels of transcripts encoding EF1α in the Zoea I and Zoea III stages were not affected by salinity (Fig. 6 shows the results of the posthoc comparison after the ANOVA was significant for salinity, stage and their interaction, Table 3). By contrast, the exposure to 40 ‰ slightly affected the expression of this gene in the Megalopa and Juvenile I. Since this effect was clearly identifiable (values at 40 ‰ were lower than at 5 ‰ and 25 ‰), we nevertheless used the EF1α as reference gene. We were especially careful with the influence that salinity might have on the ΔCt for the Megalopa and Juvenile I exposed to 40 ‰.

Table 3. *Armases miersii*. Summary of ANOVA analyses for 2^{-Ct} (EF1α) values. Symbols: MS: mean squares, Df: degrees of freedom; significant effects are indicated in bold**.**

	$Ct_{EF1\alpha}$			
	MS	**Df**	**F**	**p**
Stage (St)	3,50E-09	3	9.992	**0.000092**
Salinity (Sal)	2,97E-09	2	8.481	**0.001154**
St*Sal	1,37E-09	6	3.917	**0.005008**
Error	3,51E-10	31		

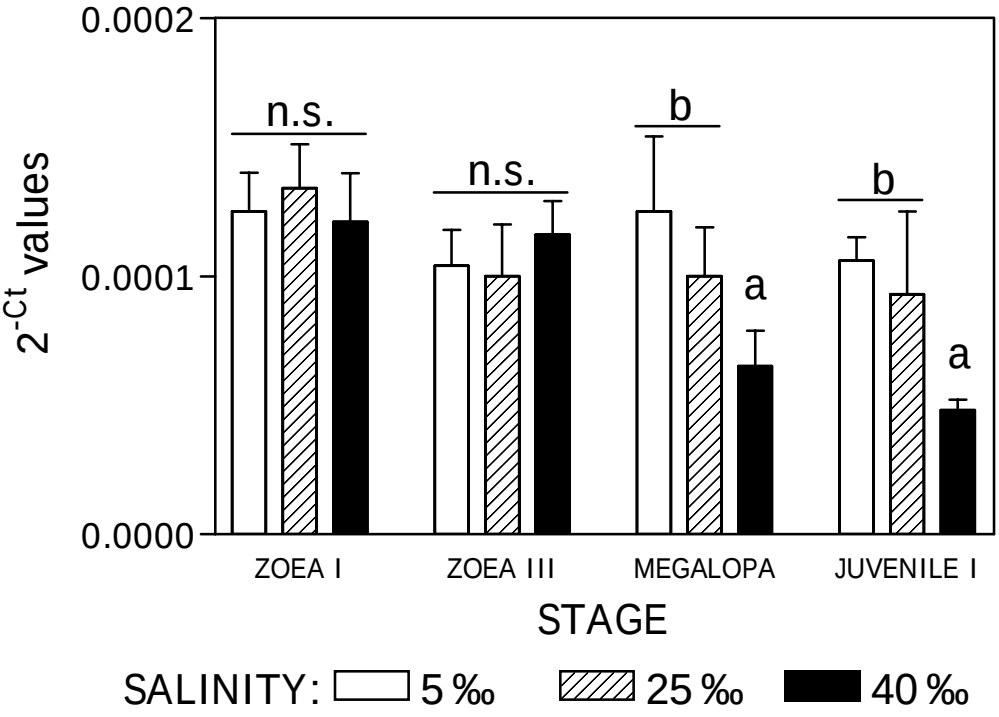

Fig. 6. *Armases miersii*. Effect of salinity and stage on the expression of Elongation Factor 1α (showed as 2^{-Ct} values). Different letters: significant differences between treatments for each stage; n.s.: no significant differences. Error bars are standard deviations.

Relative quantification of Na^+-K^+-ATPase α-subunit transcripts

The quantity of mRNA encoding Na^+-K^+-ATPase was affected by salinity and stage (Table 4). In particular, the exposure to 5 ‰ caused a significant increase in the $2^{-\Delta Ct}$ values. Thus, when stages were considered together, the expression of the gene encoding Na^+-K^+-ATPase α-subunit was higher after exposure to a low salinity (Fig. 7A). By contrast, the level of mRNA encoding the α-subunit remained constant for the optimal (25 ‰) and enhanced (40 ‰) salinities. The effect of exposure to 5 ‰ lead to an increase by 43% ($2^{-\Delta\Delta Ct}$ values) in the expression of the Na^+-K^+-

ATPase α-subunit gene in the Zoea I and Juvenile I, and by 9% and 13% in the Zoea III and Megalopa, respectively (Table 5). On the other hand, an exposure to 40 ‰ caused an increase by 32% in the Zoea I, and a decrease by 5%, 7% and 21% in the Zoea III, Megalopa and Juvenile I, respectively. All these changes, however, were statistically not significantly different (Table 5).

When combining the $2^{-\Delta Ct}$ data of the three salinities, the first zoeal instar showed the lowest expression of the α-subunit (Fig. 7B). The Zoea III exhibited a significant higher level of mRNA encoding Na^+-K^+-ATPase α-subunit. The highest levels were measured in the Megalopa and Juvenile I (Fig. 7B).

Taking into consideration all stages separately (Fig. 7C), no important effect of salinity was noted in the Zoea I. In the Zoea III, Megalopa, and more particularly in the Juvenile I, by contrast, a slight tendency to increased levels of mRNA encoding Na^+-K^+-ATPase α-subunit was observed at low salinity (Fig. 7C). The reference gene (EF1α) mRNA level was significantly lower for the Megalopa and Juvenile I exposed to 40 ‰. This might have caused that a possibly existing difference between 25 ‰ and 40 ‰ remained undetected.

Table 4. *Armases miersii*. Summary of ANOVA analyses for $2^{-\Delta Ct}$ (NaK-EF1α) values. Symbols as in Table 3.

	ΔCt (NaK-EF1α)			
	MS	**Df**	**F**	**p**
Stage (St)	0.000259	3	30.41	**2,31E-09**
Salinity (Sal)	0.000046	2	5.36	**0.010027**
St*Sal	0.000011	6	1.27	0.297457
Error	0.000009	31		

Table 5. *Armases miersii*. Modulation of Na^+-K^+-ATPase α-subunit expression after exposure to 5 ‰, 25 ‰ and 40 ‰. Significant increase compared to 25 ‰ (1.00) is indicated in bold.

	$2^{-\Delta\Delta Ct}$		
	5 ‰	25 ‰	40 ‰
Zoea I	**1.43**	1.00	1.32
Zoea III	**1.09**	1.00	0.95
Megalopa	**1.13**	1.00	0.93
Juvenile I	**1.43**	1.00	0.79

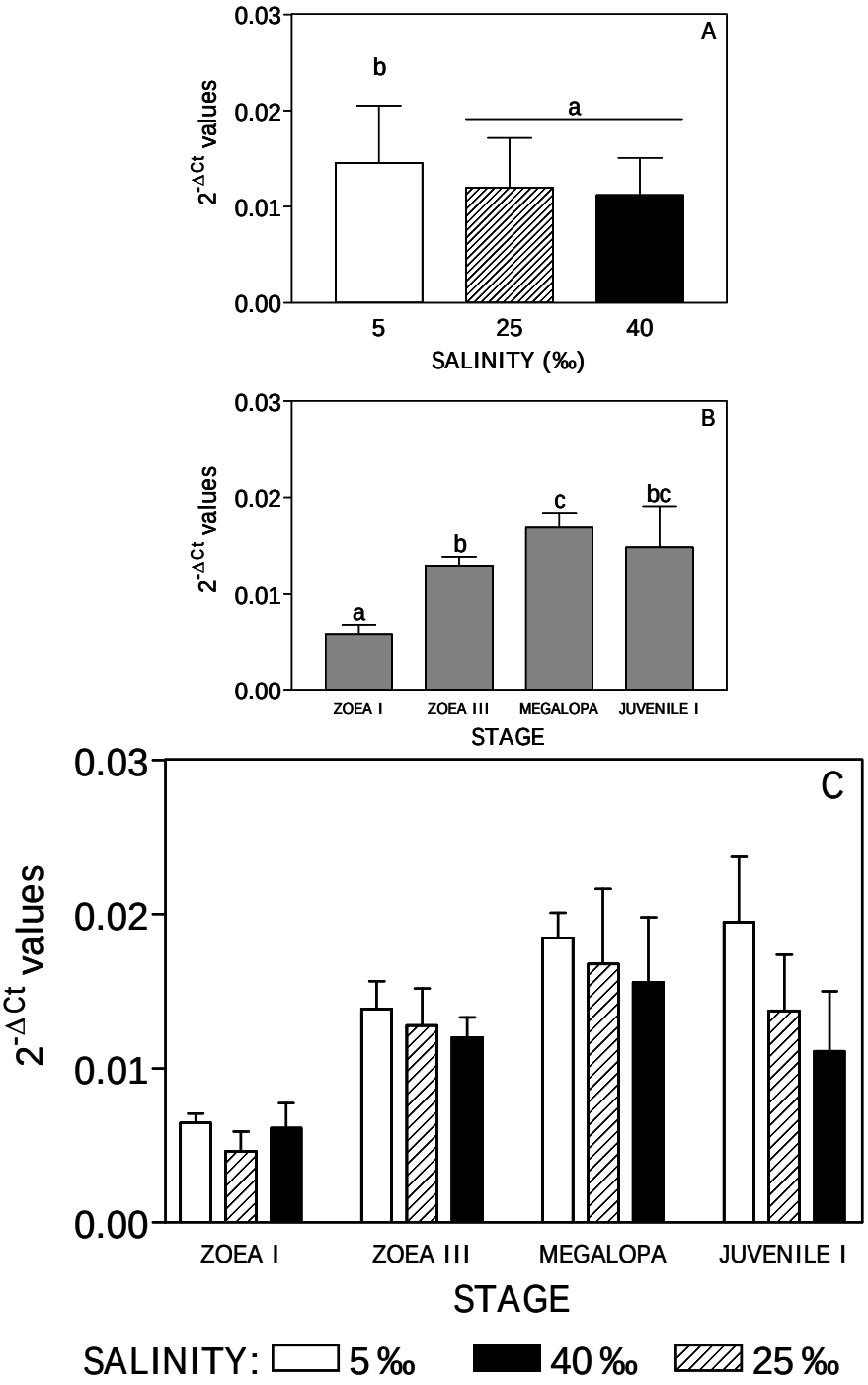

Fig. 7. *Armases miersii*. Expression of Na^+-K^+-ATPase α-subunit (showed as $2^{-\Delta Ct}$ values). A: effect of salinity (pooled stages plotted). B: effect of stage (pooled salinities plotted). C: effect of salinity at each stage (stages and salinity plotted separately). Different letters: significant differences between treatments. Error bars are standard deviations.

DISCUSSION

We provide a partial sequence for the Na^+-K^+-ATPase α-subunit isolated from larvae and juveniles of an euryhaline crab, *Armases miersii*. This subunit is

considered as highly conserved (Skou and Esmann, 1992; Lucu and Towle, 2003). This was confirmed in our study, as the partial sequence encoding the Na^+-K^+-ATPase α-subunit of *A. miersii* showed high similarity with sequences from other crustaceans, invertebrates and even vertebrates. The alignment of the amino acid sequence predicted for *A. miersii* with sequences from other species permitted to identify highly conserved regions such as transmembrane domains and the likely ATP binding site (Horisberger et al., 1991; Towle et al., 2001).

To quantify and normalize the level of mRNA expression of a target gene, it is necessary to determine the mRNA expression of an endogenous control or house-keeping gene exposed to the same experimental conditions as the target gene, i.e. a gene constitutively transcribed and supposedly unregulated within the experimental context (or at least regulated differently than the target gene). As the functions of the most common house-keeping genes, e.g. GAPDH, ribosomal proteins, actin (Sturzenbaum and Kille, 2001), are related to cytoskeleton structure and energy metabolism, it appears difficult to identify a gene whose expression is absolutely stable during larval and post-larval development. In the present study, the Elongation Factor 1α (EF1α) was considered as the internal control. Its expression appeared relatively stable during *A. miersii* development at all studied salinities and was only affected in Megalopa and Juvenile I at high salinity. EF1α has also recently been validated as a suitable invariant internal control in different studies conducted on aquatic organisms (Frost and Nielsen, 2003; Jorgensen et al., 2006; Varsamos et al., 2006).

In this study, we investigated the effect of salinity on the expression of the gene encoding Na^+-K^+-ATPase α-subunit in the osmoregulating early life-history stages of *A. miersii*. The role of the ion-transport enzyme Na^+-K^+-ATPase in hyper-osmoregulation has extensively been studied in adult crustaceans, where its activity increases after an exposure to low salinities, e.g. in *Callinectes sapidus* (Towle et al., 1976), *Carcinus maenas* (Siebers et al., 1982, 1983), *Uca minax* (Wanson et al., 1984), *Homarus gammarus* (Flik and Haond, 2000), *Chasmagnathus granulata* (Castilho et al., 2001), *Eriocheir sinensis* (Torres et al., submitted). This increase was generally documented for the posterior gills, which show typical features of ion-transporting tissues with abundant ionocytes. However, classical biochemical activity measurements do not elucidate whether the observed increase is due to a regulation

of pre-existing Na^+-K^+-ATPase molecules (e.g. post-translational processes, membrane trafficking or cell signalling), or the result of enhanced gene transcription and/or translation, due to *de novo* mRNA synthesis. In gills of the euryhaline adult crabs *Pachygrapsus marmoratus* and *Chasmagnathus granulata*, a strong increase in the expression of mRNA encoding Na^+-K^+-ATPase α-subunit was shown after acclimation to low salinities (Lucu and Towle, 2003; Spanings-Pierrot and Towle, 2003; Luquet et al., 2005). Otherwise, only scarce information is available for the molecular basis of this activity increase.

To our knowledge, there is scarce information available on the effect of salinity on the activity of Na^+-K^+-ATPase in early life-history stages of crustaceans. In the lobster *Homarus gammarus*, a transfer to low salinity did not cause an increase of the enzyme activity in larvae, but was followed by a sharp increase in postlarvae (Thuet et al., 1988). The enzyme activity tended to increase during the post-embryonic development of *LitoPenaeus japonicus*, particularly at the metamorphic transition (Bouaricha et al., 1991). Due to their small size, the measurement of enzyme activity with classical biochemical methods requires high amounts of larvae. The Q-PCR technique, however, needs fewer animals and may thus be the best tool when working with small organisms.

In our study, we focused on the possible involvement of *de novo* synthesis of mRNA encoding the Na^+-K^+-ATPase α-subunit after an exposure of larval and juvenile crabs to different salinities. The enhanced level of mRNA of this subunit after exposure to 5 ‰ indicates that hyper-osmoregulation is directly related to Na^+-K^+-ATPase activity. Furthermore, the increase of mRNA in successive stages is consistent with an increase in the hyper-osmoregulatory capacity during ontogeny (Charmantier et al., 1998), which may thus be attained by increasing levels of mRNA encoding the Na^+-K^+-ATPase. This suggests a developmentally programmed increase in the expression of the enzyme, with little modulation by salinity in the earlier stages and an increasing regulation by salinity in later life-history stages. Whether these changes are linked to the development of osmoregulatory organs, especially the gills, remains to be explored.

The response of Na^+-K^+-ATPase to increased salinity in hypo-osmoregulators varies among species. Some do not show changes in enzyme activity when exposed to concentrated media (e.g. *Carcinus maenas*: Hake and Teller, 1983; *Uca pugnax*:

Holliday, 1985; *Sesarma reticulatum*: D'Orazio and Holliday, 1985), while others show an increment (e.g. *Uca tangeri*: Drews, 1983; *Artemia salina*: Holliday et al., 1990) or a decrease (posterior gills of *Chasmagnathus granulata*: Castilho et al., 2001; *Eriocheir sinensis*: Torres et al., submitted). However, in *C. granulata* there is also a decreasing activity in the anterior gills, suggesting that the hypo-osmoregulatory capacity is, in this case, not strong enough to prevent physiological stress. In *Pachygrapsus marmoratus* acclimated to concentrated seawater, it appears that mRNA expression of Na^+-K^+-ATPase α-subunit is strongly enhanced in the biggest pair of posterior gills which may be involved in Na^+ secretion maintaining hemolymph osmolality lower than the osmolality of the external medium (Spanings-Pierrot and Towle, 2003). Further investigations are required to explain how the Na^+ is excreted. In *Armases miersii*, we did not find differences in the level of Na^+-K^+-ATPase transcripts after exposure to 25‰ or 40‰. In the zoeal stages, this should be due to lack of hypo-osmoregulatory capacity. In Megalopa and Juvenile I, possible variation in the mRNA expression may have remained undetected due to variation of the reference gene EF1α. On the one hand, a decrease in the expression of mRNA encoding Na^+-K^+-ATPase α-subunit at enhanced salinities might reduce the uptake of Na^+. On the other hand, the expression of mRNA encoding EF1α was also affected, suggesting that a slight hypo-osmoregulatory capacity was present, but this might not be strong enough to compensate osmotic stress and physiological damage. Alternatively, no variation may be found if we had used another reference gene. In this case, no modification of *de novo* synthesis of the enzyme would be involved in hypo-osmoregulation in the Megalopa and Juvenile I. The mechanism of regulation of the Na^+-K^+-ATPase in hypo-osmoregulation in *Armases miersii* remains to be elucidated.

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VII. GENERAL DISCUSSION

VII.1. Effects of salinity variation on population processes in decapod crustaceans

The topic of how the planktonic larval stages of coastal benthic invertebrates recruit to the parental habitats is subject of intensive research (Shanks, 1995; Pineda, 2000; Queiroga et al., 2006), because variations in settlement rates may cause variations in benthic population abundance and community structure (Connell, 1985, Connolly and Roughgarden 1998).

For megalopae of estuarine and semi-terrestrial crabs, the process of migration occurs through interactions between passive transport by wind-driven or tidal currents and active larval behaviour in response to physical and chemical cues (Queiroga and Blanton, 2005). Such process may be summarized in three steps (Fig. 1). As a first step, megalopae of coastal crabs are transported towards the coast by wind-driven currents. As a second step, when the larvae are near the coast or within estuaries, they are transported by selective tidal currents or other tidally-mediated flows to nursery habitats. The last step involves larval behaviour in response to chemical (e.g. maternal) cues and hydro-dynamical conditions. In this context, salinity is considered as a cue that may signal the proximity of the parental habitat type. Larvae of estuarine crabs are known to swim to the bottom or to avoid low-salinity surface layers when experiencing low salinities. This is interpreted as a behavioural response to facilitate upstream migration in estuaries (reviewed in Queiroga and Blanton, 2005).

In the theoretical framework of larval migration, salinity gradients are therefore seen as a cue, accelerating the process of migration by altering the larval behaviour. Here, I introduce salinity not as a cue, but as a stress factor that might negatively affect the process of migration. In the Megalopa of *Armases roberti*, for instance, the re-immigration into the parental habitat can be constrained by detrimental effects of hypo-osmotic stress on physiological and developmental processes. The larvae were in laboratory experiments not able to survive a direct transfer to salinities lower than 3‰, and an exposure to 5‰ caused high mortality rates (PUBLICATION 1: Torres

et al., 2006). In addition, a direct exposure to low salinities delayed metamorphosis. A gradual acclimation (i.e. stepwise exposure to decreasing salinities), however, allowed for an increasing salinity tolerance. As a result, these larvae showed higher survival rates, and even metamorphosed to the first juvenile in freshwater (Torres et al., 2006). During upstream migration, the Megalopae encounter, on one side, decreasing salinities, on the other side, increasing concentrations of adult odours. While decreasing salinities cause a delay of metamorphosis, adult odours trigger metamorphosis (Publication 2: Anger et al., in press).

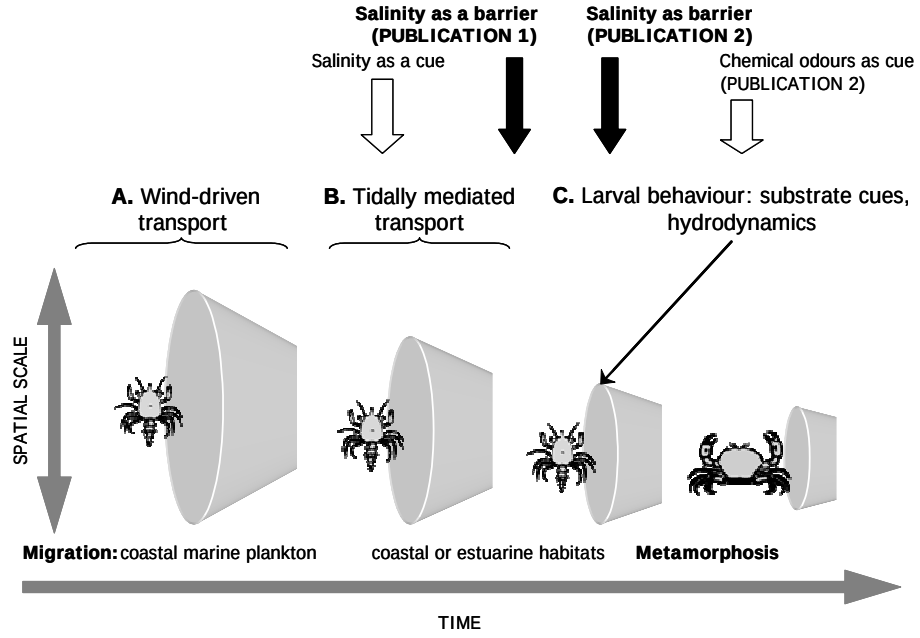

Fig. 1. Transport and behavioural processes affecting the settlement of crab larvae (adapted from Pineda 2000). **A.** Larvae are transported from several kms from the coast to the nearshore habitat by wind-driven currents; **B.** near the shore (within 1 km from the shore or within estuaries), tidally mediated transport occurs; here, salinity may act as a cue; **C.** at scales of a few meters, settlement is determined by larval behaviour in response to habitat cues and by micro-hydrodynamical processes. Bold arrows highlight the contrasting roles of salinity as a cue (white) or as a constraint (black) during the process of migration.

CONCLUSIONS:

- The upstream migration of the Megalopa of *Armases roberti* is constrained by decreasing salinity, but stimulated by adult odours.
- Gradual acclimation to reduced salinity decreases the inhibiting effects of hypo-osmotic stress, so that the Megalopa can successfully metamorphose even in freshwater.

VII.2. Effects of osmotic stress on physiological processes in larvae

Variations in salinity affect organisms through changes in the internal osmotic conditions (including both the intra- and the extracellular media). These changes may influence metabolic functions, and eventually the performance of larvae. The level of understanding of how variations in salinity affect internal osmotic conditions, metabolism and performance is variable (Fig. 2).

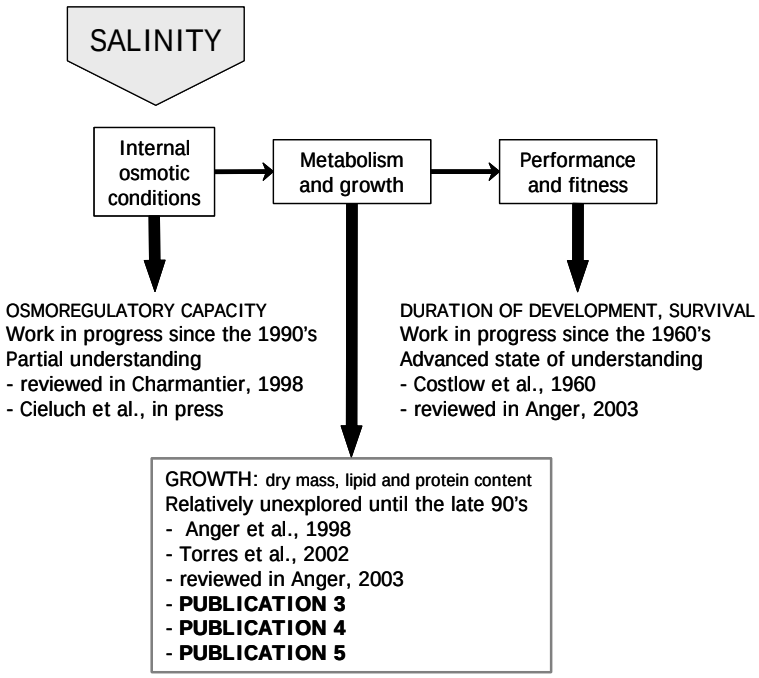

Fig. 2. A diagram illustrating connections among internal osmotic conditions, metabolism and growth, performance and fitness, and the level of knowledge about the effects of salinity on each level. The lower box highlights the contribution of the present thesis on the effects of salinity variations on larval growth in decapod crustaceans

All aquatic animals, including decapod crustaceans, exposed to variations in salinity must be able to maintain or restore their cellular volume and solute composition within a certain range. As a consequence, their cells reach a steady-state, which is in disequilibrium with the surrounding medium (i.e. the hemolymph). To ensure the balance of water and solutes, crustaceans have evolved diverse mechanisms, including osmoregulation (reviewed in Péqueux, 1995; Charmantier, 1998; Charmantier and Charmantier-Daures, 2001). The majority of crustaceans are marine osmoconformers, while estuarine and coastal species may be classified in

three groups of osmoregulators: weak hyper-regulators, strong hyper-regulators and hyper-hypo-regulators.

In larval crustaceans, the ontogenetic patterns of osmoregulation have been intensively studied since the 1990's (reviewed in Charmantier, 1998; Cieluch et al., in press). Species with larval export strategies occupy different environments during different phases of their life cycle, which is commonly reflected in the ontogeny of osmoregulation. In species retaining their larvae in the parental habitat, all life-history stages show the adult type of osmoregulation: fully marine species have osmoconforming larvae and adults, while estuarine and freshwater-inhabiting species, which do not reveal an export of their larvae to coastal marine waters, are throughout their life cycles capable of at least some osmoregulation (PUBLICATION 3). In exporting species, the capability for osmoregulation can vary greatly among successive life-history stages. There is already a partial understanding of ontogenetic changes in the osmoregulatory capacity (Fig. 1, GENERAL INTRODUCTION; Charmantier, 1998).

Studies of salinity effects on rates of larval survival and development started in the 1960's (e.g. Costlow et al., 1960, 1962, 1966) and have continued to the present days (e.g. Anger, 1991; Giménez and Anger, 2003). These studies defined the degree of euryhalinity in various species and ontogenetic stages, relationships with life-history strategies, and acclimation effects. At this level, there is a considerable body of information and understanding of salinity effects (Fig. 2).

Much less information and understanding exist on the influence of variations in salinity on metabolism and growth (Anger, 2003). This is the level addressed in the physiological section of my thesis (Fig. 2). When the stenohaline zoeae of *Cancer irroratus* were exposed to low salinities, they showed reduced rates of feeding and respiration, and low energy contents (Johns, 1982). The weakly hyper-osmoregulating Zoea I of *Carcinus maenas* showed also reduced respiration rates and biomass after an exposure to reduced salinities (Anger et al., 1998). It may be presumed that, in these cases, osmotic stress caused a decrease in the capacities and efficiencies of assimilation and conversion of nutritional energy into tissues (Anger, 2003). Modifications in the susceptibility of growth to variations in salinity, should be directly associated with species-specific or ontogenetic changes in the capability of osmoregulation. Torres et al. (2002) found that the Zoea-I stage of

stenohaline osmoconforming species showed higher sensitivity to low salinity than the same stage in weakly hyper-osmoregulating species.

Here, I focussed on three different aspects of the influence of variations in salinity on larval biomass. Firstly, I investigated effects of short-term exposure to reduced salinities on biomass in relation to patterns of life-history and ontogeny of osmoregulation (PUBLICATION 3). Secondly, I searched for general relationships between sensitivity to osmotic stress and the osmoregulatory capacity (OC) in decapod larvae (PUBLICATION 4). Finally, I focussed on cumulative effects of long-term exposure to reduced salinity (PUBLICATION 5).

Using three species of the genus *Armases* (PUBLICATION 3), I found a strong relationship between the habitat-type occupied by each larval instar and the sensitivity of growth to reduced salinity. All stages of *Armases miersii*, a crab that inhabits highly variable supratidal pools, showed consistently a high osmoregulatory capacity and low sensitivity to reduced salinities, i.e. only small changes in biomass. In *A. roberti*, both the downstream migrating Zoea I and the upstream migrating Megalopa showed a higher osmoregulatory capacity and weaker effects of low salinity on growth compared to the intermediate stages, which develop in more stable coastal waters. Finally, all larval stages of *A. ricordi*, which develop in coastal marine areas, showed comparably low osmoregulatory capacities, and consequently, the strongest detrimental effects of low salinity on biomass.

Using literature data from a larger number of species together with those from the experiments reported here, I explored the general relationships between growth, salinity tolerance, and osmoregulatory capacity (PUBLICATION 4). Consistent with Torres et al. (2002), growth was in euryhaline instars less sensitive to low salinity than in stenohaline larvae. Besides, the effect of reduced salinity on growth decreased as the osmoregulatory capacity increased. In stenohaline species, an exposure to reduced salinities may cause losses or a reduction of the rates of accumulation of both lipids and proteins, while more euryhaline species show generally only a slight decrease in the rate of accumulation of lipids.

As an additional issue, I explored cumulative effects of continuously reduced salinity on larval growth (PUBLICATION 5). Larval growth may be affected by the combination of conditions experienced at a given stage *and* by effects carried over from previous stages. For instance, low salinity during the Zoea-I stage can either

increase or decrease the survival or growth rate in the subsequent Zoea-II stage, due to acclimation or detrimental physiological effects, respectively. Carryover effects are common in decapod crustaceans (reviewed in Giménez, 2006). In the euryhaline crab *Chasmagnathus granulata*, both the Zoea I and the Megalopa are able to hyper-osmoregulate, while the intermediate zoeal stages are osmoconformers. Therefore, this species provided a useful model organism to test the effects of long-term exposure to reduced salinities on larval biomass. Detrimental effects of low salinity on the biomass of the Zoea II were prevented by hyper-osmoregulation in the Zoea I. However, the following zoeal stages were osmoconformers, suffering increasing cumulative effects of hypo-osmotic stress. The hyper-osmoregulating Megalopa was unable to revert these negative effects accumulated during the previous stages. The previous environmental conditions experienced by the larvae are thus important to explain patterns of larval survival and growth. This is still a relatively unexplored field for research.

CONCLUSIONS:

- Effects of hypo-osmotic stress on biomass are reduced or prevented by the capability of osmoregulation.
- Life-history stages occupying physically unpredictable habitats with variations in salinity must have an enhanced osmoregulatory capacity.
- Effects of long-term exposure to hypo-osmotic stress conditions depend on the ability of previous stages to hyper-osmoregulate.

VII.3. Biochemical responses to reduced, optimal, or enhanced salinities

Variations in salinity are directly related to changes in the concentrations of single osmotic effectors (e.g. Na^+, K^+, Cl^- among others), which may affect the functions of hormones, enzymes, and other vital molecules. In particular, the ion-transporting enzyme Na^+-K^+-ATPase employs energy, supplied by the hydrolysis of ATP molecules, to pump Na^+ ions into the hemolymph, in exchange for K^+ (exchange

ratio 3:2). The coordinated functions of the pump and the apical and basolateral transporters (Towle, 1990; Péqueux, 1995; Towle and Weihrauch, 2001) allow for maintaining the electrochemical gradients that are indispensable for the correct functioning of physiological systems. Thus, the Na^+-K^+-ATPase provides the major driving force for ion transport, and therefore plays a central role in the ionoregulation of crustaceans.

Histochemical evidence showed that the sodium pump is mainly found in the ion-transporting epithelia, i.e. in the posterior gills of adult brachyurans, or in the branchiostegites and the developing gills of their early life-history stages (reviewed in Péqueux, 1995; Cieluch et al., 2004; Cieluch et al., in press). The ion-transporting enzyme is located in the basolateral membrane of the ion-transporting cells, the ionocytes (Towle and Kays, 1986; Towle, 1990).

In adult crustaceans, the activity of Na^+-K^+-ATPase responds to variations in environmental salinity (for review see Lucu and Towle, 2003). The available data originate from experimental exposures of the animals to reduced salinities of several weeks (e.g. Li et al., 2006), while long-term acclimation effects (through one year) have not been studied, and only scarce information has been available for early life-history stages. Also, only few investigations dealt with effects of variations in salinity on the location of the enzyme, particularly in early life-history stages (Lignot et al., 1999; Lignot and Charmantier, 2001).

The involvement of Na^+-K^+-ATPase in the osmoregulation of juvenile *Eriocheir sinensis* during long-term exposure to different salinities was investigated by localising and measuring the activity of the ion pump in gill tissues. In agreement with previous investigations on adult brachyurans, such as *Carcinus maenas* (Siebers *et al*., 1982, 1983) and *Chasmagnathus granulata* (Castilho et al., 2001), juvenile *E. sinensis* showed after an exposure to reduced salinities an increment in the activity of Na^+-K^+-ATPase in the posterior gills. Immunolocalisation provided a plausible explanation for this increase: the posterior gills of crabs exposed to reduced salinities showed an enhanced number and a wide distribution of Na^+-K^+-ATPase molecules. In addition, the gills increased ca. 1.5 times in thickness compared to those exposed to the optimal salinity (25 ‰). In juvenile *E. sinensis*, the increase in enzyme activity is thus directly related to the multiplication of Na^+-K^+-ATPase molecules, along with further differentiation of the ion-transporting tissues, including a proliferation of

ionocytes. This response to long-term exposure to reduced salinities points to a strong relationship between the quantity of the ion-transport enzyme Na^+-K^+-ATPase and the ability of hyper-osmoregulation (Fig. 3).

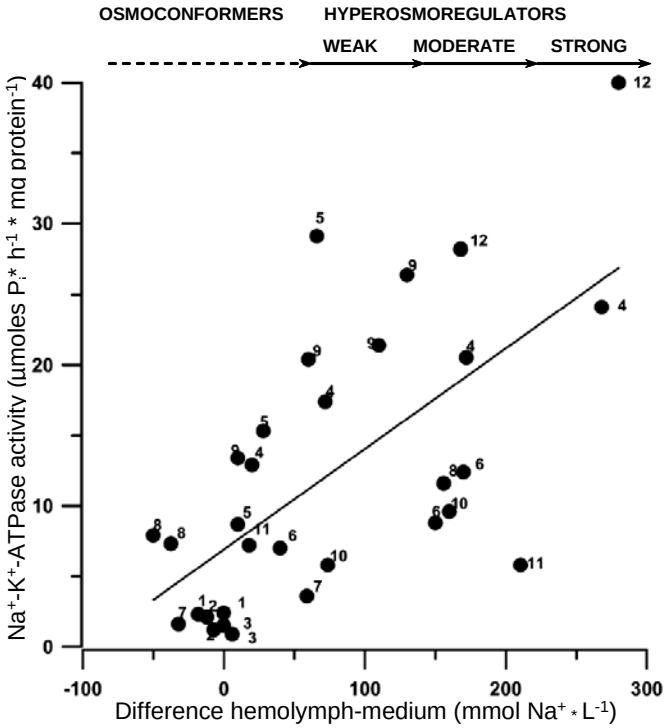

Fig. 3. Correlation between protein-specific activity Na^+-K^+-ATPase of gill homogenates and the hemolymph-to-medium sodium gradient (in mmol $Na^+ * L^{-1}$) in osmoconforming and regulating aquatic Crustacea during acclimation to dilute seawater or freshwater. Indices above circles designate species and literature sources, where enzyme activity was determined (Lucu et al., 2000). Osmoconformers: *Palinurus elephas*[1], *Maja crispata*[2], *Dromia personata*[3] (Lucu et al., 2000). Osmoregulators: *Callinectes sapidus*[4] (Neufeld et al., 1980); *Callinectes similis*[5] (Piller et al., 1995); *Carcinus maenas*[6] (Siebers et al., 1982); *Homarus gammarus*[7] (Lucu and Devescovi, 1999); *Macrobrachium olfersii*[8] (Lima et al., 1997); *Hemigrapsus nudus*[9] (Corotto and Holliday, 1996); *Uca minax*[10] (Wanson et al., 1984); *Ucides cordatus*[11] (Harris and Santos, 1993); *Uca pugnax*[12] (Holliday, 1985). Additional data for sodium gradients between seawater and hemolymph are from the following sources*: Callinectes sapidus*[4] (Colvocoresses et al., 1974); *Ucides cordatus*[11] (Martelo and Zanders, 1984), *Carcinus maenas*[12] (Zanders, 1980; Siebers et al., 1987). Star: *Eriocheir sinensis* (OC data: Cieluch et al., in press; enzymatic activity: PUBLICATION 6). After Lucu and Towle, 2003; modified.

After an exposure to enhanced salinities the activity of the enzyme in the posterior gills showed a decrease, while no effect was found in the anterior gills. The role of Na^+-K^+-ATPase in hypo-osmoregulation might be to reduce the uptake of Na^+ though a decrease in its activity. However, the response of hypo-osmoregulators to changes in salinity varies among species. For instance, *Chasmagnathus granulata* showed in the posterior gills similar changes in Na^+-K^+-ATPase activity as *E. sinensis* (Castilho et al., 2001), but it showed also a decreasing activity in the anterior gills. Some species did not show any significant changes in the enzyme activity when

exposed to concentrated media (e.g. *Carcinus maenas*: Hake and Teller, 1983; *Sesarma reticulatum*: D'Orazio and Holliday, 1985), and others showed an increase in activity (e.g. *Uca tangeri*: Drews, 1983). These inconsistent findings indicate that the mechanisms of hypo-osmoregulation in concentrated media require further comparative studies to enhance our understanding of responses to hyper-osmotic stress.

CONCLUSION:

- A proliferation of ionocytes may provide the suitable machinery to face hypo-osmotic stress, allowing for an increase in the number of Na^+-K^+-ATPase molecules, and thus, an increase of the Na^+-K^+-ATPase activity.

VII.4. Expression of mRNA coding for the transport enzyme Na^+-K^+-ATPase

Effects of variations in salinity on the activity of Na^+-K^+-ATPase have been extensively documented. However, scarce information is available about the molecular basis of the responses. Studies on the level of expression of the gene coding for the Na^+-K^+-ATPase α-subunit (i.e. the catalytic subunit) have been available only for adult crustaceans. They revealed a direct relationship between an enhanced enzyme activity after exposure to reduced salinities and an enhanced mRNA expression (Lucu and Towle, 2003; Luquet et al., 2005, Chung and Lin, 2006). Thus, the increase in Na^+-K^+-ATPase activity, which is necessary for hyper-osmoregulation is achieved by *de novo* synthesis of the enzyme. It remained unanswered whether this mechanism appeared only after metamorphosis, or if it was already present in the early life-history stages.

Classical chemical measurements of Na^+-K^+-ATPase activity require large amounts of material, which is hardly available for small larval stages. Therefore, information on this subject is scarce (Thuet et al., 1988; Bouaricha et al., 1991). New molecular techniques (Q-PCR), which require smaller amounts, have recently allowed

not only to focus on the molecular aspects but also to get more information about mechanisms involved in larval osmoregulation.

Armases miersii is, in this context, a very interesting model species, because all its life-history stages are hyper-osmoregulators. The ability of hypo-regulation appears after metamorphosis to the Megalopa stage. The Na^+-K^+-ATPase α-subunit is a highly conserved protein (Skou and Esmann, 1992). As a consequence, I found that the translated amino acid sequence of *A. miersii* yielded >95% homology with other crustaceans, and >70% with other species including *Homo sapiens* (PUBLICATION 7). Exposure to reduced salinities caused an increase in the mRNA expression coding for the Na^+-K^+-ATPase α-subunit. Thus, as in adults hyper-osmoregulation in early life-history stages is achieved in particular due to an enhanced activity of the Na^+-K^+-ATPase based on *de novo* synthesis (summarised in Fig. 4). Consistent with the ontogeny of osmoregulation, where the hyper-osmoregulatory capacity increases during development, the levels of mRNA coding for the Na^+-K^+-ATPase increased gradually in successive stages.

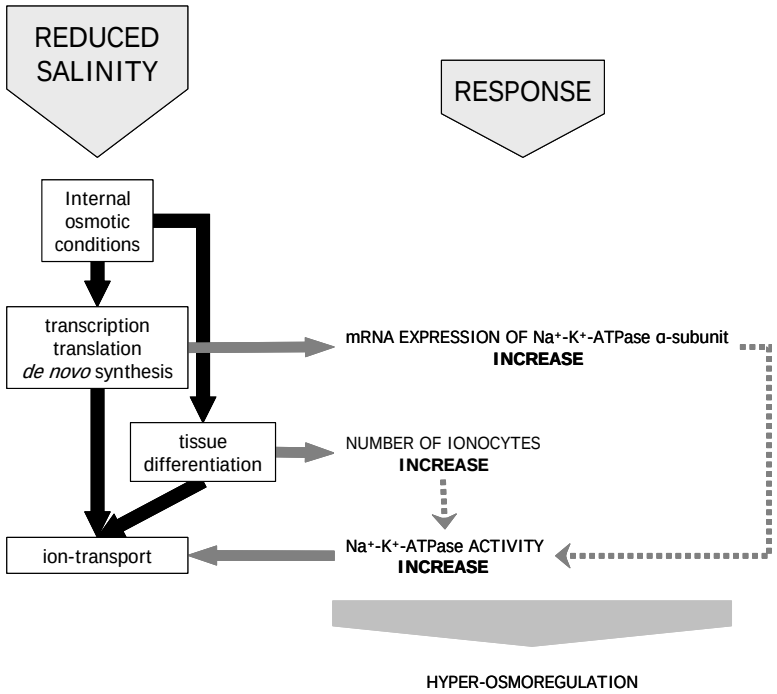

Fig. 4. Hyper-osmoregulation: tentative connections among internal osmotic conditions, molecular and cellular processes involved in the response to exposure to reduced salinity.

In contrast to the mechanisms of hyper-osmoregulation, those involved in hypo-osmoregulation are less clear. In agreement with the patterns of osmoregulation, I found that the zoeal stages I and II did not show changes in the expression levels of the α-subunit during exposure to enhanced salinities (PUBLICATION 7). In adults, inconsistent findings related to the activity of the Na^+-K^+-ATPase make it difficult to generalise (see PUBLICATION 6). In addition, the Megalopa and Juvenile I did not allow for drawing conclusions from the molecular point of view (PUBLICATION 7). It remains thus to be elucidated, which mechanisms and responses arise after exposure to enhanced salinities (summarised in Fig. 5).

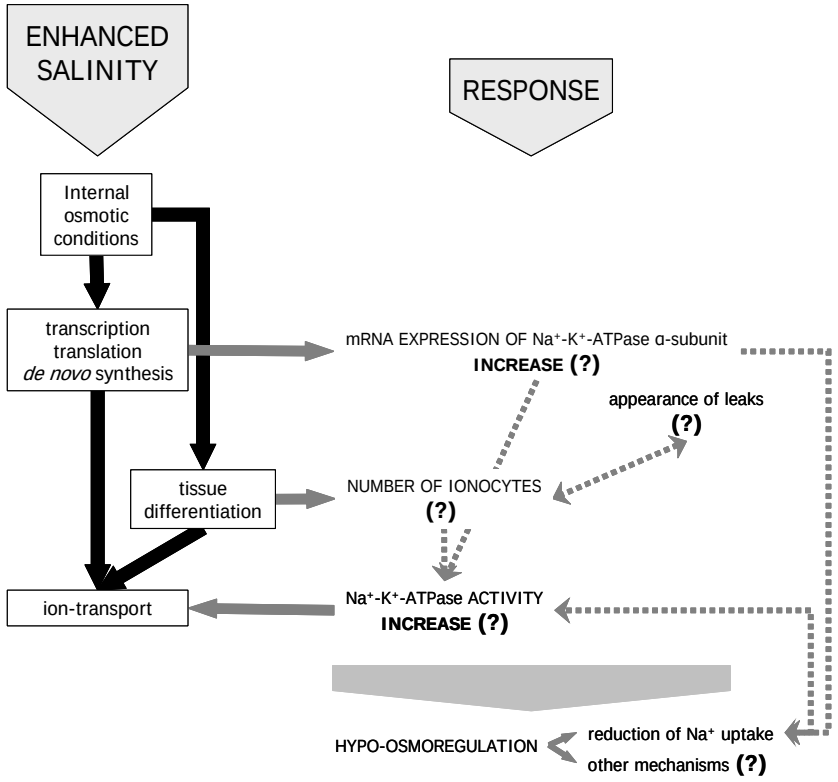

Fig. 5. Hypo-osmoregulation tentative connections among internal osmotic conditions, molecular and cellular processes involved in the response to exposure to enhanced salinity. Question marks show where further research is necessary.

CONCLUSIONS:

- The ontogenetic pattern of hyper-osmoregulation is reflected in changes in the level of mRNA expression coding for the Na^+-K^+-ATPase α-subunit.
- Hyper-osmoregulation in the early stages is achieved through *de novo* synthesis of Na^+-K^+-ATPase.

GENERAL CONCLUSIONS

Marine waters provide stable osmotic. Estuaries and coastal waters, with their characteristic variations in salinity, represent transitional habitats between limnic and marine environments. Therefore, the ability of ionoregulation was one prerequisite to invade this harsh type of environment. Among other evolutionary traits, an enhanced ion uptake through the modulation of ion-transporters allowed certain species to invade estuarine environments. In hyper-osmoregulating species, the ion-transporting tissues showed an enhanced differentiation and proliferation of ionocytes. The high degree of conservation of the amino acid sequence of Na^+-K^+-ATPase suggests that modifications in the modulation of Na^+-K^+-ATPase activity must have played a decisive role.

Successful invaders of habitats characterised by hypo-osmotic conditions must show: strong hyper-osmoregulatory capacities, lesser metabolic sensitivity to reduced salinities, strongly differentiated ion-transporting tissues (i.e. thicker gill lamellae and abundant ionocytes), a high activity of Na^+-K^+-ATPase, and an enhanced expression of mRNA encoding for the Na^+-K^+-ATPase α-subunit. In addition, the ontogenetic pattern of osmoregulation must reflect the environmental conditions that each stage must face.

VIII. PERSPECTIVES

POPULATION LEVEL

In order to better understand the organismic and physiological mechanisms underlying in the larval export strategy, more studies on the migration patterns must be conducted. For instance, each migrating stage must be investigated with respect to its reaction to variations in salinity and adult odours.

PHYSIOLOGICAL LEVEL

The metabolic response to reduced salinity should be also studied in relation to other variables such as respiration, ingestion rates.

BIOCHEMICAL LEVEL

As far as the ion-transporters are concerned, measurements of activity of Na^+-K^+-ATPase on early life-history stages are needed. In addition, histological studies on other transporters (ion-channels) may provide more information on how the ion-regulation is achieved at the cellular level.

It remains to be elucidated which role has the Na^+-K^+-ATPase in the hypo-regulation.

MOLECULAR LEVEL

The expression levels of mRNA encoding for other proteins involved in the ion-regulation might provide a better understanding of the hyper-osmoregulation at the molecular level.

In addition, it has to be explored how hypo-osmoregulation is achieved.

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Like a larvae, I was “exported” to other waters, the time to metamorphose arrived.

Gabriela Elena Torres Geninazza

Born the 25th march 1970 in Paysandú, Uruguay.

Italian and Uruguayan nationality

married, no children

EDUCATION

DEGREES

- **Oct 2002-2006:** Universität Oldenburg, Oldenburg, Germany
  - PhD project: Ecophysiological adaptations to salinity variation in early life-history stages of decapod crustaceans

- **2001:** Licenciada en Bioquímica (Degree in Biochemistry, equivalent to German Diplom), Facultad de Ciencias (FCIEN), Universidad de la República, Montevideo, Uruguay.
  - Monograph: Role of Na^+-K^+-ATPase in crustacean osmoregulation (monograph).
  - "Diplomarbeit": Effect of salinity on the biochemical composition (lipids and proteins) of first zoeae of crustacean larvae.

POSTGRADUATE COURSES

- **SEP 2001:** Aspects of the physiology and cell biology of transport epithelia.
- **OCT 2001:** Biological principles and aspects of fluorescence spectroscopy.

LANGUAGES

- SPANISH: mother tongue
- ENGLISH: First Certificate in English (Cambridge).
- FRENCH: Brevet Supérieur de Langue (Alliance française de l'Uruguay)
- GERMAN: Deutschen Sprachprüfung für den Hochschulzugang ausländischer StudienbewerberInnen - DSH (Philipps-Universität Marburg, Germany).

RESEARCH:

PUBLICATIONS

Anger K, **Torres G** & Giménez L, in press. Metamorphosis of a sesarmid river crab, *Armases roberti*: stimulation by adult odours vs. inhibition by salinity stress. Marine and Freshwater Behaviour and Physiology.

Torres G, Anger K & Giménez L, 2006. Effects of reduced salinities on metamorphosis of a freshwater-tolerant sesarmid crab, *Armases roberti*: Is upstream migration in the megalopa stage constrained by increasing osmotic stress? J. Exp. Mar. Biol. Ecol. 338, 134-139.

Giménez L, Anger K & **Torres G**, 2004. Linking life history traits in successive phases of a complex life cycle: effects of larval biomass on early juvenile development in an estuarine crab, *Chasmagnathus granulata*. Oikos: 104, 570-580.

Thatje S, Lovrich G A, **Torres G**, Hagen W & Anger K, 2004. Changes in biomass, lipid, fatty acid and elemental composition during abbreviated larval development of the sub Antarctic shrimp *Campylonotus vagans*. Journal of Experimental Marine Biology and Ecology: 301, 159-174.

Giménez L & **Torres G**, 2002. Larval growth in the estuarine crab *Chasmagnathus granulata*: the importance of salinity experienced during embryonic development, and the initial larval biomass. Marine Biology: 141, 877-885

Torres G, Giménez L & Anger K, 2002. Effect of salinity on the biochemical composition (lipids and proteins) of first zoeae of crustacean larvae. Journal of Experimental Marine Biology and Ecology: 277, 43-60

PUBLICATIONS SUBMITTED

Torres G, Boulo V, Spanings-Pierrot C, Cucchi-Mouillot P, Anger, K & Charmantier G, submitted. Effects of salinity on gene expression of Na^+-K^+-ATPase in the early life-history stages of a euryhaline crab. Submitted to Journal of Experimental Biology.

Torres G, Charmantier-Daures M, Chifflet S & Anger K. Effects of long-term exposure to different salinities on the location and activity of Na^+-K^+-ATPase in the gills of juvenile mitten crab, *Eriocheir sinensis*. Submitted to Comparative Biochemistry and Physiology.

Anger K, **Torres G** & Nettelmann U. Adaptive traits in ecology, reproduction and early life history of *Sesarma meridies*, an endemic stream crab from Jamaica. Submitted to Marine and Freshwater Research.

ABSTRACTS IN CONGRESSES

Torres G & Anger K, 2005. Effects of hypo-osmotic salinity stress on first-stage zoeal growth of estuarine marine decapod crustacean species. Fourth Crustacean Larval Conference - Sixth International Crustacean Congress, Glasgow, Scotland, UK, 18-22 July. Oral communication.

Torres G, Chifflet S & Anger K, 2005. Effects of salinity stress on the activity of Na^+-K^+-ATPase in the gills of juvenile mitten crab, *Eriocheir sinensis.* The Ecophysiology of Crustaceans - Sixth International Crustacean Congress, Glasgow, Scotland, UK, 18-22 July. Poster.

Torres G, Cieluch U, Chifflet S &Anger K, 2005. How does exposure to reduced or enhanced salinities affect the activity of Na^+-K^+-ATPase in the gills of juvenile mitten crab, *Eriocheir sinensis*?, 12. Crustaceologen-Tagung, Wilhelmshaven, Germany, 17-20 Feb. Oral communication.

Torres G, Giménez L & Anger K, 2003. Effects of salinity and exposure time on biochemical composition (proteins and lipids) of larval stages of *Homarus gammarus*. 11. Crustaceologen-Tagung, Ulm, Germany, 20-22 Feb. Poster.

Torres G, Baller C, Giménez L & Anger K, 2000. Efecto de la salinidad y el tiempo de exposición en la composición bioquímica (lípidos y proteínas) de los estadios larvales de *Chasmagnathus granulata*. IX Jornadas de la Sociedad Uruguaya de Biociencias. Solís, Uruguay, 4-7 May. Poster.

Torres G, Giménez L & Anger K, 1999. Comparative experimental study of salinity tolerance in early decapod crustacean larvae. Seventh Colloquium Crustacea Decapoda Mediterranea, Second Larval Conference, Lisbon, Portugal, 6-9 Sep. Poster.

Torres G, L Giménez & K Anger, 1999. Effects of salinity on biomass composition of larval and early juvenile *Armases miersii* (Crustacea: Decapoda). IX Crustaceologen-Tagung, Berlin, Germany, 25-28 Feb. Oral communication.

Schneider, N-L, **Torres G** & Anger, K, 1999. Kurzzeiteffekte verschiedener Salinitäten auf Biomasse und anorganischen Gehalt von Larven dreier euryhaliner Decapodenarten. IX Crustaceologen-Tagung, Berlin, Germany, 25-28 Feb. Poster.

SCHOLARSHIPS AND OTHER FUNDING

- **Dec 2005-Feb2006:** Sokrates/Erasmus Programm: Exchange Universität Oldenburg - Université Montpellier II.
- **2002-2006:** DAAD PhD research grant.
- **Sep 1999:** Financial support from Aktion Seeklar (Germany) to attend the 7th Colloquium Crustacea Decapoda Mediterranea, 2nd Larval Conference, Lisbon, Portugal, 6-9 Sep.

ACTIVITIES

Uruguay 1995-1997

Collaboration in sampling excursions corresponding to the projects:

- Benthic communities' structure and dynamics of sandy beaches of Cabo Polonio, Rocha, Uruguay.
- Biological and physical factors related to two sandy beach isopods distribution in the coast of Uruguay.

Germany 1997-1999

Physiology of marine crustacean larval development

- Effects of salinity on the biomass and inorganic content of larval and first juvenile stages of the tropical crab *Armases miersii*
- Effect of exposure time to different salinities in various crustacean species: *Chasmagnathus granulata*, *Carcinus maenas*, *Hyas araneus*, *Cancer pagurus* and *Homarus gammarus*
- Effects of salinity on the oxygen consumption of larvae of *Chasmagnathus granulata*

Collaboration in the project to repopulate the coastal area of Helgoland with European lobster, *Homarus gammarus*.

Germany 2002-2006

PhD project: Ecophysiological adaptations to salinity variation in early life-history stages of decapod crustaceans

France Dec 2005-March 2006

PhD project: Effects of salinity on gene expression of Na^+-K^+-ATPase in the early life-history stages of a euryhaline crab

EXPERIENCE IN SCIENTIFIC ADMINISTRATION:

- **2001 - 02:** Secretarial assistance at the Dean's office, Facultad de Ciencias (FCIEN), Universidad de la República, Montevideo, Uruguay.
- **2001:** Secretarial assistance at CSIC (Comisión Sectorial de Investigación Científica) office, FCIEN
- **1995 - 97:** Secretary at the office of the "Licenciatura de Bioquímica", FCIEN.

Helgoland, 18. September 2006

Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und nur die angegebenen Hilsfmittel benutzt habe.

Gabriela Torres