INTER- AND INTRASPECIFIC VARIATIONS IN REPRODUCTIVE AND DEVELOPMENTAL TRAITS OF DECAPOD CRUSTACEANS: TENTATIVE ADAPTIVE VALUE IN VARIABLE ENVIRONMENTS

Dissertation

zur Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Christian-Albrechts-Universität zu Kiel

vorgelegt von

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Kiel, 2012

Referent: Prof. Dr. Maarten Boersma

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Tag der mündlichen Prüfung: 04. Dezember 2012

Zum Druck genehmigt: 04. Dezember 2012

„Ich schaue zu, ich höre zu, die Seele halb im Meer, die Seele halb in der Erde, und mit beiden Hälften der Seele blicke ich auf die Welt“

Pablo Neruda (1904-1973)

Nobelpreis für Literatur 1971

CONTENTS

GENERAL INTRODUCTION

GENERAL INTRODUCTION

With about 15,000 species described so far, decapod crustaceans represent one of the largest taxa in the animal kingdom (Tudge 2000; Zhang 2011). The vast majority of the Decapoda is found in aquatic environments. About 90% of all decapod species live in the oceans and adjacent brackish water, and at least 1,000 species have colonized freshwater environments (Kaestner 1980). Although in much lower number, some species (< 100 species) were also able to conquer terrestrial habitats during the course of their evolution (Hartnoll 1988).

Numerous species of decapod crustaceans (e.g. lobsters, crabs, and shrimps) are subject to commercial fisheries and aquaculture activities. The world capture in marine fishing areas and inland waters was 6,102,070 tons in 2010 (FAO 2010). Among the decapod crustaceans, many species of shrimps (e.g. Penaeidae, Pandalidae and Palaemonidae), have recently also obtained great economic importance in aquaculture (New et al. 2010). In addition, shrimps are relevant for studies of evolutionary biology (Bauer 2004), for instance as model organisms for evaluating transitions from marine to freshwater habitats (Ashelby et al. 2012; Anger 2013), and for studying reproductive adaptations of species living in temperate regions with strong seasonality in environmental conditions (Anger 2001).

Most decapod crustaceans are benthic, living at the bottom of oceans, rivers and lakes, and most of these pass through complex life cycles comprising a benthic juvenile-adult and a pelagic larval phase (Anger 2001). Rather than showing a direct development from the egg to a benthic juvenile stage, they produce pelagic larvae. These planktonic stages must adapt to an environment which is different from that inhabited by the conspecific adults. Depending on reproductive patterns associated with different mechanisms of larval export or retention within the adult habitat, respectively (Strathmann 1982), decapod crustacean larvae have evolved their own evolutionary adaptations, mainly in morphology, locomotion, and physiology (Williamson 1982; Anger 2006).

In aquatic ecology, meroplanktonic larvae are considered as key components of benthic-pelagic coupling processes (Anger 2006; Kirby et al. 2007). In this context, carry-over effects of larval quality to the condition of benthic juveniles (Roughgarden et al. 1988; Giménez 2010) are subject to the interdisciplinary field of crustacean research. For example, in economically important species, investigating ontogenetic and reproductive traits provides critical information for the development of aquaculture techniques (Anger et al. 2009; Wu et

al. 2010) or for the management of sustainable fisheries (Botsford 1991; Campos et al. 2009). Also, patterns of larval dispersal, survival, and recruitment are fundamental to the structure and stability of benthic populations (Pan et al. 2011). These aspects of "supply-side ecology" also have consequences for distribution patterns, population connectivity and genetic diversity (Connolly and Roughgarden 1999; Svensson et al. 2004). In evolutionary biology, knowledge of reproductive and life-history traits is essential also for the understanding of limnic and terrestrial invasions by marine crustaceans (Dambach et al. 2012; Anger 2013). Regarding invasion biology, in particular in the context of global change, the increase of introduced species in recipient regions may be explained or predicted through developmental and ecophysiological traits of their larvae (Park et al. 2004; Rudnick et al. 2005).

Decapod crustaceans show great variation in life-history traits, which is generally considered as adaptive response to differential selective pressures on the survival of the progeny (Arthur 2000). Adaptive patterns include intraspecific variability in morphological, physiological and biochemical traits of the offspring in response to seasonal variations of temperature, salinity and food availability (Jacobs et al. 2003; Bas et al. 2007; Gebauer et al. 2010), combined effects of these environmental factors may influence growth and development (Giménez 2006), biochemical composition (Anger and Harms 1990), and other measures of physiological condition (Anger 2001).

In the present thesis, I used various decapod crustaceans (mostly shrimp) as model organisms to identify evolutionary adaptations in life-history traits, including patterns of larval development and growth of species that live under differential environmental conditions. I focused predominantly on: (1) adaptations to non-marine conditions in palaemonid shrimp with partial or fully lecithotrophic larval development (Chapters I-II); (2) reproductive adaptations of a temperate species of shrimp to seasonal variations in environmental conditions, as well as carry-over effects through successive ontogenetic stages (Chapters III-IV); (3) larval morphology of an invasive species of shrimp with an extended planktotrophic mode of development (Chapter V); (4) nutritional vulnerability of the early larval stages of a species of crab that is subject to aquaculture and fisheries activities (Chapter VI).

Model species

Macrobrachium amazonicum (Decapoda: Caridea) (Chapter I)

Shrimp assigned to the species *Macrobrachium amazonicum* (Heller 1862) have an extremely large geographic range (4,000 km across) in northern and central South America, comprising estuarine and fully limnic inland populations (Magalhães 2000) which are hydrologically isolated from each other (Hayd and Anger 2013). Significant variations in ecology, physiology, reproduction, and larval development, however, suggest an at least incipient allopatric speciation due to limited genetic exchange (Anger and Hayd 2009, 2010; Charmantier and Anger 2011). The earliest larval stages show strong lecithotrophic capacities (varying between populations). These were studied as to the biochemical basis allowing for partial independence from food.

Palaemonetes zariquieyi (Decapoda: Caridea) (Chapter II)

The freshwater shrimp *Palaemonetes zariquieyi* (Sollaud 1939) is an endemic species of the Iberian Peninsula, which inhabits environments ranging from freshwater lagoons to oligohaline channels near the Mediterranean Coast of Spain (Sanz-Brau 1983). Due to its restricted geographic distribution, *P. zariquieyi* is considered as a potentially endangered species, and thus, is under conservation management. It shows an abbreviated and fully lecithotrophic larval development with only three zoeal stages (Guerao 1993). Changes in larval biomass and chemical composition occurring during the lecithotrophic development from hatching to metamorphosis were studied under controlled laboratory conditions.

Crangon crangon (Decapoda: Caridea) (Chapters III-IV)

The brown shrimp, *Crangon crangon* (Linnaeus 1758), is a benthic key species in the North Sea ecosystem (Andresen et al. 2010; Hufnagl and Temming 2011), supporting an intense commercial fishery (ICES 2010). Its reproductive pattern is characterized by a continuous spawning season from mid-winter to early autumn (Siegel et al. 2008). During this extended period, *C. crangon* shows seasonal variations in egg size and biomass (Boddeke 1982; Paschke 1998). Embryonic and larval traits were chemically studied and compared with those of other model species of shrimp.

***Hippolyte leptocerus* (Decapoda: Caridea) (Chapter V)**

The shrimp *Hippolyte leptocerus* (Leach, 1814) show an extensive geographic and ecological distribution along the eastern Atlantic from western Ireland to Mauritania, including the Madeira and Cape Verde Islands and throughout the Mediterranean Sea and Black Sea (D'Udekem D'Acoz 1996). The morphology of the early larval stages of *H. leptocerus* is typical of species with an extended and planktotrophic mode of development. Morphological characters of the zoeal stages were described and compared with other shrimps of the genus *Hippolyte*. This study represents the first larval description of *H. leptocerus* from the eastern Atlantic and western Mediterranean.

***Maja brachydactyla* (Decapoda: Brachyura) (Chapter VI)**

The spider crab *Maja brachydactyla* (Balss, 1922) has a high economic and ecological significance, supporting fisheries along the NE Atlantic coasts (Spain, Portugal, France, Ireland and UK) (Freire et al. 2002). The high fishing pressure tolerated by populations of this crab, together with its growth and reproductive characteristics, define the species as potentially interesting for aquaculture (Andrés et al. 2007). Biochemical composition and nutritional vulnerability of the early zoea larvae of *M. brachydactyla* were investigated and compared with other decapod crustaceans that show a planktotrophic mode of larval development.

List of manuscripts

This cumulative thesis consists of six manuscripts which have been published (Manuscripts I, III, V, VI), are in press (Manuscript IV), or in preparation (Manuscript II), respectively. My contribution to each study is explained.

Manuscript I

Urzúa, Á. and Anger, K. (2011)
Larval biomass and chemical composition at hatching in two geographically isolated clades of the shrimp *Macrobrachium amazonicum*: intra- or interspecific variation?
Invertebrate Reproduction and Development 55: 236-246

The second author developed this research topic in cooperation with me. Experimental work was done by both authors, biochemical and data analyses by myself. I wrote a draft of the manuscript, which was improved by the second author.

Manuscript II

Urzúa, Á., Guerao, G., Cuesta, J., Rotllant, G., Estévez, A. and Anger, K. (2012)
The bioenergetic fuel for non-feeding larval development in an endemic palaemonid shrimp from the Iberian Peninsula, *Palaemonetes zariquieyi*
In preparation, this manuscript will be submitted to Marine and Freshwater Behaviour and Physiology.

I developed the scientific idea for this study in cooperation with all co-authors, performed the biochemical analyses, processed the data, and wrote the manuscript, which was subsequently improved in cooperation with the co-authors.

Manuscript III

Urzúa, Á., Paschke, K., Gebauer, P. and Anger, K. (2012)
Seasonal and interannual variations in size, biomass and chemical composition of the eggs of North Sea shrimp, *Crangon crangon* (Decapoda: Caridea)
Marine Biology 159: 583-599

Sampling was performed by K. Paschke (all data from studies carried out in 1996) and by myself (data from 2009). Correspondingly, the analyses of egg biomass and chemical

composition were done by the first and second author, respectively. Together with K. Anger, I developed the concept of this joint work, and I wrote the manuscript. P. Gebauer participated in complex statistical analyses of the large data sets used for this paper. All co-authors helped in the preparation of the final manuscript version.

Manuscript IV

Urzúa, Á. and Anger, K. (2012)
Seasonal variations in larval biomass and biochemical composition of brown shrimp, *Crangon crangon* (Decapoda, Caridea), at hatching
Helgoland Marine Research (In Press; DOI: 10.1007/s10152-012-0321-4)

Based upon the planning of MS III, I developed the idea of this study. The experimental work and the processing of the data were done in cooperation with the second author. I wrote the manuscript, which was improved by the second author.

Manuscript V

Guerao, G., Hernández, E. and **Urzúa, Á**. (2011)
Early zoeal development of the shrimp *Hippolyte leptocerus* (Decapoda, Caridea, Hippolytidae)
Zootaxa 2988: 53-65

I developed the research topic in cooperation with the first author. Larval analyses and taxonomic work were done by all authors. The first author wrote the manuscript, and the final version was improved by all authors.

Manuscript VI

Guerao, G., Simeó, C.G., Anger, K., **Urzúa, Á**. and Rotllant, G. (2012)
Nutritional vulnerability of early zoea larvae of the crab *Maja brachydactyla* (Brachyura, Majidae)
Aquatic Biology 16: 253-264

Rearing of larvae, sampling and chemical analyses were done by all authors. I participated in developing the experimental design (mainly done by the first author), data analyses, and manuscript writing.

CHAPTER I

Larval biomass and chemical composition at hatching in two geographically isolated clades of the shrimp *Macrobrachium amazonicum*: intra- or interspecific variation?

Ángel Urzúa and Klaus Anger

Invertebrate Reproduction & Development
Vol. 55, No. 4, December 2011, 236–246

Larval biomass and chemical composition at hatching in two geographically isolated clades of the shrimp *Macrobrachium amazonicum*: intra- or interspecific variation?

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(*Received 14 October 2010; final version received 2 February 2011*)

The shrimp *Macrobrachium amazonicum* (Heller 1862) has an extremely large geographic range (>4000 km across) in northern and central South America, comprising estuarine and fully limnic inland populations, which are hydrologically isolated from each other. Significant variations in ecology, physiology, reproduction, and larval development suggest an at least incipient allopatric speciation due to limited genetic exchange. In a comparative experimental investigation with shrimps from the Pantanal (upper Paraguay River basin) and the Amazon delta, respectively, we measured larval body size, dry weight (W), biochemical (total protein; lipid; fatty acids, FA), and elemental composition (carbon, hydrogen, nitrogen; collectively CHN) at hatching. All these early larval traits are relevant for the degree of developmental dependence on planktonic food sources. Various consistent differences were observed between the two populations: Newly hatched larvae produced by shrimps from the Amazon delta were significantly smaller and showed lower values of W, CHN, protein, and unsaturated FA compared to those from the Pantanal. On the other hand, they contained significantly higher quantities of total lipid and saturated FA and, in consequence, higher ratios of lipid:protein, C:N, and saturated:unsaturated FA. All these differences in biomass and chemical composition suggest that the larvae of the Amazon population are energetically better adapted to planktonic food limitation, which likely occurs during riverine downstream transport toward coastal marine waters, also explaining previous observations of much stronger initial starvation tolerance in larvae from the Amazon versus those from the Pantanal. The latter develop in highly productive lentic inland waters, where large body size, an early onset of feeding, and a strong musculature (indicated by a high protein content) should facilitate their role as planktonic predators and allow for fast growth. An initial independence from food (lecithotrophy in the zoea I stage) as well as a preference for oligohaline rather than fully limnic conditions observed in the Pantanal larvae are interpreted as traits that have persisted from an ancestral coastal marine clade. Altogether, consistent ontogenetic differences between shrimps from the Pantanal and the Amazon estuary support the hypothesis that the taxon *M. amazonicum* comprises a complex of closely related but separate species.

Keywords: Amazon delta; Pantanal; biochemical composition; larvae; lecithotrophy; *Macrobrachium amazonicum*; speciation

Introduction

Life-history adaptations that allow for invasions of limnic environments by marine organisms are among the top issues in evolutionary biology (e.g. Walker 1992; Lee and Bell 1999; Anger et al. 2007). Among the Crustacea, palaemonid shrimps have been particularly successful as invaders of brackish coastal lagoons, estuaries, and inland freshwater habitats, especially in tropical and subtropical regions (Bauer 2004). Showing an extremely wide geographic and ecological distribution, the palaemonid species *Macrobrachium amazonicum* (Heller 1862) may be considered as one of the most successful invaders of estuarine, riverine, and fully limnic inland waters. It ranges from the Caribbean and Atlantic coasts of South America (12°N) to northern Argentina and Paraguay (28°S), and from the eastern slopes of the Andes mountains in Ecuador, Peru and Bolivia to the Atlantic coasts of northeastern Brazil, i.e. in an area >4000 km across (Holthuis 1952; Ramos-Porto and Coelho 1990; Pettovello 1996; Odinetz Collart and Rabelo 1996; Magalhães 2000). All northern populations, which live in coastal rivers and estuaries, or in inland waters of the Amazon and Orinoco basins, are hydrologically connected to each other, allowing for some (although probably limited) gene flow. The southernmost populations, living in the upper Paraguay and Paraná river basins (La Plata system), are hydrologically connected to each other, but separated, and thus genetically isolated, from all northern populations.

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ISSN 0792–4259 print/ISSN 2157–0272 online

http://dx.doi.org/10.1080/07924259.2011.576155
http://www.tandfonline.com

As a consequence of limited genetic exchange within the vast area of distribution, *M. amazonicum* shows great regional variations in ecology, morphology, growth, reproduction, development, and physiology (for recent review, see Maciel and Valenti 2009). This suggests an incipient or already advanced speciation process, raising the question whether we are really dealing with a single, widely distributed, and highly variable species, or with various closely related but phylogenetically separate clades. Since few and inconclusive molecular genetic data have not yet allowed for resolving this question (Bastos 2002; Vergamini 2009), comparative life-history studies should reveal whether variations in reproductive and developmental traits are due to phenotypic plasticity (Pfennig et al. 2010), or represent stable (genetically fixed) characteristics of different clades. If phenotypic plasticity can be excluded as the principal explanation for variability among populations, we need more detailed morphological and molecular genetic studies to reveal whether these clades represent separate regional races or distinct species.

The occurrence of shrimps attributed to the species *M. amazonicum* in the upper La Plata system was documented already one century ago (Nobili 1896; Moreira 1913) and later repeatedly confirmed (Pettovello 1996; Bialetzki et al. 1997; Heckman 1998; Magalhães 2000; Hayd and Nakagaki 2002; Magalhães et al. 2005). In contrast to estuarine and central Amazonian populations, where life-history traits have been studied to some extent both in the laboratory and field, very little is known about those in the La Plata basin (for recent review, including ''grey literature'', see Maciel and Valenti 2009).

For a shrimp population living in the Paraná River, Bialetzki et al. (1997) provided the first ecological data (water temperature, conductivity, pH, seasonal variation in rainfalls, and fluviometric level) as well as preliminary observations on seasonal cycles of reproduction and diurnal variations in the occurrence of larvae and juveniles. With the exception of the more general ecological paper by Heckman (1998), no such data have become available for the upper Paraguay basin. This is surprising insofar, as shrimp populations living in the Pantanal (seasonally flooded wetlands; see Heckman 1998; Junk et al. 2006) in the southwestern Brazilian state of Mato Grosso do Sul are wide-spread and often dense, allowing for a commercial utilization as bait for recreational fisheries, which is the main base of the regional tourism industry (Hayd and Nakagaki 2002). The scarcely available literature suggests that shrimp populations in the upper La Plata system inhabit similar types of habitat and utilize similar food sources as those in central Amazonia (cf Walker and Ferreira 1985; Heckman 1998; Magalhães 2000).

The first life-history study on a shrimp population from the Pantanal of Mato Grosso do Sul (Anger and Hayd 2010) showed in the early postembryonic stages a high potential for food-independent larval development. This trait is based on an enhanced female energy investment into egg production, at hatching still visible as remaining yolk droplets in the larval cephalothorax. Similar to previous findings in a population from the Amazon estuary (Anger and Hayd 2009), the first zoeal stage (Z I) of the Pantanal shrimps was shown to be fully lecithotrophic (non-feeding), the second stage (Z II) is feeding but facultatively lecithotrophic (successfully developing also in absence of food), and the Z III can still survive for another few days in continued absence of food. However, the endotrophic potential of the early Pantanal larvae was significantly weaker than in the equivalent stages of estuarine shrimps, where the maximal survival time under starvation conditions was almost twice as long (14–15 versus 8–9 days). This conspicuous difference in larval dependence on food was interpreted as an adaptive pattern related to differential selective ecological conditions, under which their early larval development takes place. While shrimps living in near-coastal rivers and estuaries presumably follow an ''export strategy'' (Strathmann 1982; Anger 2001), with early development in fast flowing, strongly food-limited lotic (riverine) environments, the larvae of Pantanal shrimps are retained within the adult habitats, i.e. in lentic, highly productive shallow waters, where no planktonic food limitation should select for an enhanced female energy investment into egg production. This raised the question: What is the chemical basis of strongly differential starvation tolerance in the early larvae?

In this study, we compare the initial biomass (dry mass) and chemical composition (contents of carbon, hydrogen, nitrogen; total proteins and lipids; fatty acid profiles) of newly hatched larval shrimps from the Pantanal (P, upper Paraguay River basin) and the Amazon delta (A), respectively. For convenience, these clades are here tentatively referred to as Amazon and Pantanal ''populations'', respectively, although for the time being it must remain open, whether they actually represent isolated populations, genetically different races, or two distinct species. Larvae originating from these two populations will be denoted as ''A larvae'' and ''P larvae'', respectively. Quantifying both the variability within (among hatches) and variation between these two geographically isolated clades assigned to *Macrobrachium amazonicum*, we address two principal questions, (1) whether the two populations show consistent differences, which are larger than the variability among hatches from each population; (2) what may be the biochemical basis for previously shown differences in the early larval dependence on planktonic food availability (Anger and Hayd 2009, 2010).

Materials and methods

Collection and maintenance of shrimps

Male and ovigerous female Amazon shrimps were obtained from the Aquaculture Center (CAUNESP, Jaboticabal) of the State University of São Paulo, Brazil (for details of broodstock production and maintenance, see Morães-Valenti and Valenti 2007). The broodstock originated from a population living in estuarine tidal creeks near Belém in the Amazon Delta, northern Brazil (01°14′30″S/48°19′52″W; W. Valenti, personal communication). The shrimps were transported in cooling boxes to the Helgoland Marine Biological Laboratory (BAH), Germany, and subsequently maintained in recirculating aquaria with 30 L aerated freshwater (total ion concentration: 0.2 mg/L), a constant temperature of 29°C, a 12:12 h light:dark cycle, gravel filters, and pieces of frozen marine isopods (*Idotea* spp.) and commercial aquarium feeds (Novo Tab, JBL) provided as food (Anger et al. 2009). Females were checked twice daily for the occurrence of freshly hatched larvae. These were collected from sieves (0.3 mm mesh size) receiving the overflowing water from the aquaria.

Adult Pantanal shrimps were obtained from a research hatchery of the State University of Mato Grosso do Sul (Aquidauana, Mato Grosso do Sul), southwestern Brazil, where the shrimps were maintained at very similar conditions as at the CAUNESP, and subsequently transported to the BAH. The broodstock originated from a population living in the Rio Miranda (sampling locations at 20°8.9–10.7′S/56°30.4–30.6′W). The adult shrimps were maintained at identical conditions of food, temperature, salinity, and light, and the larvae were obtained with the same technique as in the Amazon shrimps (see above). Hence, the cultivation conditions during sexual maturation, oogenesis, and embryonic development from egg laying to larval hatching were identical in the two shrimp populations.

Measurements of larval size, biomass, and chemical composition

From four different females of each population, newly hatched larvae were taken for parallel determinations of body size, dry mass (W), elemental composition (contents of carbon, hydrogen and nitrogen; collectively CHN), and proximate biochemical composition (total lipids and proteins). For W and CHN of newly hatched larvae, data from additional 39 females were available (18 from population A, 21 from P), so that in total W and CHN data for 22 A and 25 P hatches, respectively, can be presented in this article (Table 2). These data could be pooled for each population, as the mean values for the four hatches, which were used for parallel measurements of size and proximate biochemical composition, did not significantly deviate from the average values obtained for the additional females (for statistical methods, see below).

For fatty acid determinations, we analysed newly hatched larvae from four different females from each population. These were not identical with the materials used for the other chemical measurements, because fatty acid analyses require large quantities of biomass, so that single hatches were too small to allow for parallel analyses of all chemical parameters in larvae from the same hatch (for details of methods, numbers of replicates, and larvae per replicate in the various determinations, see sections below).

Body size

Total larval body length (TL) at hatching (10 larvae per hatch) was measured to the nearest 0.01 mm as the distance from the anterior margin of the eye orbit (i.e., excluding the rostrum, which can break or vary in size) to the posterior margin of the telson, using a Leica MZ8 stereo microscope equipped with a calibrated eyepiece micrometer.

Biomass and elemental composition

Biomass (dry mass, W) and elemental composition (CHN) were measured with standard techniques (Anger and Harms 1990): larvae were briefly rinsed in distilled water, blotted on fluff-free Kleenex paper, transferred to pre-weighed tin cartridges, and stored at –20°C. Later, the samples were freeze-dried for 48 h in a vacuum dryer (Christ Alpha 1-4 LSC), and W was determined to the nearest 0.1 μg on a Sartorius SC2 ultra micro balance. Subsequently, the samples were analysed with an Elemental Vario Micro CHN Analyser using Sulphanilamide as a standard. Each measurement of larval W and CHN in hatches from different females and populations comprised five replicate determinations with five or six individuals each (depending on hatch size).

Proximate biochemical composition (total protein and lipids)

For each of the four hatches from each population, four replicate biochemical analyses were carried out with 15 larvae per replicate. The samples were gently rinsed for 10 s in distilled water, subsequently blotted on filter paper, transferred to pre-weighed 1.5 mL microcentrifuge vials, and stored frozen at –80°C. Prior to the analyses, the samples were dried for 48 h in a vacuum dryer (see above), and W was determined to the nearest 0.01 mg on a Sartorius balance (MC1 RC 210 S; capacity 210 g). Afterwards, the samples were homogenized on ice (Branson, Sonifier, Cell Disruptor

B 15), and each homogenate was divided into two aliquots for repeated protein and lipid determinations.

The protein content of the homogenate was determined using BioRad DC Protein Assay following Lowry et al. (1951), modified for microplates by Torres et al. (2007a). 25 µL homogenate was mixed with 100 µL ice-cold 20% trichloroacetic acid (TCA). After an incubation of 10 min at 4°C, the samples were centrifuged at $10.000 \times g$ for 10 min at 4°C and the supernatant was discarded. The remaining pellet was dissolved in 300 µL NaOH (1 M) and incubated, shaking at 1400 rpm for 30 min at 56°C in a thermomixer. After incubation, four 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 DC Protein Assay) in a 96-well microplate. The microplates were incubated for 15 min at room temperature in the dark, and absorbance was measured using a Multiskan Spectrum Thermo apparatus (wavelength: 750 nm). The calibration curve was obtained by dilutions of bovine serum albumin (BSA, kit: BioRad DC Protein Assay).

The total lipid content of the homogenate was determined using the sulphophosphovanillin method (Zöllner and Kirsch 1962; modified for microplates by Torres et al. 2007b). 40 µL of the homogenates were mixed with 300 µL of ice-cold $CHCl_3$:CH_3OH (2:1). After an incubation of 15 min at room temperature, the samples were centrifuged for 20 min with $10.000 \times g$ at 4°C. 180 µL of the lower phase were transferred to new tubes. These were left open to dry in a thermomixer for 90 min at 56°C, shaking at 700 rpm. The dried pellets were dissolved in 200 µL of concentrated H_2SO_4, and incubated for 10 min at 95°C, shaking at 1400 rpm in a thermomixer with closed tubes. After cooling for 20 min at room temperature, four replicates of 20 µL from each sample were distributed in two 96-well microplates. In the first plate (Blank), 300 µL of concentrated H_3PO_4 was added. In the second plate, 300 µL of vanillin solution (8 mM H_3PO_4 conc) was added. The microplates were incubated for colour development for 45 min at room temperature and subsequently measured using a Multiskan Spectrum Thermo apparatus (wavelength: 550 nm). The final values were obtained as the difference between the two plates. The calibration curve was obtained by dilutions of a standard lipid–cholesterol solution extracted from muscle tissue of adult *M. amazonicum*, homogenized, and extracted in dichloromethane:methanol (2:1; v/v) following the method described by Cequier-Sanchez et al. (2008).

Fatty acid analyses

The determination of the fatty acid composition was based on the procedures described by Malzahn et al. (2007). Four replicate samples per population with 30 larvae from each hatch were analysed. Fatty acids were measured as fatty acid methyl esters (FAMEs). Lipids were extracted from the samples by dichloromethane:methanol (2:1 vol:vol) in an ultrasound bath for 30 min. Water-soluble fractions were removed after centrifugation by washing with 0.88% KCl buffer. The water phase was removed, and the organic remainder was evaporated using nitrogen gas. The esterification was done using methanolic sulfuric acid at 70°C for 1 h. The FAMEs were washed from the methanolic sulphuric acid using *n*-hexane. Excess *n*-hexane was evaporated using nitrogen gas. FAMEs were analysed by gas chromatography (Varian CP-3800), equipped with an auto-sampler (Varian CP-8400), and temperature programming. FAME profiles were identified and quantified by the comparison of their retention time with an internal standard 23:0 FIFM (C 23:0 added to the samples at the first step of the preparation). Peak areas were determined using the Varian Galaxy software.

Statistical analyses

Statistical analyses were carried out following standard techniques (Sokal and Rohlf 1995) using SPSS ® 11.5 (Statistical Software Package). Chemical data obtained from larvae produced by different females of the respective population were analyzed with a nested two-way ANOVA, with Population (A, P) as fixed factor and Hatch as nested factor. All statistical analyses were performed on the 95% confidence level ($p < 0.05$). Normality and homogeneity of variances were tested with Kolmogorov–Smirnov and Bartlett's tests, respectively. When data did not meet these assumptions, the Scheirer–Ray–Hare extension of the Kruskal–Wallis test (non-parametric two-way ANOVA) was performed (Sokal and Rohlf 1995; Dytham 1999).

Results

Our comparative data of larval size, biomass, and chemical composition at hatching revealed numerous significant differences both among hatches from the same population and between the two populations of shrimps currently assigned to the same species, *M. amazonicum* (Amazon delta, A versus Pantanal, P; nested 2-way ANOVA). Despite significant variability among hatches, the highest and lowest mean values of size or biomass per larva generally showed no overlap between the two populations (Table 2).

Body size

Measurements of larval body size at hatching revealed that the Zoea I of the population originating

Table 1. *Macrobrachium amazonicum*, freshly hatched larvae (zoea I) from the Amazon delta (A) and the Pantanal (P).

		Min	Max	Mean	+SD	Hatch	Population
TL(mm)	A	2.47	2.64	2.57	0.08	$F_{3,28} = 12.388$*	$F_{1,60} = 173.218$***
	P	3.10	3.51	**3.28**	0.10	$F_{3,31} = 5.068$*	
W (μg)	A	55.5	59.1	57.4	1.61	$F_{3,28} = 3.671$*	$F_{1.60} = 1891.430$***
	P	77.1	81.4	**78.6**	1.92	$F_{3,31} = 20.286$*	
C (μg)	A	28.7	30.4	29.4	0.68	$F_{3,28} = 1.784$*	$F_{1,60} = 709.569$***
	P	37.7	41.4	**38.8**	1.75	$F_{3,31} = 6.080$*	
N (μg)	A	5.46	6.13	5.80	0.34	$F_{3,28} = 16.932$*	$F_{1,60} = 1501.876$***
	P	8.36	9.07	**8.63**	0.32	$F_{3,31} = 9.992$*	
H (μg)	A	4.57	4.97	4.76	0.20	$H_3 = 7.636$*	$H_1 = 26.990$*
	P	5.86	6.47	**6.05**	0.28	$H_3 = 9.096$*	
Protein (μg)	A	17.0	22.2	19.8	2.01	$F_{3,20} = 30.212$*	$F_{1,41} = 241.619$***
	P	27.4	39.1	**30.6**	5.02	$F_{3,20} = 11.632$*	
Lipid (μg)	A	6.32	9.54	**8.02**	1.21	$F_{3,20} = 69.882$*	$F_{1,41} = 378.251$***
	P	5.31	6.50	6.10	0.80	$F_{3,20} = 41.783$*	

Notes: Parallel determinations ($n = 4$ hatches per population) of body size (total length, TL), biomass (dry weight, W), elemental composition (carbon, C; nitrogen, N; hydrogen, H; collectively CHN), and proximate biochemical composition (total protein, lipid) are expressed as absolute values (μg·ind^{-1}); range (minimum – maximum) of mean values for different hatches (same population); total mean values ± SD for the two populations; separate statistical comparisons among hatches from each population and between the two populations (A versus P): nested two-way ANOVA (F) and Scheirer–Ray–Hare tests (H); significant differences are marked with asterisks (*$p < 0.05$; ***$p < 0.001$); significantly higher mean values are highlighted as bold numbers

Table 2. *Macrobrachium amazonicum*, freshly hatched larvae (zoea I) from the Amazon delta (A) and the Pantanal (P).

	A						P					
	Min		Max		Total		Min		Max		Total	
	Mean	±SD	Mean	±SD	Mean	+SD	Mean	±SD	Mean	±SD	Mean	±SD
w (μg)	54.6	0.4	68.1	0.6	61.0	3.8	66.4	2.0	84.1	1.0	**76.5*****	5.0
C (μg)	28.4	0.3	36.8	0.3	32.5	2.5	31.3	1.3	41.8	0.7	**37.5*****	2.9
N (μg)	4.76	0.07	6.53	0.10	5.95	0.43	7.27	0.35	9.14	0.17	**8.29*****	0.57
H (μg)	4.16	0.04	5.78	0.04	5.03	0.38	5.27	0.13	6.93	0.35	**6.03***	0.46
C (% W)	50.1	0.2	55.4	0.1	**53.2***	1.6	47.3	0.16	51.3	0.9	49.1	1.0
N (% W)	8.09	0.08	10.8	0.1	9.70	0.66	9.88	0.07	11.7	0.1	**10.8*****	0.4
H (% W)	7.61	0.04	8.69	0.05	**8.20***	0.28	6.94	0.09	8.75	0.22	7.90	0.45
C/N	4.32	0.03	6.56	0.02	**5.49*****	0.38	4.17	0.03	4.88	0.12	4.53	0.17
C/H	6.20	0.02	6.70	0.02	6.35	0.18	6.40	0.03	6.70	0.01	**6.51***	0.12

Notes: Initial larval dry weight (W) and elemental composition (CHN) are expressed as absolute and percentage values (in μg·ind^{-1} and % of W, respectively); for each population (A, P), mean values ± SD ($n = 5$ replicate measurements) are given for hatches with minimum and maximum biomass, respectively, as well as the grand total of all mean values with $n = 22$ hatches in population A and $n = 25$ in P, respectively; W and CHN data including those shown in Table 1, plus analyses of additional hatches (without parallel measurements of size and biochemical composition); significant differences between populations are marked with asterisks (*$p < 0.05$; ***$p < 0.001$); significantly higher mean values (comparisons between populations) are highlighted as bold numbers. In biomass parameters where significant differences between populations occur, minimum and maximum values show very little overlap; for further explanation of statistical analyses and other details, see Table 1 and Section "Material and Methods" ("Statistical analyses").

from the Amazon delta was on average significantly smaller than in larvae from the Pantanal population (mean values 2.6 ± 0.1 versus 3.3 ± 0.1 mm in A and P larvae, respectively; Table 1). No overlapping values were observed in these two groups, i.e. even the smallest P larvae exceeded the largest A larvae in size.

Dry mass (W) and elemental composition (CHN)

Corresponding with their smaller body size, A larvae also showed consistently lower mean dry mass (W) and lower quantities of CHN per individual compared to P larvae. These differences were similar in the smaller data set (parallel measurements of W, CHN, and proximate biochemical composition; using larvae from

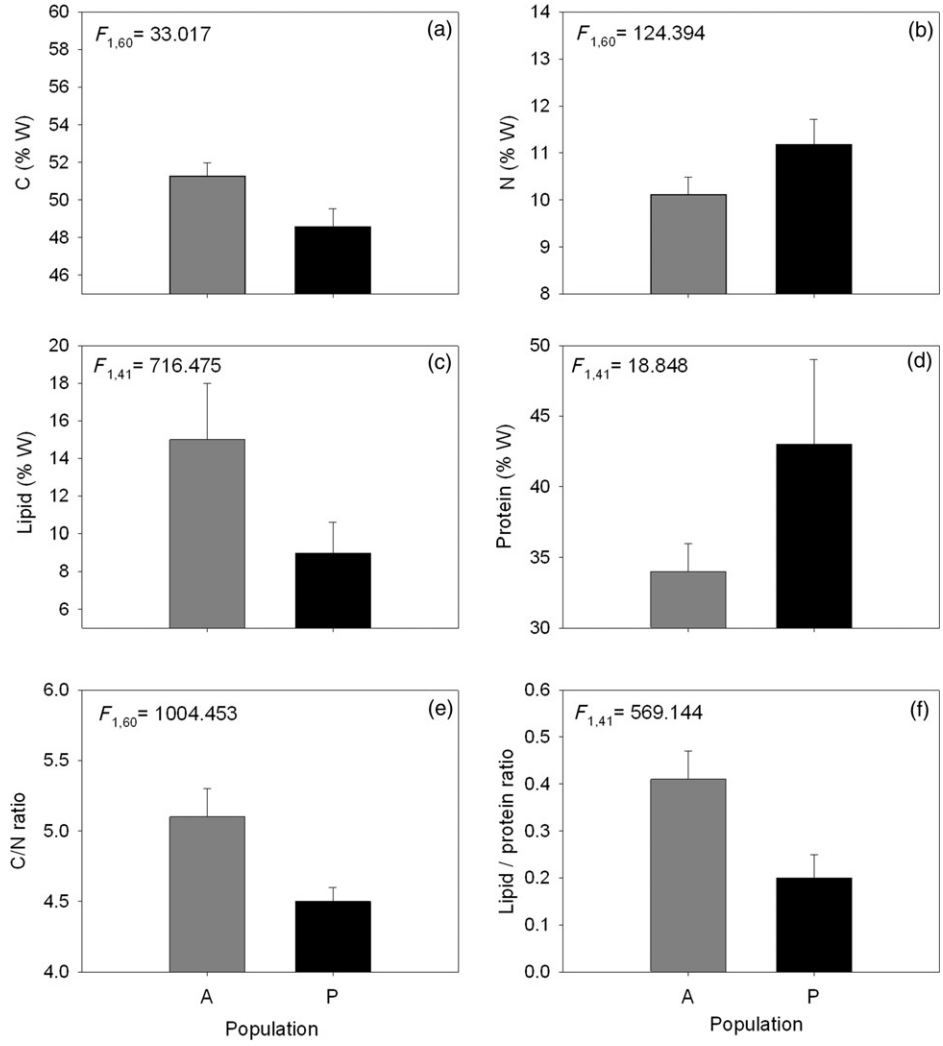

Figure 1. *Macrobrachium amazonicum*, freshly hatched larvae (zoea I) from the Amazon delta (A) and the Pantanal (P). Parallel determinations of (a) carbon, (b) nitrogen, (c) lipid, (d) protein, (e) C/N mass ratio, (f) lipid/protein ratio in $n = 4$ hatches per population (cf. Table 1); mean values ± SD, all expressed in % of dry weight (W); *F*-statistics for nested two-way ANOVA. Note: All differences were highly significant ($p < 0.001$).

$n = 4$ hatches; Table 1) and in the larger set, with W and CHN determinations only (i.e., without biochemical analyses; $n = 22$ and 25 hatches from A and P, respectively; Table 2).

In relative terms (CHN expressed in % of W), A larvae contained consistently higher proportions of C and H, but lower N values (Figure 1; Table 2). Although these differences were small, they were statistically significant, and there was hardly any overlap between the two populations (see Table 2, minimum versus maximum mean values for different hatches of each population). Also, A larvae showed a significantly higher C/N mass ratio compared to P larvae (Figure 1).

Proximate biochemical composition

Corresponding with consistent differences in body size, W and CHN per individual, the larvae from population A contained at hatching significantly less protein than those from P (20 ± 2 versus 31 ± 5 µg; Table 1). The lipid content, by contrast, showed an inverse pattern with significantly higher values found in A larvae (8.0 ± 1.2 versus 6.1 ± 0.8 µg).

Also as a percentage of W, the protein content was significantly ($p < 0.001$) lower in A than in P larvae (34 ± 2 versus 43 ± 6%), while a clearly higher lipid value was found in A larvae (15 ± 3 versus 9 ± 2%; Figure 1). Consistent with a higher lipid but lower protein content (both per individual and as a percentage of W), the lipid/protein ratio was in A larvae twice as high than in P larvae (0.41 ± 0.06 versus 0.20 ± 0.05; Figure 1; $p < 0.001$).

Fatty acid (FA) composition

Shrimp larvae from the Amazon delta and the Pantanal also differed in FA profiles as well as in their total FA

Table 3. *Macrobrachium amazonicum*, freshly hatched larvae (zoea I) from the Amazon delta (A) and the Pantanal (P).

	Fatty acid content (ng/larva)			% of total fatty acid pool		
Fatty acid	A	P	F	A	P	F
C 16:0	165 ± 12	**235** ± 51	9.048*	25.4 ± 3.31	25.3 ± 0.91	0.002^{ns}
C 16:1 n7	19 ± 0.3	**52** ± 9.0	42.660***	2.60 ± 0.67	**5.68** ± 0.64	37.395***
C 16:2 n4	2.0 ± 0.2	2.3 ± 0.2	5.615^{ns}	0.30 ± 0.03	0.26 ± 0.04	2.596^{ns}
C 16:3 n4	1.7 ± 0.1	1.0 + 0.1	18.063*	**0.22** ± 0.04	0.13 ± 0.03	12.264*
C 17:0	11 ± 0.3	13 ± 0.3	0.726^{ns}	1.69 ± 0.19	1.35 ± 0.26	4.213^{ns}
C 17:1 n7	2.3 ± 1.3	2.0 ± 0.7	0.697^{ns}	**0.40** ± 0.03	0.19 ± 0.01	3.331*
C 18:0	180 ± 8	191 ± 40	0.146^{ns}	**27.2** ± 3.02	20.2 ± 3.29	9.848***
C 18:1 n7	112 ± 25	**183** ± 24	19.066***	16.1 ± 2.79	**20.1** ± 2.52	4.422*
C 18:1 n9 (trans)	28 ± 5.3	**46** ± 6.7	19.184*	4.03 ± 0.59	4.98 ± 0.49	5.972^{ns}
C 18:1 n9(cis)	5.7 ± 0.3	1.0 ± 0.01	19.059***	**1.21** ± 0.97	0.14 ± 0.05	11.115*
C 18:2 n6 (trans)	3.7 ± 0.3	0.7 ± 0.01	18.725*	**0.80** ± 0.42	0.10 ± 0.04	1.966*
C 18:2 n6(cis)	28 ± 1.3	**71** ± 13	17.550***	3.90 ± 0.64	**7.66** ± 0.65	7.469***
C 18:3 n6	12 ± 0.7	**25** ± 1.7	3.353*	1.38 ± 0.15	**2.59** ± 0.53	6.975*
C 18:3 n3 (α-linoleic)	12 ± 3.3	10 ± 2.3	0.832^{ns}	1.68 ± 0.44	1.66 ± 0.15	1.210^{ns}
C 20:0	3.7 ± 0.7	4.0 ± 1.0	0.693^{ns}	0.58 ± 0.20	0.45 ± 0.01	1.660^{ns}
C 20:1 n9	4.7 ± 2.0	5.3 ± 0.3	0.402^{ns}	0.64 ± 0.29	0.60 ± 0.11	0.612^{ns}
C 20:2 n6	3.7 ± 1.3	**4.7** ± 0.7	3.178*	0.52 ± 0.19	0.52 ± 0.04	0.011^{ns}
C 20:4 n6 (ARA)	12 ± 2.3	11 ± 2.3	0.284^{ns}	1.54 ± 0.70	1.23 ± 0.12	0.749^{ns}
C 20:3 n3	2.0 ± 0.7	1.0 ± 0.3	5.466*	**0.27** ± 0.08	0.12 ± 0.03	10.126*
C 20:4 n3	2.0 ± 0.3	1.7 ± 0.7	1.098^{ns}	**0.31** ± 0.09	0.18 ± 0.06	6.912*
C 20:5 n3 (EPA)	22 ± 7.7	29 ± 4.7	1.056^{ns}	2.88 ± 1.29	3.12 ± 0.20	0.134^{ns}
C 22:0	2.0 ± 0.7	2.3 ± 0.3	0.118^{ns}	0.38 ± 0.03	0.25 ± 0.01	1.17S^{ns}
C 22:1 n9	2.3 ± 1.0	1.3 ± 0.3	4.315*	**0.33** ± 0.22	0.13 ± 0.06	3.587*
C 22:2 n6	3.3 ± 0.7	3.3 ± 1.0	0.031^{ns}	**0.53** ± 0.19	0.37 ± 0.14	3.616*
C 22:5 n3 (DPA)	8.0 ± 0.3	7.3 ± 0.3	0.035^{ns}	1.02 ± 0.53	0.84 ± 0.06	0.155^{ns}
C 22:6 n3 (DHA)	25 ± 5.3	17 ± 4.3	8.636*	**2.89** ± 0.26	1.83 ± 0.14	6.697*
C 24:0	5.7 ± 2.3	4.3 ± 0.01	7.575*	**0.78** ± 0.09	0.48 ± 0.09	4.796*
Total FA	680 ± 78	927± 88	10.81	100	100	

Notes: Fatty acid (FA) profiles are expressed as absolute values (ng/larva) and in % of the total FA pool; mean values ± SD; significant differences are marked with asterisks (*$p < 0.05$; ***$p < 0.001$), ns = not significant; significantly higher values highlighted as bold numbers; $n = 4$ hatches per population; nested two-way ANOVA ($F_{1,7}$).

content, which was significantly higher in larvae from population P compared to A (Table 3). The predominant FA were, in general, palmitic (16:0), stearic (18:0), and vaccenic acid (18:1 n7), each comprising 16–27% of the total FA pool. Lower values were found in palmitoleic (16:1 n7), oleic (18:1 n9, trans), linoleic (18:2 n6, cis), and eicosapentaenoic acid (20:5 n3; EPA), while other FA occurred only in traces (Table 3).

Compared to P larvae, those from A showed a lower proportion of unsaturates (43.9 ± 3.6 versus 51.9 ± 3.2 % of total FA), but a higher percentage of saturates (56.1 ± 2.6 versus 48.1 ± 3.2 %; Figure 2). In consequence, the FA pool of A larvae was characterized by a significantly higher ratio of saturates/unsaturates (1.33 ± 0.21 versus 0.93 ± 0.12; $p < 0.05$). Within the fraction of unsaturated FA, the n3/n6 ratio was significantly higher in A larvae (0.97 ± 0.03 versus 0.56 ± 0.07; $p < 0.05$).

Discussion

Our comparative biochemical study shows that phylogenetically relevant life-history traits of the South American freshwater shrimp *M. amazonicum* vary significantly between two geographically isolated populations (Amazon delta, A versus Pantanal, P). In newly hatched larvae produced under identical conditions in the laboratory, population-specific traits include differential body size, dry weight (W), elemental (CHN), and proximate biochemical composition (total proteins, lipids), as well as different fatty acid (FA) profiles. On the one hand, larvae released by females originating from the Pantanal showed consistently larger body size than A larvae, were heavier (higher W), contained greater amounts of the organically bound elements C, H, and N (collectively CHN), and had a higher protein content (both per larva and in % of W). On the other hand, P larvae contained significantly lower quantities of total lipids than A larvae. The latter difference does thus not correspond with the patterns observed in body size, W, and CHN, but it is congruent with microscopical observations showing smaller amounts of lipid droplets that remain from the egg yolk, being visible in the hepatopancreas region of the larval cephalothorax (Figure 1 of Anger and Hayd 2010). As a consequence, the relative chemical composition of larval biomass measured at

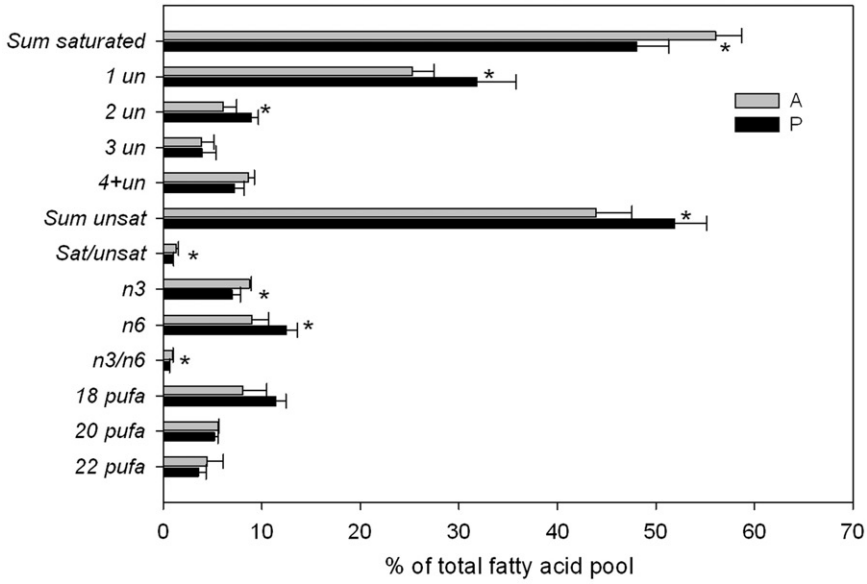

Figure 2. *Macrobrachium amazonicum*, freshly hatched larvae (zoea I) from the Amazon delta (A) and the Pantanal (P). Fatty acid composition expressed in % of the total fatty acid pool (cf. Table 3); mean values ± SD; $n = 4$ hatches per population; significant differences indicated with asterisks ($*p < 0.05$); nested two-way ANOVA ($F_{1,7}$).

hatching differed in a characteristic manner, with P larvae showing significantly lower percentage lipid and C contents (in % of W), as well as lower lipid/protein and C/N mass ratios.

As another consistent difference, we observed that the FA pool of P larvae contained a larger fraction of unsaturated FA within the total FA pool, while A larvae showed a higher percentage of saturates. While the FA profile in planktonic animals commonly reflects the composition of previously consumed food items (e.g., Aurioles-Gamboa et al. 2004; Pond et al. 2005), newly hatched and still non-feeding larvae of *M. amazonicum* (Anger and Hayd 2009, 2010) can only differ due to differential maternal nutrition (Torres et al. 2008) and/or genetic effects. Since also the adult shrimps were maintained under identical conditions of feeding, temperature, salinity etc., different FA profiles can only be explained by variation between separate populations, suggesting genetic differences. Enhanced quantities of saturated FA reserves were observed also in other decapod crustacean larvae with lecithotrophic capabilities (Kattner et al. 2003; Anger et al. 2007; Calado et al. 2007), suggesting that saturates play a particularly important role for the energy storage in eggs (for review, see Anger 2001).

Altogether, our data show that newly hatched A larvae are not only smaller and lighter than P larvae, but they also contain greater amounts of fat. In particular, their higher content of saturated FA may be considered as a metabolically available energy pool. The larger and heavier P larvae, by contrast, contain higher proportions of unsaturated FA, which typically represent structurally bound constituents of cell membranes and other essential organelles, being largely unavailable as a metabolic substrate (Kattner et al. 2003; Calado et al. 2007).

All these observations of variations in the physiological condition of newly hatched larvae are congruent with significantly stronger dependence on food availability in P larvae (Anger and Hayd 2010). Both P and A larvae show an initially high degree of endotrophic potential, however, A larvae are capable of surviving for up to 2 weeks in complete absence of food, whereas P larvae tolerate (at the same temperature) only about 1 week of starvation. This conspicuous difference in early larval dependence on food can now be explained by significantly different energy reserves, which are chemically concentrated and microscopically visible as lipid stores in the cephalothorax.

Population-specific differences in larval biochemistry and physiology (present study) as well as in larval morphology (Knott 2009; Schubert 2010) and growth patterns (Anger et al. 2009; Anger and Hayd 2010) have consistently been observed under constant and controlled conditions, and no reversal of the observed patterns has ever been detected under altered cultivation conditions (e.g. different temperatures, salinities, quality or quantity of feeding; K. Anger, unpublished data). Although phenotypic plasticity has also been observed in the larval development of both populations, causing environmentally induced variability among hatches or individual larvae from the same hatch (Knott 2009; Schubert 2010), very little overlap between size or biomass data, and complete absence of

a reversal in developmental patterns of populations A and P excludes this phenomenon as a theoretically possible explanation for differences between these two populations. This suggests that differential traits described here represent genetically fixed characteristics of isolated populations.

These indications for an advanced speciation within a monophyletic clade that still is referred to as a single species, *M. amazonicum* (Maciel and Valenti 2009), raise the question which differential habitat conditions may have selected for small larval body size but large lipid reserves at hatching (in A) versus a larger body size, higher protein content, and smaller energy reserves (in P), respectively. Compared to early A larvae, those from P also show a faster rate of growth, but this process depends in P larvae to a greater extent on food as an external energy source, while A larvae can rely on their higher internal fat reserves and seem to initially consume less food (Anger and Hayd 2010).

Enhanced lipid stores in A larvae suggest an adaptation to predictably occurring conditions of food limitation during the initial phase of larval development. Poor nutritional conditions in the plankton are likely to occur immediately after hatching in large rivers of the lower Amazon and comparable river systems in northern South America, as well as during the subsequent downstream transport to estuarine or coastal marine waters (see, e.g., Anger et al. 2009; Anger and Hayd 2009). Later larval stages, which increasingly depend on planktonic food availability, may find better nutritional conditions in estuarine waters, where plankton productivity is normally much higher than in lotic waters (for review, see Morgan 1995). This scenario of larval export is strongly supported by experimental observations showing that early A larvae survive only for a short period (maximally a few days) in freshwater, where the adult populations live, grow, and reproduce. In contrast to the conspecific adults, the larval stages thus require a moderate salt concentration for survival and development to metamorphosis (Araujo and Valenti 2007; Anger et al. 2009; earlier references cited therein).

In populations living in swamps and creeks of the Pantanal, the larvae cannot possibly be transported to brackish estuaries or coastal waters but are retained within the paternal habitat. During the development in shallow lentic inland waters, large body size, an early onset of feeding, and fast growth may allow to play a major role as consumers in highly productive limnic plankton communities, where larger larvae have access to larger prey (Morgan 1995), suggesting a shift from filter feeding toward predation. Higher protein contents in P larvae may be associated with an advanced development of swimming musculature, facilitating both their feeding activities and the escape from fish and invertebrate predators living in limnic habitats. Survival and successful development of P larvae in freshwater is possible, because they show a strong hyper-osmoregulatory capacity (Charmantier and Anger 2011). Amazon larvae, by contrast, are capable of hypo-osmoregulation (this function is absent in Pantanal larvae), allowing to tolerate high salinities, which are commonly encountered in estuaries.

While an enhanced independence from planktonic food is plausible for A larvae, it remains unclear why such an energetically expensive strategy, i.e. an enhanced female energy allocation to offspring production, is also found in P larvae (although to a lesser extent than in A larvae). In the Pantanal, planktonic food should not be a limiting factor, so that no adaptive value of initial lecithotrophy is apparent. Also, P larvae show faster development and higher survival rates at low or moderate salt concentrations (Schubert 2010), which do not occur in their natural environment (Bialetzki et al. 1997; Heckman 1998). The most plausible explanation for these unexpected observations should be a persistence of ancestral traits. Palaemonid shrimps living in freshwater, including *Macrobrachium* spp., are generally believed to originate from coastal marine ancestors (Walker 1992; Jalihal et al. 1993; Anger 2001; Murphy and Austin 2005). In *M. amazonicum*, the ancestral clade was probably split during the formation of the modern South American drainage system, when the La Plata basin was hydrologically separated from the Amazon basin. This caused an interruption of gene flow in the aquatic fauna, presumably already since the late Tertiary (for discussion and references, see Anger and Hayd 2010; Charmantier and Anger 2011).

The principal question behind this comparative study was related to the degree of similarity or divergence between two geographically separated and hydrologically isolated populations of *M. amazonicum*. Phylogenetic divergence in reproductive and developmental traits is generally considered as a crucial step in allopatric speciation within monophyletic groups. Hence, the conspicuous and consistent ontogenetic differences observed in our study are hardly compatible with the concept of intraspecific variability. This suggests that *M. amazonicum* in the Amazon delta and in the Pantanal are at least in an incipient phase of phylogenetic separation or, more likely, they may represent closely related but already separate species that have successfully adapted to differential environments, showing significant interspecific variation in relevant life-history traits. As soon as more suitable gene markers have been found and the rate of molecular evolution is better understood (Lanfear et al. 2010), this speciation event will most probably be shown also in future molecular genetic studies.

Acknowledgements

We thank Dr Liliam Hayd for transporting live shrimps from Brazil to Helgoland and IBAMA (Brasília) for the export permit. Uwe Nettelmann helped in the maintenance of animals, Bettina Oppermann and Julia Haafke in elemental analyses. Ángel Urzúa was financially supported by the Deutscher Akademischer Austauschdienst (DAAD, Bonn, Germany) and the Comisión Nacional de Ciencia y Tecnología, CONICYT (Santiago de Chile), funding this study as a part of his doctoral dissertation. The experiments comply with animal manipulation laws in Germany.

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CHAPTER II

The bioenergetic fuel for non-feeding larval development in an endemic palaemonid shrimp from the Iberian Peninsula, *Palaemonetes zariquieyi*

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Titel:

The bioenergetic fuel for non-feeding larval development in an endemic palaemonid shrimp from the Iberian Peninsula, *Palaemonetes zariquieyi*

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Abstract

Palaemonetes zariquieyi, an endemic palaemonid species of shrimp that lives in freshwater and brackish coastal habitats in northeastern Spain, shows an abbreviated, non-feeding larval development comprising only three stages. In order to identify the endogenous bioenergetic fuel that allows for food-independent development from hatching to metamorphosis, ontogenetic changes in dry weight (DW), elemental (CHN) and lipid composition (total lipids, principal lipid classes, fatty acids [FA]) were quantified under controlled laboratory conditions. Values of DW, C, H, and energy content (estimated from C data) per larva and per mass unit of DW decreased throughout the time of larval development, while the N content showed only a weak decline (suggesting strong lipid but only little protein degradation). Correspondingly, directly measured values of total lipids (both in µg/larva and in % of DW) decreased gradually, with neutral lipids remaining the predominant and most strongly used fraction. In contrast to the neutral lipids, the fraction of polar lipids per larva remained stable and, as a consequence, tended to increase as a percentage of total lipids. Our results indicate that the lecithotrophy of *Palaemonetes zariquieyi* is primarily fuelled by the utilization of carbon content and lipids (especially triacyglycerides and other neutral lipids), whereas polar lipids were preserved as structurally indispensable components (membranes) during larval growth. Additionally, other important lipid fractions, such as free fatty acids (FFA) and sterols, remained stable during larval ontogeny, while sterol wax ester/ waxes were no detected. Among the FA, palmitic (16:0), oleic (18:1n-9), linoleic (18:2n6) and eicosapentaenoic (20:5n-3) acid were predominant, showing a significant decrease during larval development. Stearic (18:0), vaccenic (18:1n-7) and arachidonic acid (20:4n-6) were found in small amounts, whereas eicosanoic (20:0) and heneicosapentaenoic acid (21:5n-3) were recorded only in juvenile stage. The abbreviated and non-feeding mode of larval development may have an adaptive value in land-locked freshwater habitats, where *P. zariquieyi* commonly lives. The patterns of reserve utilization are similar to those previously observed in other palaemonid shrimps and various other groups of decapod crustaceans with abbreviated and lecithotrophic modes of larval development, suggesting multiple convergent evolution of bioenergetic traits allowing for reproduction in food-limited aquatic environments.

Keywords: Caridea; freshwater; endemic; lecithotrophy; larval development; reproduction

Introduction

Reproductive and developmental adaptations that allow for invasions of limnic environments by marine crustaceans are among the top issues in evolutionary ecology (e.g. Lee and Bell 1999; Anger et al. 2007). Among the caridean shrimps, Palaemonidae Rafinesque, 1815 have been particularly successful invaders of brackish, estuarine and freshwater habitats (Ashelby et al. 2012). Within this family, most estuarine and limnic species belong to the genera *Macrobrachium* Spence Bate, 1868 and *Palaemonetes* Heller, 1869 (Jalihal et al. 1993; Murphy and Austin 2005).

Most palaemonid shrimps pass through complex life cycles (Bauer 2004). These comprise (1) embryogenesis inside the eggs, which are attached underneath the female abdomen, (2) a free-living pelagic, in most cases planktotrophic larval development, and (3) a benthic juvenile - adult phase that gradually leads to maturation and reproduction. In the early life-history stages, different reproductive strategies such as larval export towards the sea or retention within the adult habitat, respectively, are associated with ontogenetic changes in the tolerance of variations in environmental conditions including changes in salinity and food availability (Anger and Hayd 2009; Charmantier et al. 2011).

Studies of life history adaptations to non-marine conditions with low salinities and unpredictable planktonic food availability contribute significantly to the understanding of transitions and subsequent speciation of originally marine animals in limnic and terrestrial environments. Compared to marine and estuarine species, fully freshwater-adapted clades show significant shifts in the salinity optimum as well as tendencies towards larger egg size, a prolonged embryonic incubation period, an abbreviated mode of larval development, and facultative or complete lecithotrophy (Lee and Bell 1999). These reproductive traits have been considered as adaptations to limited or unpredictable plankton production in freshwater environments.

Abbreviated modes of larval development and lecithotrophy have evolved also in numerous palaemonid shrimps living in food-limited freshwater habitats (for recent review, see Bauer 2004; Murphy and Austin 2005; Anger in press). These ontogenetic traits involve various biochemical and physiological adaptations such as an enhanced initial energy storage (Nates and McKenney 2000; Urzúa and Anger 2011) or energy saving mechanisms (McNamara et al. 1983).

The subject of the present study, the caridean shrimp *Palaemonetes zariquieyi* Sollaud 1939, an endemic species of the eastern Iberian Peninsula, inhabits aquatic environments ranging from pure freshwater habitats to oligohaline channels, pools and lagoons along the Mediterranean coast of Spain (Zariquiey 1968; Sanz Brau 1983). Due to its restricted distribution, *P. zariquieyi* is considered as a potentially endangered species, and thus, is under conservation management. This species shows an abbreviated and lecithotrophic larval development with only three stages (Guerao 1993), which occurs in the parental habitat (Sanz 1980; Guerao 1993), where planktonic food limitation may occur (Sanz-Brau 1986).

While the ecology and physiology of adult *Palaemonetes zariquieyi* has been well studied (Sollaud 1938; Margalef 1953; Sanz-Brau 1986), there is very little information on the larval phase. This includes poor knowledge of the endogenous bioenergetic substrate that allows for food-independent development. In the present investigation, changes in larval biomass and chemical composition occurring during the lecithotrophic development from hatching to the first juvenile stage were studied under controlled laboratory conditions.

Materials and methods

Sampling and maintenance of ovigerous females and larvae

Adult shrimps of *Palaemonetes zariquieyi* were collected in February 2008 from a freshwater channel in Alicante (Spain) and transported in boxes with aerated water to the IRTA (Sant Carles de la Ràpita). In the laboratory, ovigerous females (n = 420; TL = 39 ± 3 mm) were maintained in recirculating aquariums with aerated freshwater, with constant conditions of temperature and photoperiod (18 ± 1°C, a 12:12 h light:dark), and pieces of frozen mussels (*Mytilus sp.*) and *Artemia* sp. metanauplii were provided as food.

The ovigerous females were separated individually in 40 L aquaria and checked daily for the occurrence of newly hatched larvae. These were collected and immediately transferred to individual beakers with 100 mL filtered freshwater maintained at the same conditions of temperature and photoperiod mentioned above. The water was daily changed and the moults and development time of each stage were recorded. The larval stages and first juvenile were not fed along the study due to their lecithotrophic development (Guerao 1993). A total of 329 zoeae I (ZI), 430 zoeae II (ZII), 468 zoeae III (ZIII) and 199 juveniles (J) were used for parallel biomass and chemical analyses.

Biomass and chemical composition

From seven different parental females, offspring at each development stage were taken for parallel determinations of biomass (dry weight, DW), elemental composition (contents of carbon, hydrogen and nitrogen; collectively CHN), and lipid composition (total lipids, lipid classes and fatty acids).

Dry weight and elemental composition

Dry weight (W) and elemental composition (CHN) were measured with standard techniques (Anger and Harms 1990): larvae were briefly rinsed in distilled water, blotted on fluff-free Kleenex paper, transferred to pre-weighed tin cartridges, and stored at –20°C. Later, the samples were freeze-dried for 48 h in a vacuum dryer (Christ Alpha 1-4 LSC), and W was determined to the nearest 0.1 mg on a Sartorius SC2 ultra micro balance. Subsequently, the samples were analysed with an Elemental Vario Micro CHN Analyser using Sulphanilamide as standard. The energy content was estimated from the carbon data (Salonen et al. 1976).

Lipid composition

Total lipid content from each larval stage was quantified gravimetrically after extraction in chloroform/methanol (2:1 vol:vol) and evaporation of the solvent under nitrogen gas (Folch et al. 1957). Total lipids were stored (10 mg mL^{-1}) in chloroform / methanol (2:1 vol:vol) containing 0.01% butylated hydroxytoluene at -20°C for subsequent analyses of lipid class and fatty acid composition.

Lipid class determination and separation was performed by high-performance thin-layer chromatography (HPTLC) following the method described by Olsen and Henderson (1989). After separation, bands were identified by charring the plates at 100°C for 30 min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (vol:vol) H_3PO_4 and quantified by scanning densitometry using a GS 800 Calibrated Densitometer (Bio-Rad Laboratories Inc, USA). Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalyzed transmethylation using 2 ml of 1% H_2SO_4 in methanol plus 1 mL toluene (Christie 1982) and thereafter extracted twice using isohexane/diethyl ether (1:1 vol:vol) (Ghioni et al. 2002) and purified on TLC plates. FAME were separated and quantified by gas–liquid chromatography on a Trace GC (Thermo Fisher Scientific Inc, USA) using a flame ionization detector and on column injection. Individual methyl esters were identified by

comparison to known standards (Supelco 37 FAME mix 47885-U), and quantified by means of the response factor to the internal standard 21:0 fatty acid, added prior to transmethylation, using a Chrompack software (Thermo Electron, UK).

The area of the fat droplets, in the hepatopancreas region, was calculated using a stereo microscope (Olympus SZX2- ILLB) equipped with a calibrated eyepiece micrometer and a digital camera. The images obtained were digitalized with the CELL (Olympus) image analysis software.

Statistical analyses

Statistical analyses were performed with standard methods (Sokal and Rohlf 1995) using the statistic software package STATISTICA 8 (StatSoft). Differences in biomass and biochemical composition between stages were tested by one-way ANOVA. Significant differences were analyzed with a multiple comparison test (Student-Newman-Keuls). All tests were run on the 95 % confidence level ($p < 0.05$). Normality and homogeneity of variances were tested with Kolmogorov–Smirnov and Levene's tests, respectively. When the data did not meet the assumptions, the non-parametric Kruskal–Wallis and Dunn´s multiple comparison test were applied.

Results

Larval stage and development time

Palaemonetes zariquieyi during the ontogenetic development showed 3 zoeal stages prior to first juvenile, the average development time from zoea I (ZI) to first juvenile (J) was 10 ± 2 days.

Biomass and elemental composition

Larval biomass and elemental composition revealed conspicuous changes during the ontogeny. The absolute values of DW, C, H and energy decreased significantly throughout the ontogenetic development, with maximum average values observed in ZI and minimum average values in J (ca. 500 vs. 440 µg W · ind^{-1}, 265 vs. 220 µg C · ind^{-1}, 39 vs. 30 µg H · ind^{-1} and 11 vs. 8 Joule · ind^{-1}; cf. ZI vs. J) (Figure 1a, b, d, e). The N values per larva did not show significant differences during the larval development (Figure 1c).

Lipid composition

The relative composition expressed in % of DW showed similar tendencies as observed in absolute values. Thus, the percentage of C (53 vs. 46%, cf. ZI vs. J), H (8 vs. 7%, cf. ZI vs. J), energy content (22 vs. 18 J mg DW^{-1}, cf. ZI vs. J) and C/N ratio (5 vs. 4.4, cf. ZI vs. J) decreased gradually and significantly from ZI until J (ANOVA, p <0.05; Figure 2a, c, d, e), whereas the percentage of N remained stable with average values of about 10.5% (Figure 2b).

Total lipids

Lipid content gradually decreased during the ontogeny (Figures 3 and 4), thus in the hepatopancreas region of the cephalotorax (Figure 4), the size of lipid droplets gradually decreased from ZI (0.701 µm²) to ZIII (0.377 µm²). Consistently with these microscopy observations, the average lipid values in the first zoea were twice the values observed in the juvenil (90 µg · ind^{-1} vs. 45 µg · ind^{-1}; $p < 0.05$) (Figure 3a). Relative lipid composition content also decreased significantly throughout the larval development, with maximum percentage in ZI and minimum values in JI (ca. 17% vs. 10%; $p < 0.05$) (Figure 3b).

Lipid class

Total neutral lipids (NL) were always higher in average percentages than total polar lipids (PL) (78% vs. 22%; $p < 0.05$). PL increased during the ontogeny, from 22% in ZI until 36% observed in J. In contrast, NL showed an opposite pattern, with average values decreasing from 78% until 64% in ZI and J, respectively ($p < 0.05$) (Table 1).

Triacylglycerols (TAG) and cholesterol (CHOL) were always in higher amounts than the rest of NL. TAG decreased significantly during larval development (54% vs. 33%; ZI vs. J, respectively) whereas CHOL increased from 18% in ZI until 24% in J. Free fatty acids (FFA) occurred in low quantities and remained stable along larval development, with average values of 6%. On the other hand, other neutral lipids, such as sterol esters/waxes, were not detected along the ontogenetic development in this species (Table 1).

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the predominant polar lipids. Both increased during the ontogeny with minimum PC percentages recorded in Z I and maximum in J stage (9% vs. 17%; $p < 0.05$) (Table 1). Some PLs such as phosphatidylserine + phosphatidylinositol (PS+PI) and lyso-phosphatidylethanolamine (LysoPE) were found in

small amounts (2% and 1%, respectively), while other occurred only in traces e.g. sphingomyelins (SM) (Table 1).

Fatty acid composition (FAs)

The total saturated fatty acids (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) significantly decreased along the development. The total PUFA value was higher than total values observed in MUFA and SFA. Within the total fatty acid pool, saturated and monounsaturates fatty acids dominated throughout larval development, followed by the fractions of PUFA (Table 2). The most representative FAs, were in general, palmitic (16:0), oleic (18:1n-9), linoleic (18:2n6) and eicosapentaenoic acid (20:5n-3). Stearic (18:0), vaccenic (18:1n-7) and arachidonic acid (20:4n-6) were found in small amounts, while other FAs, e.g. eicosanoic (20:0) and heneicosapentaenoic acid (21:5n-3) were recorded only in ZIII and J (Table 2).

Total content of PUFA n-6 and n-3, decreased significantly from ZI (15.96 ± 1.6 PUFAn-6; 21.78 ± 1.6 PUFA n-3, µg · mg W^{-1}) to J (9.53 ± 1.4 ω-6; 12.14 ± 1.4 ω-3, µg · mg W^{-1}) (Table 2). 18:2n-6 (LA) was the most abundant n-6 PUFA showing higher values in ZI than in J (13.71 ± 1.2 vs. 7.83 ± 0.6 µg · mg W^{-1}) ($p < 0.05$). 20:5n-3 (EPA) was the predominant n-3 PUFA showing maximum values in ZI (13.39 ± 1.6 µg · mg DW^{-1}) and minimum in J (7.82 ± 1.5 µg · mg W^{-1}) (Table 2).

Discussion

In palaemonid shrimps, the types of larval development are associated with exportation or retention strategies in the adult habitat, i.e. extended or abbreviated development, both in response to planktonic food availability. In the case of *Palaemonetes zariquieyi*, as well as in other freshwater palaemonid shrimps (see Table 3), larval development is abbreviated and consists of three larval stages prior to metamorphosis.

Abbreviated development in decapod crustacean larvae is normally associated with high quantities of lipid reserves (Anger and Moreira 2004; Kattner et al. 2003; Thatje and Mestre 2010), that allow the larvae a relative independence of food (Anger 2001). The results of the present study, in terms of biomass and chemical composition of the early life history stages of *Palaemonetes zariquieyi* provide evidence for lecithotrophy in this species. This food - independent larval development is based on high initial organic reserves, mainly

consisting of enhanced lipid stores remaining from the egg (for comparison with planktotrophic larvae, see Anger 2001).

Biomass components (DW, C, H) and C/N ratio decreased from hatching to metamorphosis, with N remaining relatively stable during development. These results suggest an independence of external energy resources in the larvae of *P. zariquieyi*. A decrease in C is related to the utilization of internal lipid reserves during larval development in the absence of food. In contrast N content, linked to proteins and thus structural components, was conserved during development. The reduction in C/N ratio, considered as a measure of lipid/protein ratio, could result from larvae using more lipids than proteins, which in turn are utilized in developmental processes and larval growth. Similar patterns of changes in biomass and chemical composition during ontogeny have been reported in other species of crustacean decapods with lecithotrophic larval development (*Lepidophthalmus louisianensis* Schmitt, 1935: Nates and Mc Kenney 2000; *Lithodes santolla* Molina, 1782 and *Paralomis granulosa* Jacquinot, 1847: Kattner et al. 2003; *Sesarma curacaoense* De Man, 1892 and *Armases miersii* Rathbun, 1897: Anger and Schultze 1995).

In decapod crustacean larvae, the lipid composition reflects changes in developmental state, nutritional condition, and effects of environmental factors (Andrés et al. 2010, Urzúa and Anger 2011). Among the lipids, TAG, PLs and free sterols usually constitute the predominant lipid fractions (Arts et al. 2009). NLs, mainly TAG, are a major energy source during periods of food limitation, while phospholipids and sterols change relatively little under suboptimal nutritional conditions (Anger 2001; Arts et al. 2009). According to the results observed in the present study, both microscopic observations and chemical analyses showed that the lipid reserves are gradually utilized in the absence of food. In *P. zariquieyi,* similarly as reported in lecithotrophic larvae of other decapod crustaceans (Nates and Mc Kenney 2000; Katnner et al. 2003), the utilization of lipids was closely related to that of NLs (in particular TAG), which decreased from ZI to J, whereas PLs showed the opposite pattern. These results indicate that while NLs were used as an energy source, PLs were deposited as structural components (membranes) during larval growth. Additionally, other important lipid fractions, such as free fatty acids (FFA) and sterols, remained stable during larval ontogeny, while sterol wax esters/waxes were not detected. An increase in the relative proportion of CHOL was observed from ZI to J as a consequence of total lipid, NL and TAG reduction. While NLs and TAG were reduced from 78 and 54% in ZI to 64 and 33% in J, respectively, CHOL was conserved in similar amounts along larval development (from 2.38 mg g^{-1} DW in

ZI to 1.57 mg g^{-1} DW in J) showing the important role that CHOL plays for good growth and survival of crustaceans (Sheen 2000).

The fatty acid composition of larval stages was characterized by the high content of palmitic, oleic, LA and EPA comprising over 50% of the total fatty acids. These fatty acids are common in caridean shrimps with abbreviated larval development (Thatje et al. 2004; Calado et al. 2010). The high content of stearic acid is explained by its predominance in membrane phospholipids (Kattner et al. 1994; Wehrtmann and Graeve 1998). Whereas, the high proportion of oleic, LA and EPA indicates that larval development in this species is supported by the lipid material derived from the female. These fatty acids are considered essential fatty acids for crustaceans with high influence on larval survival, development, and growth (Anger 2001; Calado et al. 2005; Nghia et al. 2007). During larval development PUFA, especially n-3 series, were preferentially conserved in larval tissue, compared to SFA and MUFA mostly used for energy (see Table 2).

During their larval development in the adult habitat (retention strategy: Strathmann 1982), *P. zariquieyi* shows conspicuous life-history adaptations: (1) abbreviated larval development, (2) high larval biomass, (3) high initial lipid content and (4) lecithotrophy. This flexibility should have an adaptive value in freshwater environments (e.g. lagoons, rivers and upper estuaries), where the physical and nutritional conditions may be unpredictable and plankton production temporally and spatially patchy. The peculiar habitats of these shrimps do not provide enough planktonic food items, and, during the course of paleographic formation of such habitats, food limitation must have selected for full lecithotrophy in the larval stages. In this context, the question is how *P. zariquieyi* arrived to this freshwater habitat, and which paleogeographic scenario drove the invasion and subsequent segregation of populations, favouring finally the speciation. Probably the populations of *P. zariquieyi* were separated from co-specific marine populations during the Messinian salinity crisis (i.e. desiccation of the Mediterranean: Krigsjman et al. 1999; Garcia et al. 2011), and some populations remained isolated in inland waters without connections with marine waters. Under this scenario of retention of larval stages within parental habitats, *P. zariquieyi* may have developed adaptations of early life history traits (e.g. abbreviated development and lecithotrophy) in response to non marine conditions.

In palaemonid shrimps, including some marine species, the earliest larval stages frequently show a tendency of independence from planktonic food (see Bauer 2004; Ituarte et

al. 2005; Calado et al. 2007; Anger and Hayd 2010), and numerous other aquatic Decapoda also pass through non-feeding or facultatively lecithotrophic developmental phases. Therefore, this lecithotrophy may be explained as adaptations to specific life styles (Anger 2001). Future comparative studies should investigate physiological mechanisms and adaptive implications, which will enhance our understanding of the life history evolution of crustaceans in general.

Acknowledgements

We thank Marta Sastre at IRTA for her help during the biochemical analysis of the larvae; Julia Haafke and Bettina Opperman for the elemental analyses. AU was financially supported by the Deutscher Akademischer Austauschdienst (DAAD, Bonn, Germany) and the Comisión Nacional de Ciencia y Tecnología, CONICYT (Santiago de Chile), funding this study as a part of his doctoral dissertation. This work was partially supported by the Ministry of Science and Research to GG (post-doctoral fellowship; INIA). The experiments comply with animal manipulation laws in Germany.

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Legend of figures and tables

Figure 1. *P. zariqueiyi*. Dry weight (DW) and elemental composition (CHN) during the larval development (Zoea I, II and III) and first Juvenil (J): (A) dry weight, (B) carbon, (C) nitrogen, (D) hydrogen and (E) energy content, all expressed in absolute values (µg per individual or joule per individual). ANOVA (*F*-values) and significance level (*p*), mean values ± SD. Different lower case letters indicate significant differences among stage of development (after SNK test)

Figure 2. *P. zariqueiyi*. Relative chemical composition during the larval development (Zoea I, II and III) and first Juvenil (J): (A) carbon, (B) nitrogen, (C) hydrogen, (D) C/N ratio and (E) energy content, all expressed as percentage of dry weight (W) or joule per mg W^{-1}. ANOVA (*F*-values), Kruskal–Wallis (*H*) and significance level (*p*), mean values ± SD. Different lower case letters indicate significant differences among stage of development (after SNK or Dunn´s test)

Figure 3. *P. zariqueiyi.* Lipid content during the larval development (Zoea I, II and III) and first Juvenil (J), (A) expressed in µg · ind^{-1} and (B) in % of dry weight. ANOVA (*F*-values) and significance level (*p*), mean values ± SD. Different lower case letters indicate significant differences among stage of development (after SNK test)

Figure 4. *P. zariqueiyi.* Lipid droplets in the hepatopancreas region of the cephalotorax during the larval development (Zoea I, II and III)

Table 1. *P. zariqueiyi.* Lipid class composition, expressed in mg/g and in % total lipids, during larval development (Zoea I, II and III) and first Juvenil (J). Data is shown as mean ± SD. Different lower case letter in a row represent significant differences among developmental stages (ANOVA, SNK test, $p < 0.05$). Total Polar (Total PL): sum of sphingomyelins (SM), lysophosphatidylcholine (LysoPC), phosphatidylcholine (PC), phosphatidylserine + phosphatidylinositol (PS+PI), Phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (LysoPE); Total Neutral (Total NL): sum of Cholesterol (CHOL), free fatty acids (FFA), Tryacylglycerols (TAG) and Sterol ester/waxes (SE+W)

Table 2. *P. zariqueiyi.* Changes in the fatty acid (FA, µg FA · mg W^{-1}) during larval development (Zoea I, II and III) and first Juvenil (J) Data is shown as mean ± SD. Different lower case letter in a row represent significant differences among developmental stages (ANOVA, SNK test, $p < 0.05$). SFA (Saturated FA): sum of 14:0, 15:0, 16:0, 18:0 and 20:0; MUFA (Monounsaturated FA): sum of 16:1n-9, 18:1n-9, 18:1n-7 and 20:1n-9; PUFA n-6 (Polyunsaturated FA n-6): sum of 18:2n-6, 18:3n-6, 20:3n-6, 20:4n-6, 22:5n-6; PUFA n-3 (Polyunsaturated FA n-3): sum of 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, 21:5n-3, 22:5n-3, 22:6n -3; TOTAL PUFA: sum of PUFA n-3 and PUFA n-6.

Table 3. Comparison between habitat and number of larval stages of *Palaemonetes* species

Figures and Tables

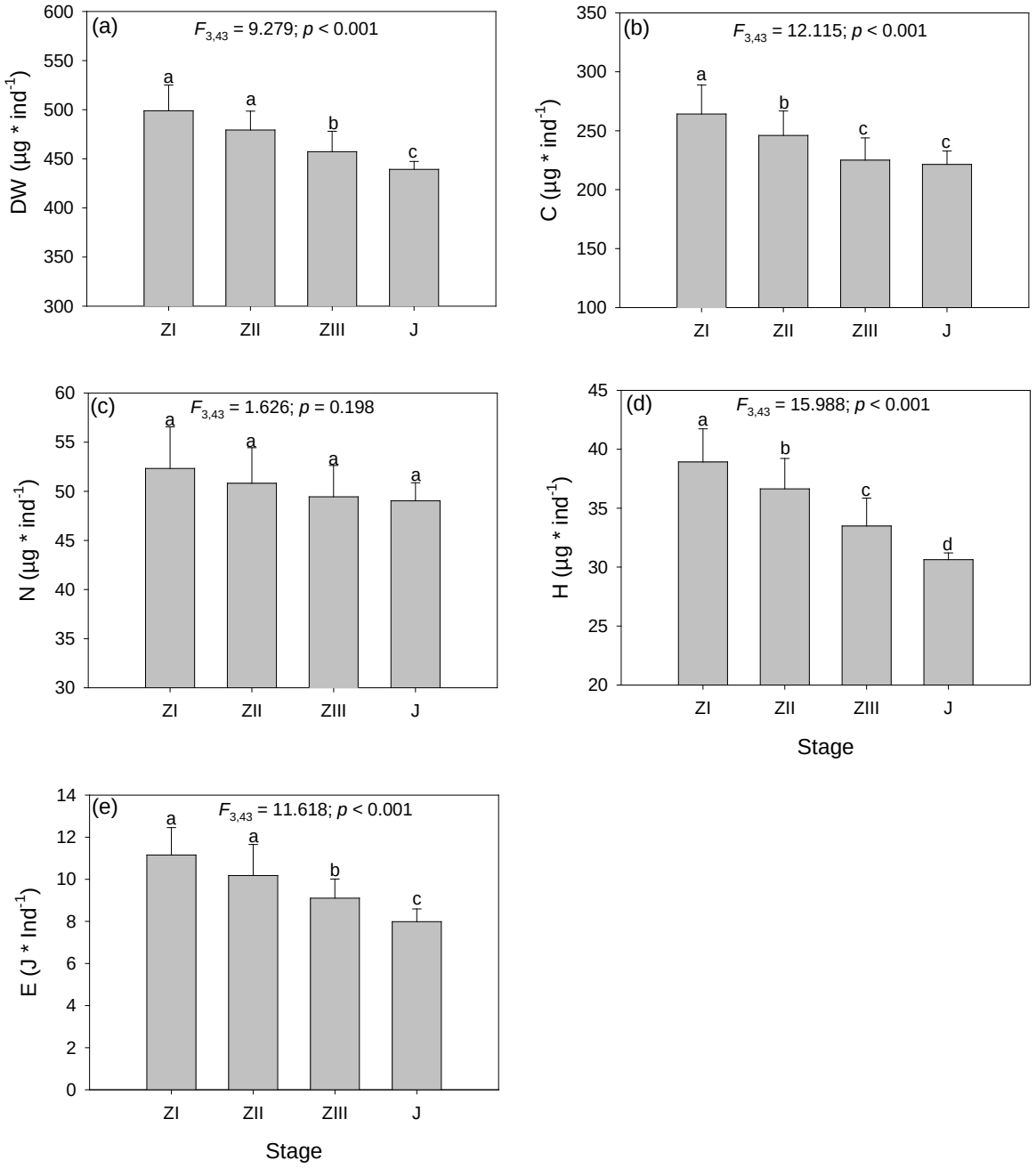

Figure 1

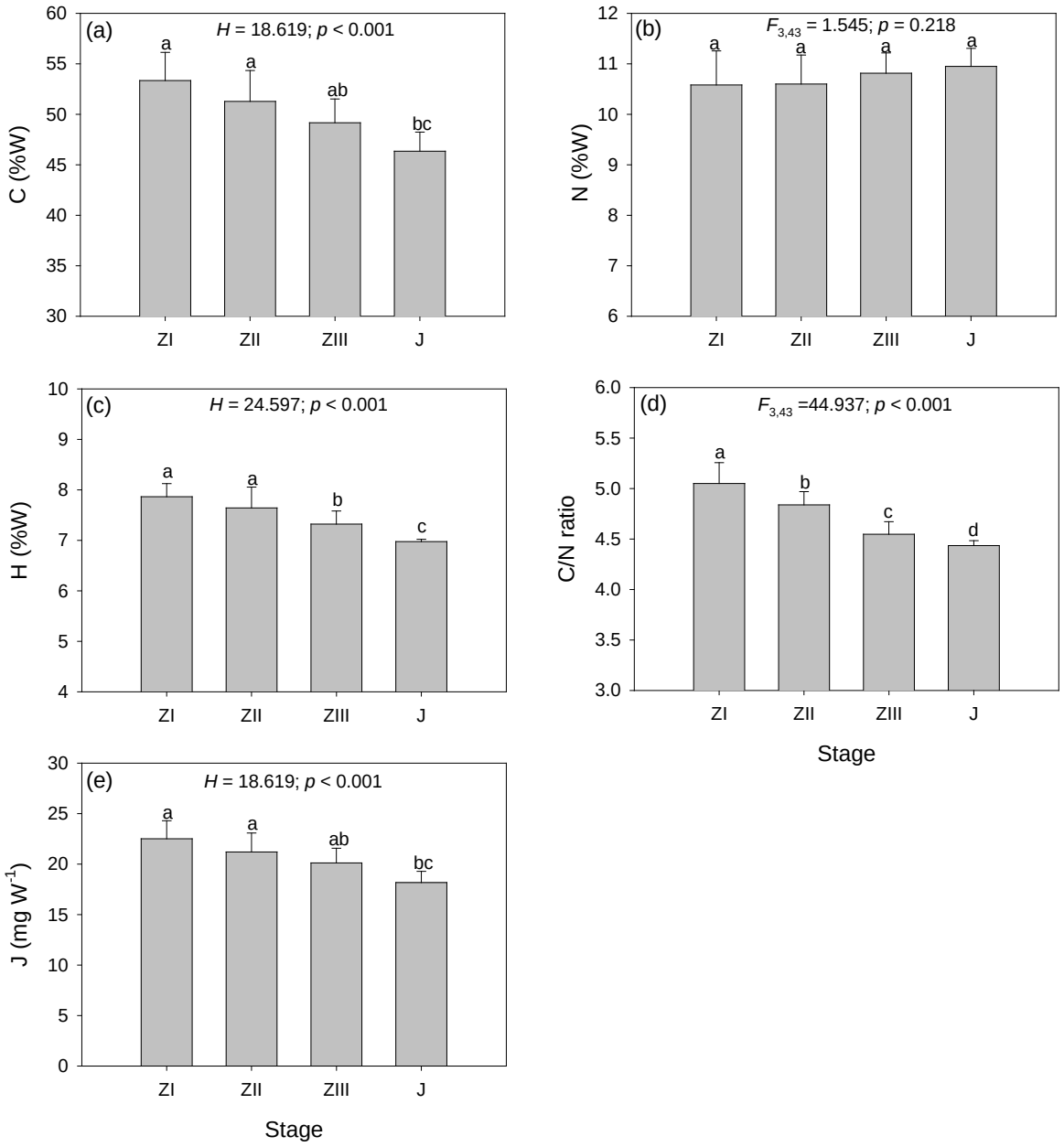
(a)
H = 18.619; p < 0.001
C (%W)
(b)
F3,43 = 1.545; p = 0.218
N (%W)
(c)
H = 24.597; p < 0.001
H (%W)
(d)
F3,43 =44.937; p < 0.001
C/N ratio
(e)
H = 18.619; p < 0.001
J (mg W-1)
ZI
ZII
ZIII
J
Stage

Figure 2

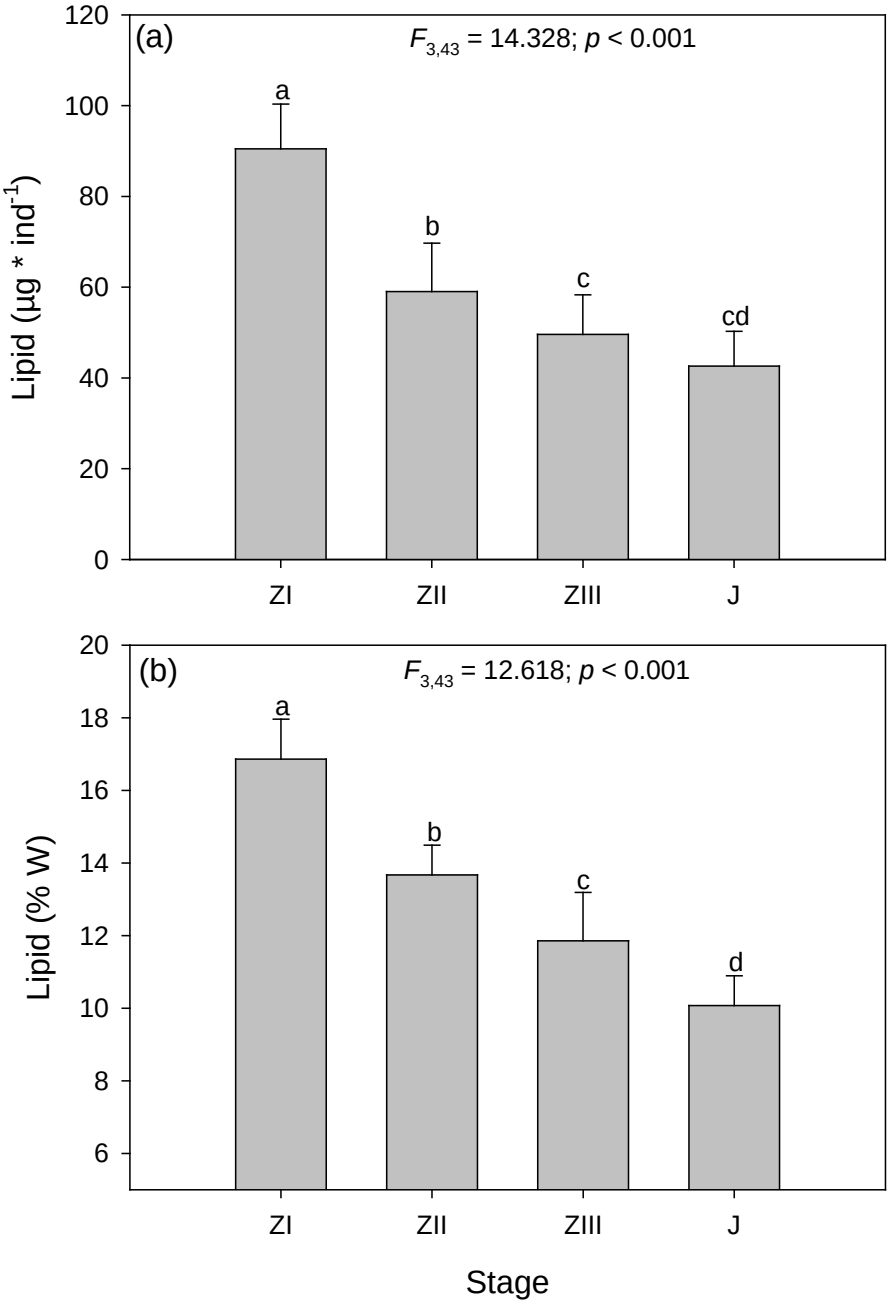
(a)
$F_{3,43}$ = 14.328; p < 0.001
Lipid (µg * ind^{-1})
120
100
80
60
40
20
0
a
b
c
cd
ZI
ZII
ZIII
J
(b)
$F_{3,43}$ = 12.618; p < 0.001
Lipid (% W)
20
18
16
14
12
10
8
6
a
b
c
d
ZI
ZII
ZIII
J
Stage

Figure 3

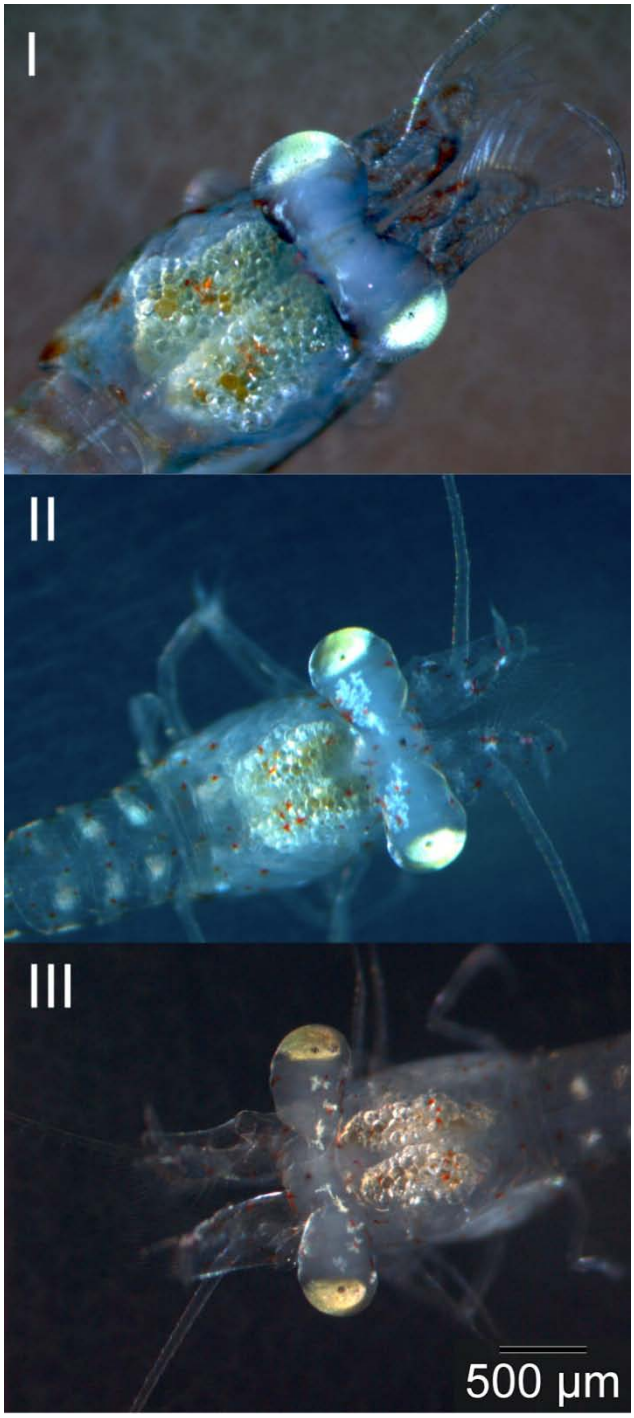

Figure 4

Table 1

Lipid class	ZI	ZII	ZIII	J
Total lipids ($mg \cdot g^{-1} \cdot DW$)	168.6	136.7	118.6	100.7
SM	0	0	0.49± 0.002[a] 0.41%	0.43± 0.001[a] 0.42%
PC	16.27 ± 0.009[a] 9.65%	12.17± 0.004[a] 8.90%	14.53 ± 0.001[b] 12.25%	17.27 ± 0.003[c] 17.15%
PS+PI	4.47 ± 0.007[a] 2.65%	3.66 ± 0.001[b] 2.68%	2.87 ± 0.002[b] 2.42%	4.09 ± 0.001[c] 4.06%
PE	13.89 ± 0.002[a] 8.24%	11.56 ± 0.004[a] 8.46%	10.10 ± 0.001[a] 8.52%	12.73 ± 0.009[b] 12.64%
LysoPE	2.07 ± 0.001[a] 1.23%	1.75± 0.003[a] 1.28%	0.75 ± 0.001[b] 0.63%	1.68 ± 0.003[c] 1.67%
Total PL	36.99 ± 0.021[a] 21.94%	29.46 ± 0.014[b] 21.55%	28.71 ± 0.001[b] 24.21%	36.17 ± 0.016[c] 35.92%
CHOL	30.38± 0.002[a] 18.02%	26.64 ± 0.003[b] 19.49%	26.71 ± 0.022[c] 22.52%	24.66 ± 0.008[c] 24.49%
FFA	9.96 ± 0.004[a] 5.91%	8.86 ± 0.002[b] 6.48%	7.98 ± 0.004[b] 6.73%	6.60 ± 0.001[b] 6.55%
TAG	91.31± 0.006[a] 54.16%	71.73 ± 0.009[b] 52.47%	55.16 ± 0.005[c] 46.51%	33.25 ± 0.025[d] 33.02%
Total NL	131.6 ± 0.012[a] 78.06%	107.2 ± 0.014[a] 78.44%	89.86 ± 0.03[b] 75.77%	64.52 ± 0.034[c] 64.07%

Table 2

Fatty acid composition	ZI	ZII	ZIII	J
Total lipids (mg g^{-1} DW)	168.6	136.7	118.6	100.7
µg FA mg $lipid^{-1}$	530.96 ± 102	691.52 ± 78	581.90 ± 86	545.38 ± 60
14:0	1.31 ± 0.1[a]	1.68 ± 0.09[b]	1.27 ± 0.11[a]	0.91 ± 0.07[c]
15:0	0.80 ± 0.09[a]	1.09 ± 0.06[b]	0.64 ± 0.04[c]	0.57 ± 0.1[c]
16:0	16.38 ± 1.2[a]	18.55 ± 0.9[b]	12.98 ± 0.8[c]	10.12 ± 1.1[d]
18:0	3.06 ± 0.1[a]	2.93 ± 0.12[a]	2.79 ± 0.9[b]	2.72 ± 0.8[b]
20:0	0	0	0.29 ± 0.01[c]	0.27 ± 0.06[c]
SFA	21.54 ± 1.8[a]	24.24 ± 1.6[b]	18.15 ± 1.2[c]	14.59 ± 1.1[d]
16:1n-9	7.40 ± 0.9[a]	9.23 ± 0.7[b]	3.99 ± 0.9[a]	3.24 ± 1.2[a]
18:1n-9	18.43 ± 1.8[a]	25.58 ± 2.6[b]	13.23 ± 3.2[c]	8.21 ± 1.4[d]
18:1n-7	4.70 ± 1.9a	3.9 ± 0.8[a]	5.30 ± 1.2[a]	5.27 ± 0.8[a]
20:1n-9	0.25 ± 0.1[a]	0.30 ± 0.1[b]	0.10 ± 0.06[c]	0.09 ± 0.05[c]
MUFA	30.78 ± 2.8[a]	39.01 ± 2.1[b]	22.63 ± 1.8[c]	16.85 ± 3.1[d]
18:2n-6	13.71 ± 1.2[a]	12.97 ± 0.9[a]	11.12 ± 1.1[b]	7.83 ± 0.6[c]
18:3n-6	0.50 ± 0.1[a]	0.55 ± 0.1[a]	0.27 ± 0.2[b]	0.20 ± 0.1[c]
20:3n-6	0.12 ± 0.1[a]	0.17 ± 0.2[b]	0	0
20:4n-6	1.63 ± 0.2[a]	2.26 ± 0.3[b]	1.27 ± 0.1[c]	1.24 ± 0.1[c]
22:5n-6	0	0	0.26 ± 0.09[a]	0.18 ± 0.02[b]
PUFA n-6	15.96 ± 1.6[a]	15.95 ± 1.4[a]	12.91 ± 0.9[b]	9.53 ± 1.4[c]
18:3n-3	2.81 ± 1.2[a]	2.19 ± 1.1[a]	1.55 ± 0.8[b]	1.0 ± 0.4[c]
18:4n-3	0.19 ± 0.04[a]	0.14 ± 0.08[b]	0.05 ± 0.01[c]	0.04 ± 0.01[c]
20:4n-3	0	1.277 ± 0.9[a]	0.13 ± 0.04[b]	0.10 ± 0.01[b]
20:5n-3	13.39 ± 1.6[a]	14.47 ± 1.8[a]	10.05 ± 0.9[b]	7.82 ± 1.5[c]
21:5n-3	0	0	0.11 ± 0.01[a]	0.07 ± 0.02[b]
22:5n-3	0.81 ± 0.2[a]	1.26 ± 0.6[b]	0.55 ± 0.1[c]	0.18 ± 0.09[d]
22:6n-3	4.58 ± 1.2[a]	3.45 ± 0.9[b]	3.37 ± 0.7[b]	2.93 ± 0.6[b]
PUFA n-3	21.78 ± 1.6[a]	21.51 ± 1.2[a]	15.81 ± 0.9[b]	12.14 ± 1.4[c]
PUFA	37.75 ± 2.2[a]	37.46 ± 1.9[a]	28.73 ± 2.9[b]	21.67 ± 2.4[c]

Table 3

Species	Distribution	Habitat	Larval stages	References
P. antennarius H. Milne Edwards, 1837	Mediterranean lagoons	Freshwater	3	Falciai and Palmerini (2001)
P. antrorum Benedict, 1896	North America	(Freshwater-troglobitic)	3	Strenth (1976)
P. argentinus Nobili, 1901	Atlantic and Caribbean coasts of South America	Estuarine	9	Menú-Marque (1973)
P. atrinubes Bray, 1976	Australia	Estuarine	7	Bray (1976)
P. australis Dakin, 1915	Australia	Freshwater	3	Bray (1976)
P. carteri Gordon, 1935	Amazon and Orinoco River basins	Freshwater	3	Pereira and Garcia (1995)
P. cummingi Chace, 1954	North America (Florida, West Indies)	Freshwater	3	Dobkin (1971)
P. hobbsi Strenth, 1994	North America (Northeastern Mexico)	Freshwater	3	Rodríguez-Almaraz et al. (2010)
P. ivonicus Holthuis, 1950	Amazon basins	Freshwater	3	Magalhaes (1986)
P. kadiakensis Rathbun, 1902	Pacific coast of North America	Estuarine	5-8	Broad and Hubschman (1963)
P. mercedae Pereira, 1986	South America-Amazon and Orinoco	Freshwater	1	Magalhaes (1988)
P. mexicanus Strenth, 1976	North America- Mexico	Freshwater	3	Rodríguez-Almaraz et al. (2010)
P. paludosus Gibbes, 1850	North America-South Carolina	Freshwater	3	Dobkin (1963)
P. pugio Holthuis, 1949	Atlantic coast of North America (Maine – Gulf of Mexico)	Estuarine	10	Broad (1957)
P. varians Leach, 1813	Europe, North Africa	Estuarine	5	Fincham (1979)
P. vulgaris Say, 1818	Atlantic coast of North America (Gulf of St. Lawrence)	Estuarine	10	Sollaud (1923)
P. zariquieyi Sollaud, 1938	European waters (Eastern Spain)	Freshwater	3	Guerao (1993)

CHAPTER III

Seasonal and interannual variations in size, biomass and chemical composition of the eggs of North Sea shrimp, *Crangon crangon* (Decapoda: Caridea)

Ángel Urzúa, Kurt Paschke, Paulina Gebauer and Klaus Anger

Mar Biol (2012) 159:583–599
DOI 10.1007/s00227-011-1837-x

ORIGINAL PAPER

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Received: 26 April 2011 / Accepted: 1 November 2011 / Published online: 15 November 2011

Abstract In the shrimp *Crangon crangon*, an important fishery resource and key species in the southern North Sea, we studied temporal variations in size, biomass (dry weight, *W*) and chemical composition (C, N, protein and lipid) of eggs in an initial embryonic stage. Data from 2 years, 1996 and 2009, consistently revealed that egg size and biomass varied seasonally, with maxima at the beginning of the reproductive season (January), decreasing values throughout spring, minima in June–July, and a slight increase thereafter. This cyclic pattern explains why "Winter eggs" are on average larger and heavier than "summer eggs". Using a modelling approach, we estimated the duration of oogenesis in relation to seasonally changing seawater temperatures. According to an additive model of multiple explanatory variables, the C content per newly laid egg showed in both years a highly significant negative relationship with day length ($r^2 = 0.38$ and 0.40, respectively; $P < 0.0001$), a weak positive relationship with temperature ($r^2 = 0.08$ and 0.09; $P < 0.05$), and a weak negative relationship with phytoplankton biomass ($r^2 = 0.11$ and 0.12; $P < 0.05$) at the estimated time of beginning oogenesis. Phenotypic plasticity in initial egg size and biomass is interpreted as an adaptive reproductive trait that has evolved in regions with strong seasonality in plankton production and periods of larval food limitation. In contrast to biomass per egg, the percentage chemical composition remained similar throughout the reproductive period. Both the absolute and percentage values also showed significant interannual variations, which caution against generalizations based on short-term studies of reproductive traits of *C. crangon* and other species of shrimp.

Communicated by X. Irigoyen.

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Introduction

Life-history patterns are generally considered as adaptive traits, because they are shaped by selection pressures on the survival of offspring. This selection basically depends on intraspecific variation, which may be considered as "the origin of evolutionary novelties" (Arthur 2000). Intraspecific variation in life-history traits is quite common in marine invertebrates (Hines 1986a, b; Hadfield and Strathmann 1996). One of the central questions in this context is whether it is advantageous to invest limited energy in the production of many small or rather in fewer but larger eggs (Stearns 1992; Levin and Bridges 1995; Moran and McAlister 2009). Large eggs generally develop more slowly (Steele and Steele 1975), but give rise to more advanced and larger larvae (Clarke 1993; Jalihal et al. 1993; Murphy and Austin 2005). The production of large eggs generally reflects an enhanced maternal energy investment at the cost of reduced fecundity, that is lower offspring number (Smith and Fretwell 1974; Allen et al. 2008). In temperate and high-latitude marine invertebrates, this reproductive trait

has often been interpreted as an evolutionary adaptation to a mismatch between short seasonal periods of planktonic food availability and a prolonged larval development duration enforced by low temperatures (e.g. Anger et al. 2003; Kattner et al. 2003; Thatje et al. 2004; Marshall et al. 2008).

Seasonal effects have been identified as an important source of intraspecific variation in the reproduction of temperate marine invertebrates, in general (Ghiselin 1987). Among the Crustacea, intraspecific variability in reproductive traits such as egg size has been observed not only as a presumable response to seasonal variations in environmental conditions (Boddeke 1982; Bas et al. 2007), but also between years (Kattner et al. 1994; Ouellet and Plante 2004), and in latitudinal as well as bathymetric gradients (e.g. Wehrtmann and Kattner 1998; Lardies and Castilla 2001; Thatje et al. 2004; Brante et al. 2004; Laptikhovsky 2006; Fischer and Thatje 2008).

Among the Decapoda, seasonal changes have been observed in the number and size of newly produced eggs (e.g. Díaz 1980; Bas et al. 2007) as well as in the biochemical composition of embryos (O'Leary Amsler and George 1984; Jacobs et al. 2003; Bas et al. 2007) and, as a consequence, in the physiological condition of early larvae (Ouellet and Allard 2002; Paschke et al. 2004; Gebauer et al. 2010). Since the size and quality of eggs and newly hatched larvae may also affect the developmental success of subsequent phases of the life cycle (Giménez et al. 2004, 2006, 2010; Pechenik 2006; Harrison et al. 2011), seasonal variation in offspring quality may be relevant not only for supply-side ecology in general (Underwood and Keough 2001; Pan et al. 2011), but also for aquaculture (e.g. Arcos et al. 2003; Wu et al. 2010) and fisheries management of crustaceans (Botsford 1991; Fischer et al. 2009).

Seasonal variation in reproductive traits has been observed also in the subject of the present study, the North Sea shrimp (or "Brown Shrimp"), *Crangon crangon* (Linnaeus 1758), especially in egg size (Boddeke 1982) and early larval starvation resistance (Paschke et al. 2004). This northeastern Atlantic species shows a large latitudinal distribution, ranging from 34°–67°N (Abelló et al. 1988; González-Gordillo et al. 2001; Gunnarsson et al. 2007; Viegas et al. 2007; Campos et al. 2010). In the shallow areas of the southern North Sea ecosystem, it is considered as a key species playing an important role both as a prey for demersal fishes and as a predator of benthic and planktonic organisms (e.g. Plagmann 1939; Tiews 1970; Boddeke 1971; Kuipers and Dapper 1984; Spaargaren 2000; Oh et al. 2001; Andresen and van der Meer 2010). Also, it is subject to intense commercial fisheries in coastal waters of Germany, The Netherlands, Denmark, Belgium, and Great Britain. Its annual catches exceed 32,000 metric tons, 80% of which are landed in Germany and The Netherlands combined (ICES 2009).

The reproductive pattern of this species is in the southern North Sea characterized by continuous spawning throughout the year (Temming and Damm 2002; Oh and Hartnoll 2004; Siegel et al. 2008). During this extended reproductive period, *Crangon crangon* has been reported to produce fewer but larger "winter eggs" and more numerous but smaller "summer eggs" (Havinga 1930; Boddeke 1982; Neudecker and Damm 1992; Paschke 1998; Oh and Hartnoll 2004). However, it has remained unclear whether these actually represent two distinct cohorts, or if they belong to a more gradual, possibly cyclic pattern of seasonal change. In the present study, we therefore studied seasonal variations in egg size and biomass with a higher temporal resolution, sampling eggs in intervals of 1–2 months, attempting also to explain previously observed seasonal variations in larval quality at hatching (Paschke et al. 2004). Moreover, we are joining here data collected in two different years (1996, 2009), considering interannual variability as a potentially confounding source of variation in reproductive traits. As another aspect of the present study, we explore relationship between egg size, dry weight, elemental and biochemical composition (measured as contents of carbon, nitrogen, protein and lipid) and the energy content estimated from these constituents.

Materials and methods

Sampling and maintenance of ovigerous females

In two different years and throughout most of the reproductive season of *Crangon crangon* in the southern North Sea (February-September, 1996; January–September, 2009), adult shrimps were periodically sampled from a population living in the lower Elbe estuary (54°03′–54°04′ N; 8°18′–8°24′ E; ca 13 m depth), employing bottom trawls of research vessels "Uthörn" and "Aade". No samples were taken in October–December due to unavailability of ship time.

Sexually mature individuals were transferred to the Helgoland Marine Biological Station. In the laboratory, they were placed in aerated flow-through seawater aquaria (simulating ambient conditions with similar temperatures and salinities as in the field), and fed daily with pieces of frozen marine isopods (*Idotea* spp). Ovigerous females (44–58 mm total body length) with bright white-yellow egg masses were isolated, and samples of initial egg size were removed from the outer layer of the egg mass using a fine forceps. Only eggs in an early blastula stage (microscopically identified by uniform distribution of yolk and

absence of cleavage; see Meredith 1952; Oh and Hartnoll 2004) were used for the determination of egg size and biochemical composition (for numbers of females and eggs analysed, see following sections).

Previously unpublished data from 1996 were obtained from a research project that was partially presented in a PhD dissertation (Paschke 1998). Data of sea surface temperature and phytoplankton biomass were obtained from the long-term monitoring programme "Helgoland Roads" (Wiltshire et al. 2008).

Size and volume of eggs

Length (or larger diameter, D_1) and width (smaller diameter, D_2) of 30 eggs per female were measured under a stereo microscope (1996: Olympus SZH equipped with a calibrated eyepiece micrometer; 2009: Olympus SZX2-ILLB equipped with a calibrated eyepiece micrometer and a digital camera). The images obtained from 2009 samples were digitalized using a CELL (Olympus) image analysis software. For both years, the egg volume (V) was calculated using the formula for oblate spheroids (Turner and Lawrence 1979):

$$V = \left(\pi \cdot D_1^2 \cdot D_2\right)/6.$$

Determinations of dry mass (W) and elemental composition (C, N)

Biomass (measured as dry weight, W) and elemental composition (carbon, nitrogen; C, N) were determined with standard techniques (Anger and Harms 1990). Briefly, the eggs were rinsed in distilled water, blotted on fluff-free Kleenex paper, transferred to pre-weighed tin cartridges, and stored at −20°C. Later, the samples were freeze-dried for 48 h in a vacuum dryer (1996: Finn-Aqua Lyovac GT2E; 2009: Christ Alpha 1-4 LSC), and W was determined to the nearest 0.1 µg on an ultra micro balance (1996: Mettler UMT2; 2009: Sartorius SC2). Subsequently, the samples were analysed with a CHN Analyser using Sulphanilamide as a standard (1996: Fisons Elemental Analyser EA 1108; 2009: Elemental Vario Micro).

Each measurement of W, C and N comprised 5 replicate samples with 30–32 eggs per female (depending on clutch size). Hydrogen was measured as well, but is not considered in this paper; H data are closely correlated with those of C and thus, generally show very similar patterns (Anger 2001). The energy content of egg biomass was estimated from C data (Salonen et al. 1976) and, independently, from the major biochemical constituents lipid and protein (Winberg 1971). In the latter estimates, carbohydrates are not considered, as this fraction contributes only a minor part to egg biomass (ca. 3% of W; see Jaeckle 1995; Moran and McAlister 2009).

Proximate biochemical composition (total protein, lipid)

For each female, four replicate biochemical analyses were carried out with $n = 50$ eggs each. The samples were gently rinsed for 10 s in distilled water, subsequently blotted on filter paper, transferred to pre-weighed 1.5-ml microcentrifuge vials, and stored frozen at −80°C. Prior to the analyses, the samples were dried for 48 h in a vacuum dryer (see above), and W was determined to the nearest 0.01 mg on a Sartorius balance (MC1 RC 210 S; capacity 210 g). Afterwards, the samples were homogenized with 100 µl of Milli-Q ultrapure water and sonicated (Branson, Sonifier, Cell Disruptor B 15) with 5 strokes of 5 s on ice, then each homogenate was divided into two aliquots for repeated protein and lipid determinations (proximate biochemical composition).

The protein content of the homogenate was determined using a BioRad DC Protein Assay following Lowry et al. (1951); in the 2009 study, we used a slightly modified method for microplates (Torres et al. 2007). 25 µl homogenate was mixed with 100 µl ice-cold 20% trichloroacetic acid (TCA). After incubation for 10 min at 4°C, the samples were centrifuged at 10,000*g* for 10 min at 4°C and the supernatant was discarded. The remaining pellet was dissolved in 300 µl NaOH (1 M) and incubated shaking at 1,400 rpm for 30 min at 56°C in a thermomixer. 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 DC Protein Assay) in a 96-well microplate. The microplates were incubated for 15 min at room temperature in the dark, and absorbance was measured using a Multiskan Spectrum Thermo apparatus (wavelength: 750 nm). The calibration curve was obtained by dilutions of bovine serum albumin (BSA, kit: BioRad DC Protein Assay).

Total lipid content of the homogenate was determined using the sulphophosphovanillin method (Zöllner and Kirsch 1962; modified for microplates by Torres et al. 2007). 40 µl of the homogenates were mixed with 300 µl of ice-cold $CHCl_3/CH_3OH$ (2/1). After an incubation of 15 min at room temperature, the samples were centrifuged for 20 min at 10,000*g* and 4°C; 180 µl of the lower phase were transferred to new tubes. These were left open to dry in a thermomixer for 90 min at 56°C, shaking at 700 rpm. The dried pellets were dissolved in 200 µl of concentrated H_2SO_4, and incubated for 10 min at 95°C, shaking at 1,400 rpm in a thermomixer with closed tubes. After cooling for 20 min at room temperature, 4 replicates of 20 µl from each sample were distributed in two 96-well microplates. In the first plate-A (Blank), 300 µl of concentrated H_3PO_4, were added. In the second plate-B, 300 µl of vanillin solution (8 mM H_3PO_4 conc) was added.

The microplates were incubated for 45 min at room temperature for colour development and subsequently measured using a Multiskan Spectrum Thermo apparatus (wavelength: 550 nm). The final values were obtained as the difference between the two plates (A-B). The calibration curve was obtained by dilutions of a standard lipid solution extracted from muscle tissue of adult *Crangon crangon*, following the method described by Folch et al. (1957) and slightly modified by Cequier-Sánchez et al. (2008), which used dichloromethane:methanol as solvent for lipid extraction.

Estimating the time and environmental conditions at the onset of oogenesis

Since cell size and other characteristics of crustacean eggs are determined long before the extrusion of the eggs under the female abdomen, they may be influenced by environmental conditions prevailing at the onset of oogenesis inside the ovaries (Meusy and Payen 1988; Wenner and Kuris 1991). In order to evaluate whether our data of egg size and biomass measured at the beginning of embryogenesis were correlated with environmental conditions at the time when oogenesis had begun, we first estimated for each day of egg laying the theoretical time of the preceding onset of oogenesis. Our modelling approach was based on the following assumptions: Since no quantitative data for the duration of oogenesis inside the ovary of *Crangon crangon* in relation to temperature are available, we assumed that this species shows total spawning, that is the ovary is completely emptied when eggs are laid, and a new ovarian cycle begins concomitantly, while the extruded and subsequently fertilized eggs begin to develop externally through embryogenesis (Oh and Hartnoll 2004; Siegel et al. 2008). Hence, the duration of oogenesis should be similar to that of embryonic development.

Temming and Damm (2002) described the duration (D, in days) of embryonic development as a function of water temperature (T, °C): $D = 1031.34 \cdot T^{-1.354}$. This regression equation was based on previously published data from Havinga (1930), Tiews (1954), Meixner (1969), and Wear (1974). Assuming that oogenesis duration is similar to that of embryogenesis and that both processes show a similar dependence on T, this regression was then applied to estimate the duration of oogenesis in relation to water temperature.

Taking a simulation modelling approach that Anger (1983), Miller and Tande (1993), and Temming and Damm (2002) used to estimate embryonic and larval development durations under variable temperature conditions in the field, we inserted in the regression (see above) for each calendar day (d = 1…365; where 1 = 1 January, 365 = 31 December) a T value recorded in the German Bight (Wiltshire et al. 2008; interpolated, where necessary). This provided us with an estimate of the theoretical duration (D) of oogenesis at a given T. From this result, we then calculated the fraction of the expected duration of ovarian development (1/D) for 1 day with a given T. Going backward in time, these calculations were then repeated for each successive calendar day prior to egg laying, until the summation of these developmental fractions ($\sum$1/D) reached a value of 1.0 (=100%). This provided the theoretical day of the beginning of oogenesis. Miller and Tande (1993) termed this simulation procedure "cumulative fractional completion of development".

As a second step in our modelling approach, we determined the corresponding values of day length and water temperature. As an additional environmental factor that potentially may influence egg size at, or already before, the beginning of oogenesis, we included in our model also phytoplankton biomass, which was used as a proxy for food availability. Assuming that this factor may affect reproductive processes through a longer period prior to the onset of oogenesis, we calculated a mean value for phytoplankton biomass observed through a fortnight period prior to the theoretical day of the onset of oogenesis. Data for seawater temperature and phytoplankton biomass measured at that time near Helgoland were taken from Wiltshire et al. (2008).

As a third step, our data of initial egg size and biomass (at the beginning of embryogenesis) were statistically analysed in relation to conditions of day length, temperature and phytoplankton biomass prevailing at the time when oogenesis had begun, and when egg size and biomass were biologically determined (Wenner and Kuris 1991).

Data analysis

Statistical analyses were performed with standard methods based on Sokal and Rohlf (1995) and Zuur et al. (2007), using the statistics software packages STATISTICA 8 (StatSoft) and Brodgar 2.6.6. All statistical analyses were performed on the 95% confidence level ($P < 0.05$). Normality and homogeneity of variances were tested with Kolmogorov–Smirnov and Levene's tests, respectively. When data did not meet the assumptions, nonparametric Mann–Whitney test was carried out.

Data of seasonal variations in size, biomass and chemical composition of eggs were tested for the two different years (1996, 2009) with a Generalized Additive Model (GAM) using the function based on the R-mgcv package (Wood 2006). This GAM model allows evaluating the nature of the relationship between one explanatory variable (in this case calendar day) and various dependent variables (egg parameters).

Temperature and day length were estimated for the theoretical day of beginning oogenesis. For food

availability, we calculated the average phytoplankton biomass for 2 weeks preceding the beginning of oogenesis (for modelling, see above). The effects of these environmental factors on egg biomass (taking the carbon content as a proxy for total organic matter) were analysed separately for each year, applying an additive model of multiple explanatory variables:

$$Y_i = \alpha + f_1(\mathrm{T}_i) + f_2(\mathrm{DL}_i) + f_3(\mathrm{PB}_i) + \varepsilon_i$$

with Y = C content (μg egg^{-1}), T = temperature (°C), DL = day length (h), PB = Phytoplankton biomass (mg C m^{-3}); α = intercept, $f_{1,2,3}$ = different smoothing functions, ε = error term, i = observed data, σ^2 = variance, where $\varepsilon_i \sim$ n (0, σ^2). Analyses of normality, outliers and collinearity were performed with standard methods according to Zuur et al. (2007). Interannual differences in average size and biomass of eggs were tested using two-way ANOVA with season and year as factor levels. Differences in average size of females between seasons and years were tested with two-way ANOVA as well. Subsequently, the effect of female size on egg size was tested with regression analysis.

Results

Seasonal variations in size, biomass and biochemical composition of early eggs

Females with newly laid eggs were found throughout the period of sampling, that is from mid-January or late February (2009 and 1996, respectively) until the end of September. According to the GAM model, the relationship between the explanatory variable (calendar day) and the dependent variables (egg size and biomass) showed conspicuous seasonal variations in both years of our study (Figs. 1, 2, 3). The smoothing function showed a maximum level consistently at the beginning of the egg-laying season (January–February), gradually decreasing in spring and early summer, reaching a minimum approximately in the middle of the season (June–July), and increasing trends thereafter (Figs. 1, 2, 3). As a consequence, eggs laid during winter were in both years on average larger than those laid in summer. Consistent with their larger size (expressed as egg volume; Tables 1, 2), eggs that were laid at the beginning of the reproductive season ("winter eggs") showed in both years also considerably higher contents of W, C and N per egg (Tables 1, 2). In 2009, for example, maximum C values in "winter eggs" reached twice the minimum values observed in "summer eggs" (ca. 14 vs. 7 μg ind^{-1}; cf. calendar days 30 vs. 150–210; Table 2).

The quantities of proximate biochemical constituents (total lipids and proteins per egg) showed similar tendencies as the values of W, C and N (Fig. 3; Tables 1, 2). Again, a maximum level was found at the beginning of the reproductive season (January–March), with average values of about 3.9 μg lipid and 13.1 μg protein per egg. Gradually decreasing values were measured until June–July, with minimum levels of about 2.4 μg lipid and 10.7 μg protein. An increase occurred subsequently in late summer, reaching average values of 3.5 μg lipid and 12.2 μg protein per egg (Table 2).

While all absolute values of egg size and biomass differed significantly among calendar days (all $P < 0.001$), the relative composition of egg biomass [elemental and biochemical fractions in % of W; energy in J (mg W)$^{-1}$] showed no significant differences throughout the reproductive period (Fig. 3; Tables 1, 2).

Due to a fairly constant chemical composition, the amounts of biomass per egg depended mainly on egg volume ($r^2 = 0.72$, 0.67, 0.65, 0.62 and 0.60 for W, C, N, lipid and protein, respectively; all $P < 0.001$; for graphical illustration, using W and C as examples, see Fig. 4a, b). Also, the energy content estimated from independent measurements of either elemental (C) or biochemical composition (total lipid and protein) showed similar seasonal patterns, again with maximum values at the beginning of the reproductive season and minimum values in the middle of the season ($P < 0.001$; cf. calendar days 60 vs. 210; Tables 1, 2). Energy values estimated independently from biochemical and C data were significantly correlated, although the former estimates tended to be higher (Fig. 4c).

The average total body length of ovigerous females was not significantly different between either years or seasons, ranging from 44 to 58 mm (two-way ANOVA, $F = 1.726$; $P = 0.242$), and the egg volume was not related with female body size ($r^2 = 0.0118$; $P = 0.126$). However, the average number of eggs per female (fecundity) was significantly lower in winter than in summer (1860 ± 142 vs. 2556 ± 192 in pooled data from January to May vs. all later months, respectively; $U = 1062$; $P < 0.01$).

Interannual variations

The average values of egg volume and biomass as well as elemental and biochemical composition (in μg per egg) differed significantly not only between seasons but also between the 2 years of our study, 1996 and 2009. These interannual variations showed significant interactions with the factor season (Table 3), however, without showing consistent patterns in the various parameters of egg size and biomass per egg. While eggs laid in winter (see calendar day 60) were generally similar in the 2 years of the study, eggs laid in spring-summer (cf. calendar days 120–150) tended to show lower average W, C and N values in 2009 as compared to 1996 (Tables 1, 2).

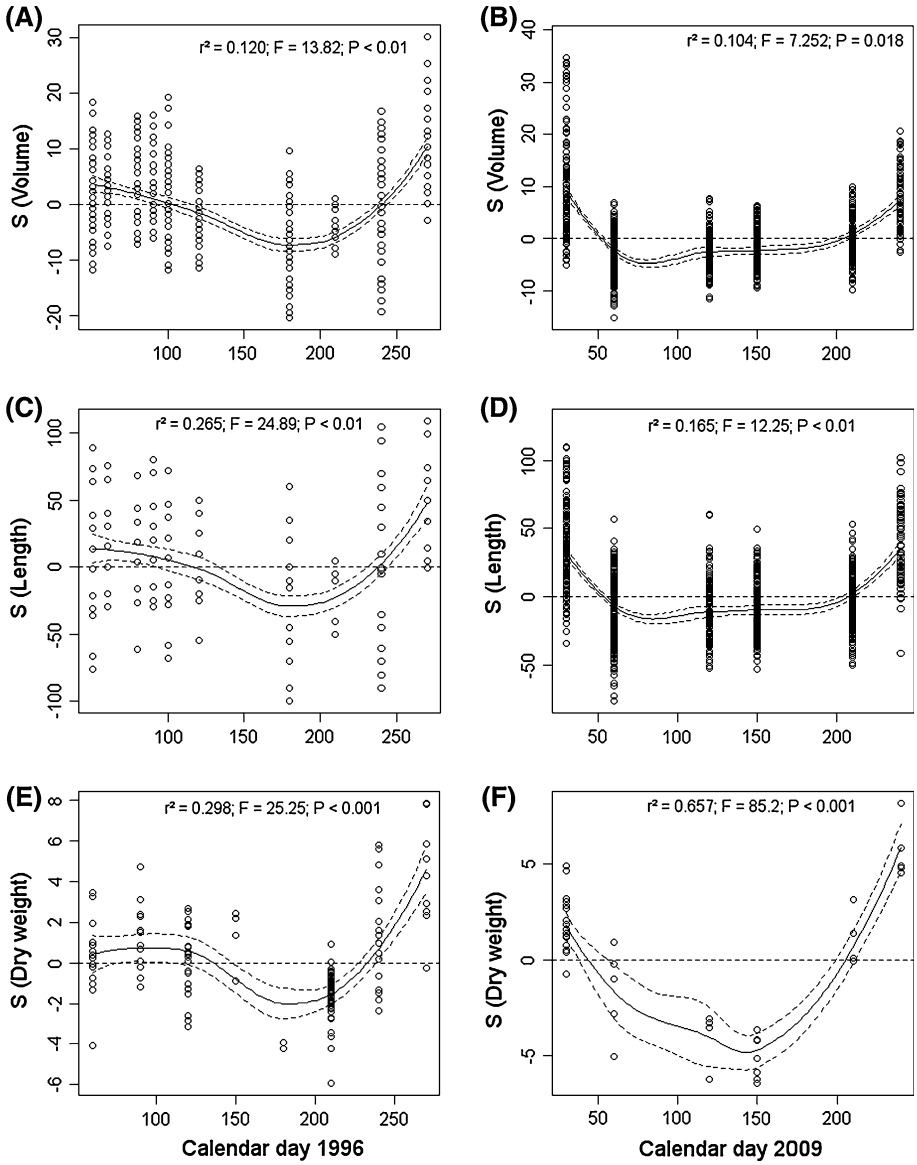

Fig. 1 *Crangon crangon*, eggs in an early blastula stage; comparison of egg parameters measured in two different study years (1996, 2009). Smoothing function (S) obtained by a Generalized Additive Model, GAM, for egg volume (mm^3 10^{-3}; graphs **a**, **b**), length (mm; **c**, **d**), and dry weight (μg egg^{-1}; **e**, **f**). These plots allow evaluating relationships between an explanatory variable (calendar day, *x*-axis) and the adjusted residuals (*y*-axis) of dependent variables (volume, length, dry weight). Solid line: estimated smoothing function; dotted lines: 95% confidence intervals. Total variance is quantified by values of r^2, the significance of the smoothing function by values of *F* and *P*; dots represent mean values per female, with numbers of ovigerous females, $n = 242$ (**a**), 212 (**b**), 238 (**c**), 204 (**d**), 102 (**e**), and 76 (**f**); for numbers of eggs analysed per female for determinations of egg size and biomass, see Methods section

As in the absolute values of egg size and biomass, also the mass-specific C, N, lipid and protein values (expressed in % W) showed significant differences between the 2 years as well as interactions with the factor season (Table 3). For example, the percentage C values were during the whole sampling period significantly higher in 1996 than in 2009 (Tables 1, 2).

Onset and duration of oogenesis

Our estimates of the onset and duration of oogenesis (for modelling, see above, Methods section) varied greatly due to seasonal variation in water temperature. Abrupt changes in the duration of oogenesis estimated for eggs laid in May–June (coinciding with rapidly increasing spring temperatures; Fig. 5) suggest that two principal periods of egg production may be distinguished for convenience, with "winter eggs" being laid from January to May and "summer eggs" from June to October. According to our model, winter eggs developed in the ovaries for about 64 ± 5, 93 ± 6, 116 ± 4, 122 ± 3, and 86 ± 9 day (periods estimated for egg-laying during the months of January–May, respectively). This implies that their oogenesis had already begun in autumn (October–November) of the previous year, taking about 3–4.5 months until egg laying occurred. Summer eggs by contrast, passed theoretically through much shorter oogenesis periods of only 38 ± 5, 26 ± 2, 22 ± 1, and 20 ± 1 day, for the months of June–September, respectively. Their development in the ovaries should thus have taken place during the period from May to late August.

Like seasonal variation, also interannual differences in the duration of oogenesis were related to water temperature (Fig. 5). The time spans estimated for winter eggs

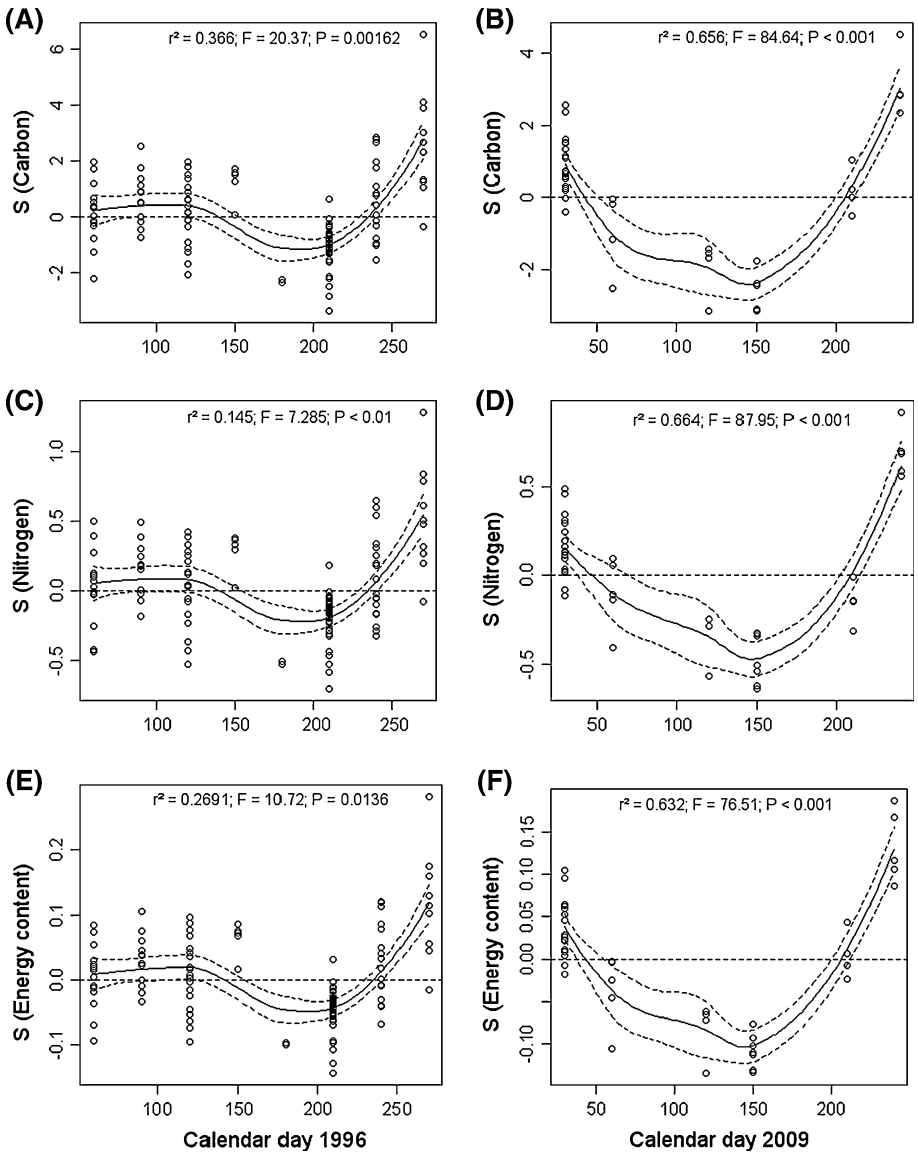

Fig. 2 *Crangon crangon*. Smoothing function (S) obtained by GAM for carbon (μg egg^{-1}; **a**, **b**), nitrogen (μg egg^{-1}; **c**, **d**), and energy content [J egg^{-1}, estimated from carbon data (Salonen et al. 1976); **e**, **f**]; numbers of ovigerous females, $n = 102$ (**a**), 76 (**b**), 102 (**c**), 76 (**d**), 102 (**e**), and 76 (**f**); for further explanations, see Fig. 1

produced in a cold year, 1996, reached up to about 172 days, while much shorter durations were estimated for 2009, reaching maximally around 122 days (comparisons between years: $U = 3948$; $P < 0.01$).

Relationships between egg biomass and previous environmental factors at the onset of oogenesis

The environmental factors temperature (T) and day length (DL) determined for the theoretical day at the onset of oogenesis, as well as the factor phytoplankton biomass (PB) averaged over a fortnight period prior to the onset of oogenesis (cf. Fig. 5c, d) exerted significant effects on egg biomass (using the carbon content as a proxy for total organic matter per egg) measured at the onset of embryogenesis. An additive model of multiple explanatory variables indicated that in both years the C content of newly laid eggs showed a highly significant negative relationship with DL, a weak positive relationship with T, and a weak negative relationship with PB (Table 4). About 40% of the variation in early egg biomass can be explained by variation in DL (coefficients of determination $r^2 = 0.38$ and 0.40, respectively, for the 2 years of our study). By contrast, the factor T contributed less than 10% to the variation in egg C ($r^2 = 0.08$ and 0.09) and factor PB contributed about 12% ($r^2 = 0.11$ and 0.12).

Discussion

The North Sea shrimp, *Crangon crangon*, a commercially exploited and ecologically important decapod crustacean, shows continuous breeding throughout the year, with a minimum frequency of ovigerous females in late autumn

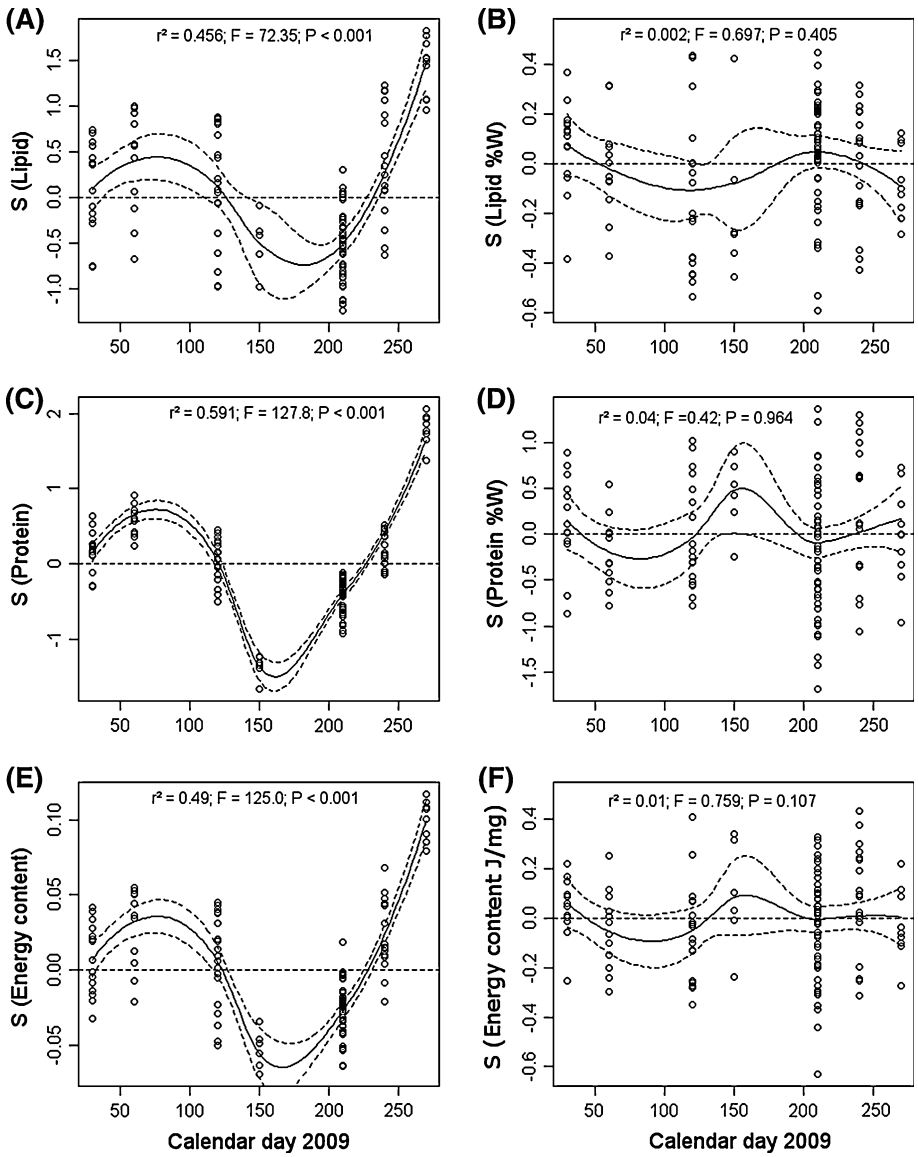

Fig. 3 *Crangon crangon*. Smoothing function (S) obtained by GAM for lipid [**a** (μg egg^{-1}), **b** (%W)], protein [**c** (μg egg^{-1}), **d** (%W)], and energy content [estimated from lipid and protein data (Winberg 1971); **e** (J egg^{-1}), **f** (mass-specific energy content J mg W^{-1})]; numbers of ovigerous females in all cases $n = 126$; for further explanations, see Fig. 1

(Temming and Damm 2002; Paschke et al. 2004; Siegel et al. 2008; Hufnagl et al. 2010; present study). Assuming a short period of no or very little egg-laying activity near the end of the year, we may tentatively define here January as the time of beginning of the "reproductive season".

In the German Bight (Siegel et al. 2008; present study) as well as in Dutch waters (Havinga 1930) and in the Irish Sea (Oh and Hartnoll 2004), this species seems to produce larger "winter eggs" and smaller "summer eggs" (Havinga 1930; Boddeke 1982; Criales 1985; Linck 1995; Paschke 1998; Temming and Damm 2002; Campos et al. 2009; present study). In both years of our study, however, our data revealed that these seasonal changes in reproductive traits actually follow cyclic patterns rather than indicating the existence of two clearly distinct cohorts or broods, which are commonly distinguished for convenience to facilitate seasonal comparisons. This cyclic pattern comprises maximum values of egg size and organic biomass constituents (C, N, lipid and protein per egg) during the beginning and, again, towards the end of the reproductive season, while minimum values occur in the middle of this extended period. Similar patterns have been observed also in some species of brachyuran and anomuran crabs from temperate regions (Bas et al. 2007; P. Gebauer, unpublished data), suggesting that such patterns might be more widespread among Decapoda living in regions with strong seasonality.

Large eggs are generally considered to reflect a high maternal energy investment into offspring, although egg size alone can be a poor predictor of the actual energetic content of embryonic biomass (Moran and McAlister 2009). In *Crangon crangon*, the relative chemical composition (contents of elemental and biochemical constituents in % of W) did not vary seasonally. This indicates that

Table 1 *Crangon crangon,* eggs in an early blastula stage

Calendar day 1996										
Egg parameters	60	90	120	150	180	210	240	270	*F*	*P*
V (mm^3 10^{-3})	41.9 ± 5.47^{a}	41.1 ± 6.42^{a}	35.3 ± 4.70^{b}	34.6 ± 2.35^{b}	29.0 ± 4.38^{c}	31.7 ± 3.34^{bcd}	33.8 ± 3.80^{bcde}	42.9 ± 7.53^{af}	42.416	<0.001
L (mm)	0.46 ± 0.03^{a}	0.46 ± 0.04^{a}	0.44 ± 0.02^{a}	0.43 ± 0.06^{b}	0.40 ± 0.03^{c}	0.41 ± 0.03^{bcd}	0.42 ± 0.03^{bcde}	0.46 ± 0.04^{af}	16.78	<0.001
W (μg)	19.3 ± 1.84^{a}	19.9 ± 1.70^{a}	18.3 ± 1.78^{a}	17.1 ± 1.36^{a}	13.3 ± 0.20^{b}	15.5 ± 1.22^{b}	17.8 ± 2.62^{abc}	20.6 ± 2.69^{ad}	20.789	<0.001
C (μg)	10.5 ± 1.07^{a}	10.8 ± 0.94^{a}	10.1 ± 1.14^{a}	9.40 ± 0.67^{a}	7.81 ± 0.06^{b}	8.42 ± 0.69^{b}	9.82 ± 1.42^{abc}	11.6 ± 1.94^{ad}	20.868	<0.001
N (μg)	2.17 ± 0.26^{a}	2.26 ± 0.19^{a}	2.09 ± 0.27^{a}	1.92 ± 0.14^{a}	1.59 ± 0.01^{b}	1.78 ± 0.15^{b}	2.03 ± 0.30^{abc}	2.43 ± 0.38^{ad}	17.121	<0.001
E (J) [C]	0.44 ± 0.04^{a}	0.46 ± 0.04^{a}	0.43 ± 0.05^{a}	0.41 ± 0.02^{a}	0.31 ± 0.01^{b}	0.35 ± 0.03^{b}	0.41 ± 0.06^{abc}	0.49 ± 0.08^{ad}	20.438	<0.001
Lipid (μg)	3.84 ± 0.62^{a}	3.60 ± 0.34^{a}	ND	ND	2.41 ± 0.27^{b}	2.35 ± 0.34^{b}	ND	ND	26.042	<0.001
Protein (μg)	12.9 ± 2.09^{a}	12.7 ± 2.05^{a}	ND	ND	9.87 ± 1.39^{b}	9.79 ± 1.69^{b}	ND	ND	27.224	<0.001
E (J) [B]	0.78 ± 0.03^{a}	0.76 ± 0.02^{a}	ND	ND	0.63 ± 0.01^{b}	0.61 ± 0.02^{b}	ND	ND	24.221	<0.001
C (%W)	54.8 ± 0.73	54.6 ± 0.38	54.8 ± 1.28	55.0 ± 0.80	54.6 ± 0.34	54.2 ± 0.75	54.2 ± 0.71	54.3 ± 0.51	0.761	0.121
N (%W)	11.3 ± 0.27	11.3 ± 0.20	11.4 ± 0.49	12.0 ± 0.14	11.1 ± 0.27	11.4 ± 0.23	11.4 ± 0.23	11.3 ± 0.14	0.863	0.1
C/N ratio	4.81 ± 0.09	4.80 ± 0.06	4.82 ± 0.09	4.75 ± 0.02	4.89 ± 0.09	4.72 ± 0.06	4.78 ± 0.09	4.77 ± 0.06	0.946	0.09
E (J mg W^{-1}) [C]	23.2 ± 0.84	23.3 ± 0.24	23.4 ± 1.22	24.9 ± 0.54	23.3 ± 0.22	23.0 ± 0.48	23.3 ± 0.47	23.1 ± 0.33	0.916	0.09
Lipid (%W)	16.2 ± 0.76	16.7 ± 0.4	ND	ND	15.9 ± 0.90	15.7 ± 0.6	ND	ND	1.115	0.07
Protein (%W)	65.8 ± 0.80	66.5 ± 0.9	ND	ND	65.3 ± 0.62	64.8 ± 0.9	ND	ND	1.062	0.08
E (J mg W^{-1}) [B]	21.4 ± 0.21	21.6 ± 0.19	ND	ND	21.8 ± 0.14	21.7 ± 0.18	ND	ND	0.892	0.11

Seasonal variations in egg size, biomass and chemical composition in 1996. Egg parameters: volume (*V*), length (larger diameter, *L*), dry weight (*W*), contents of carbon (C), nitrogen (N), energy estimated from carbon data [E (C), lipid, protein, and energy estimated from lipid and protein data E (B)]; all values in μg egg^{-1} or J egg^{-1}, and in % of W or J mg W^{-1}, respectively; C/N mass ratio; mean values ± SD; ANOVA (*F*) and significance level (*P*); different lower case letters in a row represent significant differences between calendar days (SNK tests), *ND* no data available

Table 2 *Crangon crangon*. Seasonal variations in egg size, biomass and chemical composition in 2009; for further explanations, see Table 1

Calendar day 2009									
Egg Parameters	30	60	120	150	210	240	270	*F*	*P*
V ($mm^3 \times 10^{-3}$)	49.8 ± 8.49^{a}	35.1 ± 3.78^{b}	33.7 ± 3.72^{b}	31.8 ± 3.24^{b}	37.5 ± 3.7^{bc}	45.7 ± 4.02^{ad}	ND	182.59	<0.001
L (mm)	0.47 ± 0.03^{a}	0.42 ± 0.02^{b}	0.41 ± 0.02^{b}	0.40 ± 0.02^{b}	0.43 ± 0.01^{bc}	0.47 ± 0.02^{ad}	ND	130.693	<0.001
W (µg)	27.5 ± 1.47^{a}	22.1 ± 1.34^{b}	15.7 ± 0.48^{c}	12.4 ± 1.09^{d}	14.8 ± 1.47^{cde}	17.8 ± 1.09^{cf}	ND	148.01	<0.001
C (µg)	14.2 ± 0.77^{a}	11.2 ± 0.99^{b}	8.23 ± 0.80^{c}	6.55 ± 1.47^{d}	7.28 ± 0.64^{cde}	9.14 ± 0.83^{cf}	ND	159.881	<0.001
N (µg)	2.92 ± 0.16^{a}	2.46 ± 0.19^{b}	1.79 ± 0.14^{c}	1.43 ± 0.14^{d}	1.35 ± 0.12^{de}	1.99 ± 0.14^{cf}	ND	145.447	<0.001
E (J) [C]	0.58 ± 0.03^{a}	0.47 ± 0.04^{b}	0.34 ± 0.03^{c}	0.27 ± 0.02^{d}	0.30 ± 0.02^{cde}	0.39 ± 0.04^{cf}	ND	140.059	<0.001
Lipid (µg)	3.94 ± 0.49^{a}	4.03 ± 0.55^{a}	3.33 ± 0.62^{b}	2.50 ± 0.32^{c}	2.44 ± 0.38^{c}	2.66 ± 0.63^{c}	3.57 ± 0.30^{abd}	54.51	<0.001
Protein (µg)	13.1 ± 0.26^{a}	13.2 ± 0.19^{a}	12.0 ± 0.27^{b}	10.3 ± 0.14^{c}	10.7 ± 0.20^{c}	10.9 ± 0.23^{c}	12.2 ± 0.21^{bd}	148.621	<0.001
E (J) [B]	0.76 ± 0.02^{a}	0.77 ± 0.02^{a}	0.71 ± 0.03^{b}	0.64 ± 0.01^{c}	0.63 ± 0.01^{c}	0.63 ± 0.01^{c}	0.73 ± 0.01^{bd}	127.077	<0.001
C (%W)	51.6 ± 0.49	52.3 ± 0.61	52.3 ± 0.23	51.9 ± 1.51	53.2 ± 0.89	52.2 ± 1.46	ND	0.962	0.1
N (%W)	10.6 ± 1.10	11.4 ± 0.13	11.4 ± 0.14	11.4 ± 0.24	10.9 ± 0.08	11.3 ± 0.12	ND	1.086	0.087
C/N ratio	4.73 ± 0.07	4.57 ± 0.03	4.58 ± 0.07	4.61 ± 0.07	4.63 ± 0.24	4.67 ± 0.17	ND	0.971	0.09
E (J mg W^{-1}) [C]	21.3 ± 0.26	21.8 ± 0.38	21.8 ± 0.15	21.6 ± 0.95	22.4 ± 0.57	21.7 ± 0.93	ND	1.096	0.08
Lipid (%W)	15.4 ± 0.18	15.3 ± 0.20	15.3 ± 0.33	15.3 ± 0.37	15.4 ± 0.23	15.4 ± 0.23	15.3 ± 0.14	0.775	0.16
Protein (%W)	65.0 ± 0.49	64.6 ± 0.38	64.6 ± 0.57	65.1 ± 0.38	64.3 ± 0.67	64.7 ± 0.71	64.3 ± 0.51	1.08	0.08
E (J mg W^{-1}) [B]	21.7 ± 0.12	21.6 ± 0.15	21.6 ± 0.19	21.7 ± 0.19	21.6 ± 0.22	21.7 ± 0.22	21.5 ± 0.14	0.759	0.1

seasonal variation in egg size is, in this species, associated with changes in quantitative (size-related) rather than qualitative traits. As a consequence, the amounts of W and organically bound elemental (C, N) and biochemical constituents (total lipid, protein) per egg are positively related with egg volume. Since the quantity of organic biomass is closely correlated also with the energy content, larger eggs reflect an enhanced maternal energy investment per embryo.

The energy content of shrimp eggs may indirectly be estimated from either elemental (Salonen et al. 1976) or proximate biochemical analyses (Winberg 1971). However, it appears that figures based on C data tend to severely underestimate the actual energy content (Fig. 4c). Estimates obtained from biochemical data should thus be more realistic, although also these values are still slightly too low, because minor constituents such as carbohydrates, chitin nucleic acids etc. are neglected. In cases where the energy concentration (Anger et al. 2002) is known, rough estimates of the energy content per egg are possible also from egg volume. In newly laid eggs of *Crangon crangon*, the average energy concentration (estimated from biochemical data) was 11.4 ± 0.7 J mm^3. This is well within the range of values recorded in early eggs of various other caridean shrimp species (7–18 J mm^3, based on C data; Anger et al. 2002).

Several decapod crustaceans including the American and European lobster (Attard and Hudon 1987; Ouellet and Plante 2004; Moland et al. 2010) as well as the coconut crab (Sato and Suzuki 2010) show a positive correlation between egg size and female body size. In *Crangon crangon*, no such relationship was detected, however, this may be due to a small size range of ovigerous females found in the southern North Sea rather than representing a taxon-specific trait. If shrimps from the northern and southernmost limits of the large geographical distribution range of this species would be compared (extending also the size range), a dependence of egg size on female body size might be observed as well, at least among populations.

Our findings of intraspecific variations in the size and biomass of newly laid eggs of *Crangon crangon* raise the question whether those seasonal changes in reproductive traits have an adaptive value for this species. Since egg size *per se* has no obvious selective advantage, a tentative explanation requires the consideration of "latent" or "carry-over effects" (Harrison et al. 2011), which can bridge successive life-history phases. In particular, we may expect that variations in size, biomass and energy content of eggs in an early stage of embryonic development should influence larval quality at hatching (Giménez 2006, 2010). This includes variations in the degree of larval dependence on external (planktonic) food sources (Paschke et al. 2004; Gebauer et al. 2010), the number of larval stages (Criales and Anger 1986; Wehrtmann 1991), and eventually, the chances of survival in the plankton (Morgan 1995). In *C. crangon,* seasonal variations in egg volume and biomass translate to differential larval size and biomass at hatching. Compared with larvae originating from summer eggs, those hatching from winter eggs show reduced nutritional vulnerability (Paschke et al. 2004), tend to be larger, and require less stages to reach the first juvenile stage (Criales 1985; Linck 1995). The present study suggests that

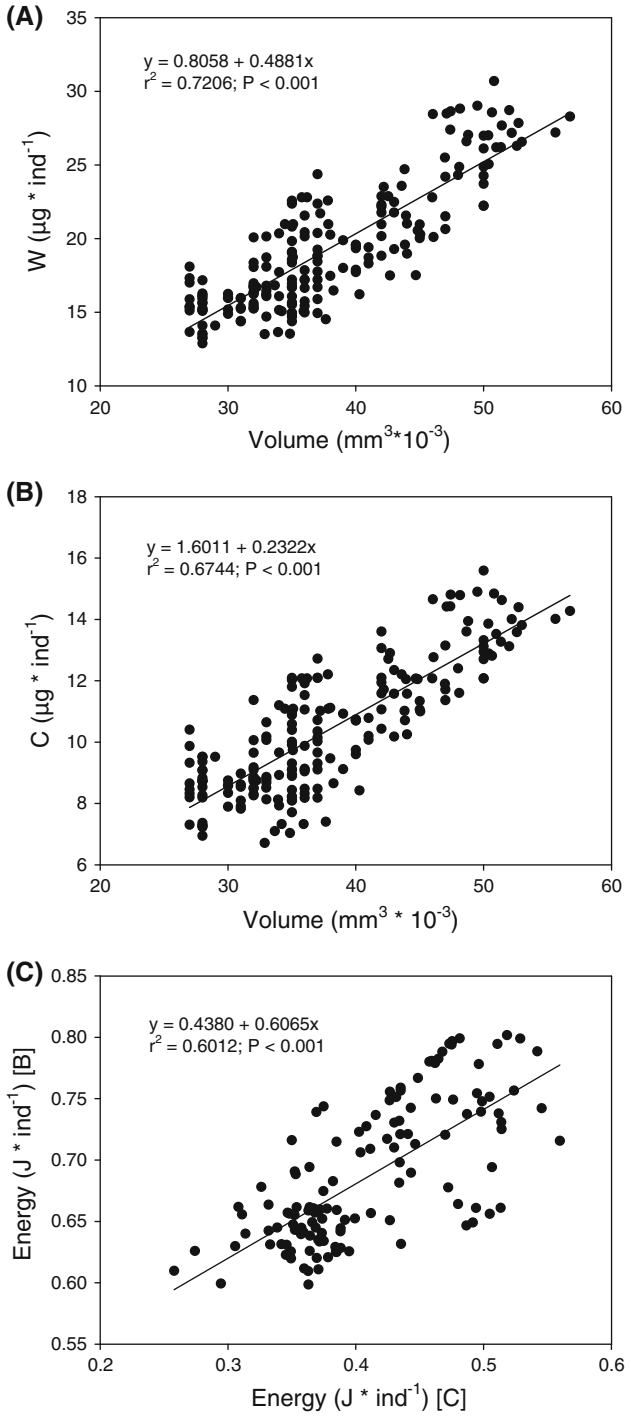

Fig. 4 *Crangon crangon*. Linear correlations between egg volume and W (**a**), egg volume and C content (**b**), and (**c**) between energy estimated from carbon data (**c**; cf. Fig. 2) and energy estimated from lipid and protein data (**b**; cf. Fig. 3); coefficients of determination (r^2), significance level (P); number of ovigerous females analysed, $n = 148$ (**a**) (**b**), 126 (**c**)

stronger starvation resistance in laboratory-reared winter larvae (Paschke et al. 2004) was due to higher initial contents of C, lipid, protein, and energy.

In the field, enhanced energy reserves carried over from the embryonic phase through hatching should improve larval survival under conditions of low or unpredictable planktonic food availability in the pelagic environment. Poor nutritional conditions occur in the southern North Sea typically (i.e. predictably) during late winter and early spring (Wiltshire et al. 2008) and may thus represent selective forces that have favoured the evolution of phenotypic plasticity in egg size. An enhanced female energy allocation in eggs produced during autumn and winter may allow for a stronger starvation resistance in larvae hatching during the first few months of the new reproductive season (January to May), when poor nutritional conditions coincide with long development duration due to low temperatures (Criales and Anger 1986; Temming and Damm 2002). During late spring and throughout summer (June to September), by contrast, smaller summer eggs are produced, while temperatures and plankton densities are increasing or remaining at high levels (Wiltshire et al. 2008; Tian et al. 2009). This allows for an enhanced fecundity (Henderson and Holmes 1987; present study), an efficient exploitation of planktonic food resources by the planktotrophic larvae, and probably, high rates of survival (Morgan 1995), growth and development (Temming and Damm 2002). Similar results of intraspecific variation in the energy investment per egg in response to environmental factors have been observed in calanoid copepods. For example in *Calanus helgolandicus*, egg size is inversely related to food availability and fecundity, with increasing numbers of smaller eggs produced during periods of high food availability (Pond et al. 1996; Jónasdottir et al. 2005).

In summary, a pronounced seasonality of plankton productivity in temperate regions such as the southern North Sea may have selected in *Crangon crangon* for the evolution of a recurrent seasonal pattern of intraspecific variation in the reproductive energy investment per offspring. It would therefore be interesting to compare reproductive traits among various populations within the large climatic range of distribution of this species. This may show if similar seasonal variations in egg size and biomass occur also in environments with weaker seasonality, for example in southwestern and southern Europe including the Mediterranean, where *C. crangon* is a common inhabitant of coastal marine communities (Abelló et al. 1988; Drake et al. 1998; González-Gordillo et al. 2001; Viegas et al. 2007).

If we accept that seasonal patterns of variation in reproductive traits have an adaptive value in strongly seasonal environments, then this raises the question which physical or chemical signals from the environment can be perceived by *Crangon crangon* as external cues that could trigger hormonally controlled changes in the energy allocation per egg (Bomirski and Klęk 1974; Sastry 1983;

Table 3 *Crangon crangon*

Egg parameters	Factor	*df*	MS	*F*	*P*
V (mm^3 10^{-3})	Season	1	822.066	13.980	<0.01**
	Year	1	1275.096	21.685	<0.01**
	Season × year	1	4125.2	70.157	<0.01**
	Error	681	58.801		
L (mm)	Season	1	0.0189	17.586	<0.01**
	Year	1	0.0151	13.981	<0.01**
	Season × year	1	0.0535	49.534	<0.01**
	Error	653	0.00108		
W (μg)	Season	1	826.217	75.984	<0.01**
	Year	1	54.698	5.030	<0.05*
	Season × year	1	234.123	21.531	<0.05*
	Error	175	10.874		
C (μg)	Season	1	221.015	67.136	<0.01**
	Year	1	42.2	12.82	<0.05*
	Season × year	1	53.443	16.234	<0.05*
	Error	175	3.292		
N (μg)	Season	1	9.533	70.095	<0.01**
	Year	1	2.590	19.044	<0.05*
	Season × year	1	2.818	20.730	<0.05*
	Error	175	0.136		
E (J)	Season	1	0.398	67.230	<0.01**
	Year	1	0.0655	11.064	<0.05*
	Season × year	1	0.0864	14.605	<0.05*
	Error	175	0.00592		
Lipid (μg)	Season	1	43.826	88.828	<0.01**
	Year	1	22.407	18.651	<0.05*
	Season × year	1	28.201	57.901	<0.05*
	Error	178	0.487		
Protein (μg)	Season	1	69.923	125.987	<0.01**
	Year	1	20.281	36.542	<0.05*
	Season × year	1	29.146	52.515	<0.05*
	Error	178	0.555		
C (%W)	Season	1	1.378	1.292	0.257^{ns}
	Year	1	191.193	179.35	<0.01**
	Season × year	1	5.838	5.476	<0.05*
	Error	175	1.066		
N (%W)	Season	1	0.251	2.460	0.122^{ns}
	Year	1	3.298	32.33	<0.05*
	Season × year	1	0.670	6.568	<0.05*
	Error	175	0.102		
C/N ratio	Season	1	0.0673	6.889	0.105^{ns}
	Year	1	0.301	30.808	<0.01**
	Season × year	1	0.0336	3.439	<0.05*
	Error	175	0.00977		
E (J mg W^{-1})	Season	1	0.167	0.338	0.562^{ns}
	Year	1	76.374	154.603	<0.01**
	Season × year	1	2.815	5.698	<0.05*
	Error	175	0.494		

Table 3 continued

Egg parameters	Factor	*df*	MS	*F*	*P*
Lipid (%W)	Season	1	1.50	3.177	0.142^{ns}
	Year	1	39.26	83.177	<0.05*
	Season × year	1	7.361	15.595	<0.05*
	Error	178	0.472		
Protein (%W)	Season	1	0.601	2.293	0.185^{ns}
	Year	1	9.152	34.931	<0.05*
	Season × year	1	1.740	6.641	<0.05*
	Error	178	0.262		

Interannual variations in egg size, biomass and chemical composition: two-way ANOVA evaluating differences in egg parameters (see Table 1) between seasons and years; asterisks: significant differences (*$P < 0.05$; **$P < 0.01$)

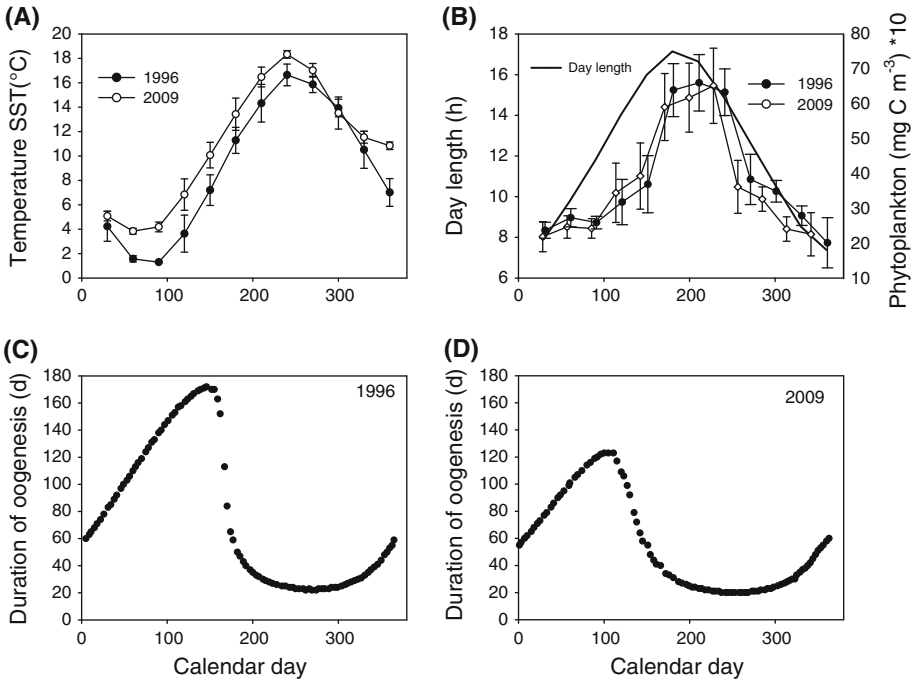

Fig. 5 *Crangon crangon*. Seasonal variations in environmental factors and duration of oogenesis: (**a**) temperature (°C), mean values ± SD; (**b**) day length (h) and phytoplankton biomass (mg C m^{-3})·10, mean values ± SD; (**c**, **d**) duration of oogenesis (**d**) estimated for 1996 and 2009, respectively (for modelling, see "Materials and methods" section)

Meusy and Payen 1988). According to an additive model of multiple explanatory variables, decreasing day length during autumn may be the principal factor inducing the production of bigger winter eggs, whereas its increase in spring may stimulate the production of smaller summer eggs.

In addition to seasonal changes in egg size, our data also indicated interannual variations, not only in the size and biomass per egg but also in the relative composition of embryonic biomass. These findings should caution against generalizations based upon short-term studies of reproductive traits in *Crangon crangon* and other shrimp species. Higher egg biomass and percentage C values observed during the period April–May of a colder year (1996 vs. 2009; see Fig. 5a; Tables 1, 2) seem to suggest that different average water temperatures might be responsible for interannual variations in reproductive traits. However, explanations for variations among years remain difficult as long as comparable data are available for only 2 years. Future long-term comparisons may reveal the underlying causes, eventually allowing for an enhanced predictability of intraspecific variations in egg size and biomass.

Interannual variation in the estimated duration of oogenesis was caused by differences in average water temperature. In a cold winter (January-March 1996, temperatures ~1–3°C), oogenesis took much longer (172 ± 8 days) than in a mild winter (2009–2010; 4–6°C; 122 ± 3 days). The delay in oogenesis of winter eggs may be explained by a transitory period of diapause at very low temperatures, as described also for embryogenesis in various other species of crustaceans (Wear 1974, Petersen and Anger 1997; Webb et al. 2007). In *Crangon crangon*, such a developmental arrest seems to occur at temperatures

Table 4 *Crangon crangon*

Parametric coefficients	Estimate	Std error	*t* value	r^2	*F*	*P*
Intercept						
1996	9.01228	0.71036	12.689	–	–	<0.0001
2009	12.7108	0.68020	18.677	–	–	<0.0001
Day length						
1996	−0.26416	0.02895	−9.125	0.38	83.26	<0.0001
2009	−0.65406	0.05653	−11.57	0.4	133.87	<0.0001
Temperature						
1996	0.31323	0.04773	6.563	0.08	43.07	<0.05
2009	0.39857	0.04317	9.234	0.09	85.26	<0.05
Phytoplankton biomass						
1996	−0.05866	0.00983	−5.924	0.11	32.96	<0.05
2009	−0.12977	0.02871	−4.711	0.12	22.18	<0.05

Additive model of multiple explanatory variables evaluating the effects of environmental factors (temperature, day length and phytoplankton biomass) estimated for the time at the onset of oogenesis in 1996 and 2009 on egg biomass (expressed as carbon content, μg C egg^{-1}); for model and parameters, see "Materials and methods" section; estimated parameters, standard errors, *t* values, coefficients of determination (r^2), *F* values, significance level (*P*); total variance explained by the model: 57% and 61%, for 1996 and 2009, respectively

below ~4°C, probably both in oogenesis and embryogenesis (for discussion see Wear 1974, Paschke 1998).

Since *Crangon crangon* is a key species in coastal benthic communities and target of a high-value trawl fishery in the southern North Sea, variations in its population dynamics have immediate effects on both the coastal marine food web and the commercial exploitation of this species, whose landings are consistently highest in autumn (ICES 2009). As differences in the energy provision of early eggs may subsequently affect the larval and later life-history stages, seasonal variations in egg size have implications also for fishery models estimating the relative contributions of different cohorts to recruitment and production of adult benthic populations and thus, to commercial fisheries (Oh et al. 1999; Spaargaren 2000; Temming and Damm 2002; Viegas et al. 2007; Siegel et al. 2008; Campos et al. 2009).

Acknowledgments We thank the crews of R.V. "Uthörn" and "Aade" for capture and transporting live shrimps to Helgoland, Uwe Nettelmann helped in the maintenance of the animals; Cornelia Püschel, Bettina Oppermann and Julia Haafke made elemental analyses. We also thank two anonymous reviewers for constructive criticism and helpful suggestions. AU and KP were financially supported by the Deutscher Akademischer Austauschdienst (DAAD, Bonn, Germany). AU also thanks the support of the Comisión Nacional de Ciencia y Tecnología, CONICYT (Santiago de Chile), funding this study as a part of his doctoral dissertation. The experiments comply with animal manipulation laws in Germany.

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CHAPTER IV

Seasonal variations in larval biomass and biochemical composition of brown shrimp, *Crangon crangon* (Decapoda, Caridea), at hatching

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Helgol Mar Res
DOI 10.1007/s10152-012-0321-4

ORIGINAL ARTICLE

Seasonal variations in larval biomass and biochemical composition of brown shrimp, *Crangon crangon* (Decapoda, Caridea), at hatching

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Received: 26 April 2012 / Revised: 13 July 2012 / Accepted: 17 July 2012

Abstract The "brown shrimp", *Crangon crangon* (Linnaeus 1758), is a benthic key species in the North Sea ecosystem, supporting an intense commercial fishery. Its reproductive pattern is characterized by a continuous spawning season from mid-winter to early autumn. During this extended period, *C. crangon* shows significant seasonal variations in egg size and embryonic biomass, which may influence larval quality at hatching. In the present study, we quantified seasonal changes in dry weight (W) and chemical composition (CHN, protein and lipid) of newly hatched larvae of *C. crangon*. Our data revealed significant variations, with maximum biomass values at the beginning of the hatching season (February–March), a decrease throughout spring (April–May) and a minimum in summer (June–September). While all absolute values of biomass and biochemical constituents per larva showed highly significant differences between months ($P < 0.001$), CHN, protein and lipid concentrations (expressed as percentage values of dry weight) showed only marginally significant differences ($P < 0.05$). According to generalized additive models (GAM), key variables of embryonic development exerted significant effects on larval condition at hatching: The larval carbon content (C) was positively correlated with embryonic carbon content shortly after egg-laying ($r^2 = 0.60$; $P < 0.001$) and negatively with the average incubation temperature during the period of embryonic development ($r^2 = 0.35$; $P < 0.001$). Additionally, water temperature ($r^2 = 0.57$; $P < 0.001$) and food availability (phytoplankton C; $r^2 = 0.39$; $P < 0.001$) at the time of hatching were negatively correlated with larval C content at hatching. In conclusion, "winter larvae" hatching from larger "winter eggs" showed higher initial values of biomass compared to "summer larvae" originating from smaller "summer eggs". This indicates carry-over effects persisting from the embryonic to the larval phase. Since "winter larvae" are more likely exposed to poor nutritional conditions, intraspecific variability in larval biomass at hatching is interpreted as part of an adaptive reproductive strategy compensating for strong seasonality in plankton production and transitory periods of larval food limitation.

Keywords *Crangon crangon* · Eggs · Larvae · Biochemical composition · Seasonal variations · Carry-over effects · Southern North Sea · Food availability

Introduction

Marine organisms with a complex life cycle develop through a series of different ontogenetic stages including embryonic, larval, juvenile and adult phases, which are linked to each other (Giménez 2006; Podolsky and Moran 2006). In invertebrates and fish, variability in the density of adult populations has mainly been attributed to fluctuations in brood size and to variations in growth and mortality during earlier life-history stages (McCormick and Hoey 2004; Marshall and Keough 2006).

The environmental conditions experienced during the embryonic phase have been recognized as important factors influencing larval performance, and therefore, indirectly also later benthic life-history phases of marine invertebrates

Communicated by H.-D. Franke.

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Published online: 31 July 2012

(Grosberg and Levitan 1992; Giménez 2010). In all pleocyemate decapod crustaceans, the eggs are carried under the abdomen of the females and thus experience the parental environmental conditions prevailing in the habitat. Newly laid eggs contain all the energy that is necessary for embryonic development (Jaeckle 1995), and therefore, the initial larval biomass depends on both these initial energy reserves and their subsequent utilization during embryogenesis (Anger 2001). Also, it is influenced by physico-chemical factors experienced during embryogenesis, for example, temperature (Wear 1974; Fischer et al. 2009), oxygen (Fernandez et al. 2006) and salinity (Giménez and Anger 2001), so that previous environmental conditions may also affect the larval capacity to face starvation during periods of planktonic food limitation (Paschke et al. 2004; Calado et al. 2007).

In the pelagic marine environment, crustacean larvae may seasonally be exposed to food limitation and temperature changes, and combined effects of both factors may influence their growth and development (Anger 2001). As an adaptation to seasonal variation in food availability, planktotrophic larvae may therefore seasonally vary in their energy content at hatching, showing enhanced endogenous reserves during periods of low plankton productivity (Anger 2001, 2006). In temperate and high-latitude marine invertebrates, this reproductive trait has been interpreted as an evolutionary adaptation to a mismatch between short seasonal periods of planktonic food availability and a prolonged larval development at low temperatures (e.g. Anger et al. 2003).

Environmental effects on sets of reproductive traits of organisms can have important consequences on populations. For a better understanding of the ecology of species with complex life cycles, we need to study how the various life-history phases are connected to each other. One important link is between the embryonic and the larval phase. In this context, the larval performance can be related to maternal input, conditions experienced during embryogenesis, and to environmental factors prevailing at hatching or thereafter (Giménez 2006). The combination of these pre- and post-hatching factors may explain larval survival and, consequently, affect also later phases of the life cycle (Roughgarden et al. 1988; Giménez 2010). Hence, the quality of early larval stages may be relevant not only for the settlement and recruitment success in the field (Pan et al. 2011), but also for aquaculture (Racotta et al. 2003) and fisheries management (Campos et al. 2009).

The subject of this study is the generally well studied and commercially important "brown shrimp", *Crangon crangon*. This species is very common and abundant in the shallow areas of the German Bight (Siegel et al. 2008), plays an important role in the energy transfer within marine food webs (Pihl and Rosenberg 1984; Campos et al. 2009) and sustains an important fishery with annual captures exceeding 35,000 tons (ICES 2010). The complex life cycle of *C. crangon* comprises a benthic juvenile–adult and a pelagic larval phase (Tiews 1970; Hufnagl and Temming 2011), which are tightly linked (Daewel et al. 2011; Viegas et al. 2012). It is thus necessary to also investigate traits of the early life-history phases, which may influence the population dynamics.

The reproductive pattern of *C. crangon* is characterized by continuous egg-laying from mid-winter throughout spring, summer and early autumn. During this extended period, this species shows significant seasonal variations in size and biomass of newly laid eggs (Boddeke 1982; Urzúa et al. 2012). However, the question remains, whether seasonal variations in egg biomass directly translate to variations in larval quality at hatching, as suggested by studies of larval tolerance of food limitation (Paschke et al. 2004). We therefore explored relationships between previously published data of biomass and chemical composition of eggs (Urzúa et al. 2012), water temperature during embryogenesis (Wiltshire et al. 2010) and larval biomass at hatching, which may indicate "latent" or "carry-over effects" between successive early life-history phases (Harrison et al. 2011).

Materials and methods

The methodology used in this study is described only briefly, as it is largely the same as explained in details in a recent paper dealing exclusively with the embryonic phase of *C. crangon* (Urzúa et al. 2012).

Sampling and maintenance of ovigerous females

Throughout most of the reproductive season of *C. crangon* in 2009 (January–September), shrimps were periodically sampled from a population living in the lower Elbe estuary (54°03′–54°04′N; 8°18′–8°24′E; ca 13 m depth) employing bottom trawls of research vessel "Uthörn". Adult individuals were transferred to the Helgoland Marine Biological Station. No samples were taken in October–December due to unavailability of ship time. In the laboratory, ovigerous females ($n = 32$; 48–54 mm total body length) were isolated and placed in aerated flow-through seawater aquaria with similar temperatures and salinities as in the field, until newly hatched larvae were found in sieves (0.2 mm mesh size) receiving the overflowing water from the aquaria.

Total length (TL), dry weight (*W*), elemental composition (CHN) and proximate biochemical composition (total protein and lipid) of newly hatched larvae

Newly hatched larvae were taken in regular intervals (approximately monthly) for parallel determinations of

total length (TL), dry weight (W), elemental composition (contents of carbon, hydrogen and nitrogen; collectively CHN) and proximate biochemical composition (total protein and lipid), which were measured with standard techniques (for recent description of details, see Urzúa and Anger 2011; Urzúa et al. 2012). The energy content was estimated from biochemical data (Winberg 1971), because conversions from CHN data (Salonen et al. 1976) tend to underestimate the energy content more strongly than conversions from biochemical composition (Anger 2001; Urzúa et al. 2012).

Statistical analyses

Statistical analyses were performed with standard methods (Sokal and Rohlf 1995) using the statistics software packages STATISTICA 8 (StatSoft) and Brodgar 2.6.6. All tests were run on the 95 % confidence level ($P < 0.05$). Normality and homogeneity of variances were tested with Kolmogorov–Smirnov and Levene's tests, respectively. Data of seasonal variations in biomass and biochemical composition of newly hatched larvae were tested using a two-level nested ANOVA, with month as fixed factor and hatch (or female) as nested factor. Significant differences were analyzed with a multiple comparison test (Student–Newman–Keuls). Relationships between egg size and larval biomass at hatching were tested with regression analysis.

Generalized additive models (GAM)

We applied generalized additive models based on the R-mgcv function (Zuur et al. 2007) with various explanatory variables to determine, in a first step (GAM I), the influence of embryonic physiology (pre-hatching factors) on initial larval biomass. As a second step (GAM II), we evaluated relationships between larval quality and environmental parameters prevailing at the time of hatching (for more details, see Urzúa et al. 2012). Normality, outliers and collinearity were checked following Zuur et al. (2007).

Effects of pre-hatching factors (GAM I)

Data of initial egg biomass and mean temperature during the period of embryonic development were taken from Urzúa et al. (2012), and the effects of these factors on larval carbon content (used as a proxy for total organic matter) at hatching were tested with the following model:

$$Y_i = \alpha + f_1(\mathrm{EB}_i) + f_2(\mathrm{TE}_i) + \varepsilon_i \quad (1)$$

with Y = larval carbon (µg larva^{-1}), EB = initial egg biomass (C, µg egg^{-1}), TE = mean temperature during the period of egg development (°C), α = intercept, $f_{1,2}$ = smoothing functions, ε = error term, i = observed data, σ^2 = variance, where $\varepsilon_i \sim n\ (0, \sigma^2)$.

Environmental conditions prevailing at the time of hatching (GAM II)

As explanatory variables, we included in this model the mean water temperature and phytoplankton carbon (as a proxy for food availability) measured through a fortnight period prior to the day of larval hatching (data from Wiltshire et al. 2010). The relationships between these environmental parameters and larval quality at hatching were explored with the equation:

$$Y_i = \alpha + f_1(\mathrm{TH}_i) + f_2(\mathrm{PB}_i) + \varepsilon_i \quad (2)$$

with Y = larval carbon (µg larva^{-1}), TH = mean temperature during a fortnight period prior to hatching (°C), PB = mean phytoplankton biomass during a fortnight period prior to hatching (mg C m^{-3}); other parameters as above (Eq. 1).

Results

Seasonal variations in offspring size and female energy investment in egg production

Measurements of egg volume (Urzúa et al. 2012) revealed significant differences between seasons, with higher average values observed in winter than in summer (44.3 ± 3.26 vs. 34.5 ± 1.66 mm^3 $\times$ 10^{-3}; $F = 242.76$; $P < 0.05$). Corresponding with larger "winter eggs", larvae hatching in late winter and early spring were on average larger than those hatching in late spring and summer (total length, 2.80 ± 0.12 vs. 2.10 ± 0.06 mm; $F = 122.56$; $P < 0.05$).

While the average number of eggs produced per female was significantly lower in winter than in summer (1842 ± 86 vs. 2526 ± 108; $F = 82.94$; $P < 0.05$), the female energy investment in egg production (calculated as number of egg multiplied by the energy content per egg) did not show significant seasonal differences (1484 ± 86 vs. 1518 ± 44 J; $F = 1.22$; $P = 0.18$).

Seasonal variations in larval dry weight (W) and elemental composition (CHN) at hatching

Periodical samples of newly hatched larvae revealed that all values of biomass showed significant differences among months (Table 1). Maximal values were measured at the beginning of the larval hatching season in late winter (February–March), decreasing values during spring (April–May) and minimum values in summer (June–September)

Table 1 *Crangon crangon*, newly hatched larvae

Biomass parameters	Factor	SS	*df*	MS	*F*
W (μg)	Month	2303.63	7	329.09	297.69***
	Hatch (month)	209.89	24	8.75	7.91*
	Error	106.13	96	1.11	
	Total	2619.64	127		
C (μg)	Month	451.692	7	64.527	281.23***
	Hatch (month)	42.972	24	1.791	7.80*
	Error	22.027	96	0.229	
	Total	516.691	127		
N (μg)	Month	38.3862	7	5.4837	299.67***
	Hatch (month)	2.8055	24	0.1169	6.39*
	Error	1.7568	96	0.0183	
	Total	42.9484	127		
H (μg)	Month	14.0591	7	2.0084	363.88***
	Hatch (month)	1.0340	24	0.0431	7.81*
	Error	0.5299	96	0.0055	
	Total	15.6230	127		
Lipid (μg)	Month	8.1994	7	1.1713	323.39***
	Hatch (month)	0.6374	24	0.0266	7.33*
	Error	0.5795	160	0.0036	
	Total	9.4163	191		
Protein (μg)	Month	182.220	7	26.031	1011.6***
	Hatch (month)	2.697	24	0.112	4.4*
	Error	4.117	160	0.026	
	Total	189.034	191		
E (J)	Month	0.63117	7	0.090167	388.65***
	Hatch (month)	0.06995	24	0.002914	12.56*
	Error	0.03722	160	0.000232	
	Total	0.73834	191		
C (% W)	Month	56.5	7	8.1	10.8*
	Hatch (Month)	109.5	24	4.6	6.1*
	Error	72.1	96	0.8	
	Total	238.1	127		
N (% W)	Month	34.69	7	4.96	36.88*
	Hatch (month)	6.87	24	0.29	2.13*
	Error	12.90	96	0.13	
	Total	54.46	127		
H (% W)	Month	53.864	7	7.695	163.7**
	Hatch (month)	10.731	24	0.447	9.5*
	Error	4.512	96	0.047	
	Total	69.107	127		
C/N ratio	Month	0.970	7	0.139	14.8*
	Hatch (month)	0.809	24	0.034	3.6*
	Error	0.896	96	0.009	
	Total	2.675	127		
Lipid (% W)	Month	4.987	7	0.712	23.9*
	Hatch (month)	5.124	24	0.214	7.2*
	Error	4.765	160	0.030	
	Total	14.877	191		

Table 1 continued

Biomass parameters	Factor	SS	*df*	MS	*F*
Protein (% W)	Month	32.8	7	4.7	37*
	Hatch (month)	8.4	24	0.3	3*
	Error	20.3	160	0.1	
	Total	61.4	191		
E (J*mg W^{-1})	Month	30.00	7	4.29	15.8*
	Hatch (month)	31.23	24	1.30	4.81*
	Error	43.02	160	0.27	
	Total	104.25	191		

Two-level nested ANOVA evaluating differences in dry weight, elemental composition and proximate biochemical composition among months and hatches nested within months; interaction between these factors; biomass parameters: (*W*) dry weight, (*C*) contents of carbon, (*N*) nitrogen, (*H*) hydrogen, C/N ratio, lipid, protein and (*E*) energy; significant differences marked with *asterisks* (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)

(Figs. 1, 3). Hence, larvae hatching during winter were heavier than those hatching in summer (Fig. 1a). Consistent with higher dry weight, "winter larvae" showed also higher contents of CHN per individual (Fig. 1b–d). Their average biomass reached twice the values recorded in "summer larvae" (11 vs. 6 µg C; 3 vs. 1.5 µg N; 1.8 vs. 1 µg H; cf. February vs. July) (Fig. 1).

While all absolute values of biomass per larva showed highly significant differences among months (all $P < 0.001$), values expressed in percent of dry weight showed only marginally significant differences ($P < 0.05$) (Table 1). For example, carbon and nitrogen (expressed in % W) remained relatively stable at average levels of about 37 and 10 %, respectively (Fig. 2).

Seasonal variations in the proximate biochemical composition of larval biomass

The biochemical composition (total lipid and protein per larva) and energy content (J per larva, estimated from biochemical data) showed similar patterns as dry weight and elemental composition (Fig. 3). Again, a maximum level was found in February–March, with average values of about 1.3 µg lipid, 8 µg protein and 0.7 J per larva.

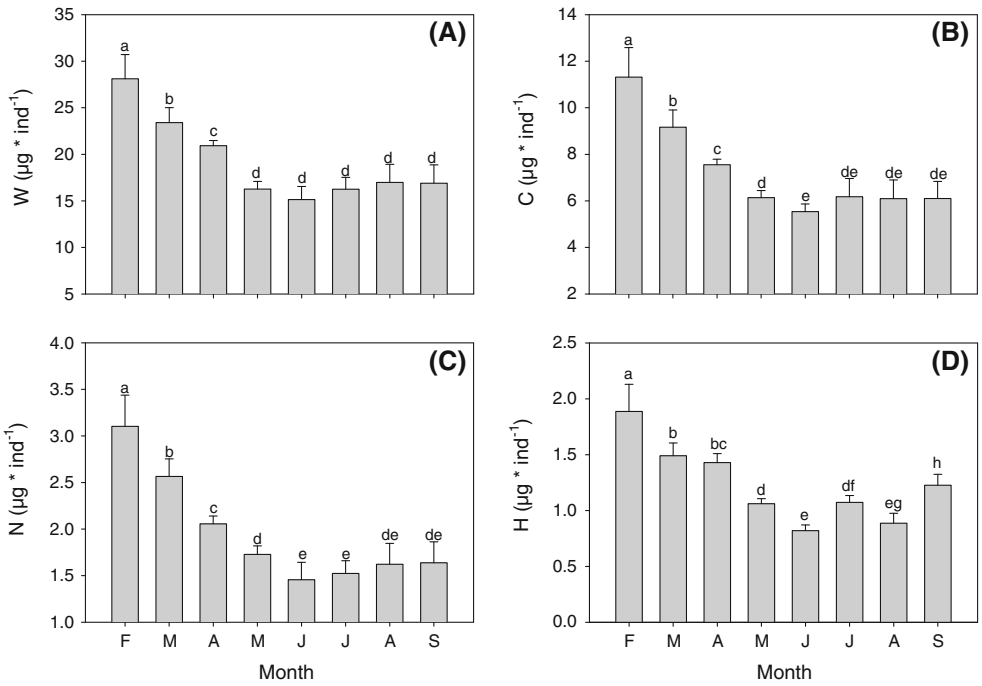

Fig. 1 *Crangon crangon*, newly hatched larvae. Seasonal variations in dry weight (*W*) and elemental composition (CHN): **a** dry weight, **b** carbon, **c** nitrogen and **d** hydrogen (all expressed in µg ind^{-1}); mean values ± SD. *Different lower case letters* indicate significant differences among months (after SNK test)

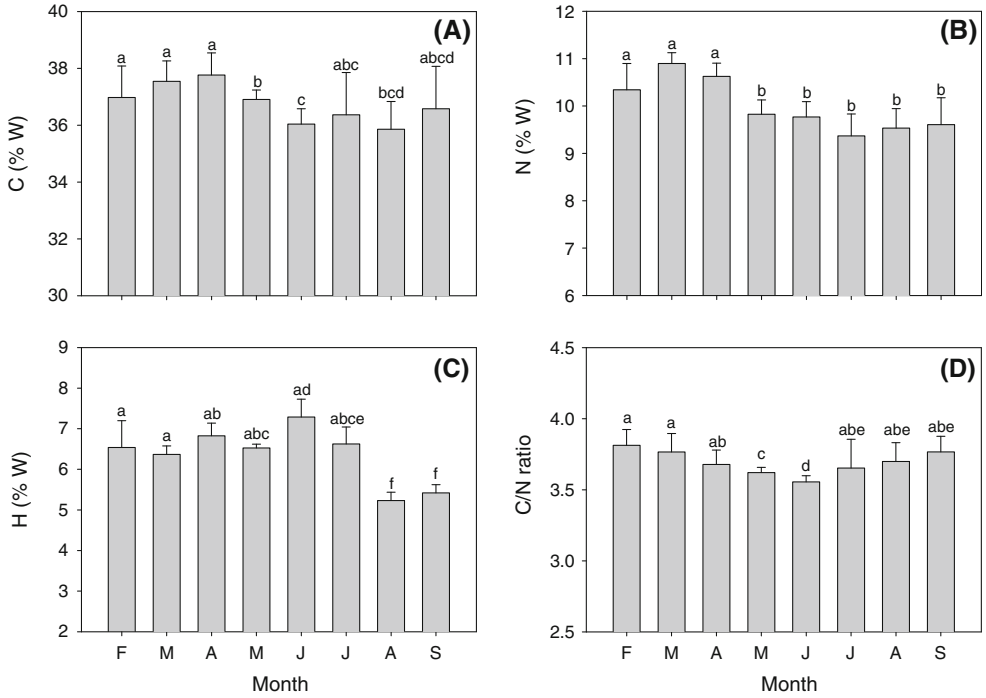

Fig. 2 *Crangon crangon*, newly hatched larvae. Seasonal variations in elemental composition: **a** carbon, **b** nitrogen and **c** hydrogen (all expressed in % of W); **d** C/N ratio; mean values ± SD. *Different lower case letters* indicate significant differences among months (after SNK test)

Decreasing levels were measured from April to June, with minimum values about 0.7 µg lipid, 5 µg protein and 0.45 J. A slight increase occurred subsequently between July and September, reaching values of about 0.8 µg lipid, 6 µg protein and 0.5 J (Fig. 3). All values of lipid, protein and energy content per larva differed significantly among months (all $P < 0.001$) (Table 1).

Compared to the absolute biomass values, the relative biochemical composition and energy content (expressed in % of W or J mg W^{-1}, respectively) showed only marginally significant seasonal variations throughout the reproductive period ($P < 0.05$) (Table 1), with average values of about 5.7 % lipid, 46 % protein and 18.5 J mg W^{-1} (Fig. 3).

Relationships between egg size and elemental composition of newly hatched larvae

The elemental composition (CHN) and energy content (E, estimated from biochemical data) of newly hatched larvae (values in µg or J per individual, respectively) were positively correlated with egg size ($r^2 = 0.71$, 0.73, 0.68 and 0.75 for C, N, H and E, respectively; all $P < 0.001$, Fig. 4). Seasonal variations in egg size are thus propagated to larval biomass at hatching, so that larvae hatching from larger "winter eggs" showed higher CHN and energy values than those hatching from smaller "summer eggs".

Effects of pre-hatching factors

According to generalized additive models (GAM) of multiple explanatory variables, a combination of key factors related to embryonic physiology exerted significant effects on the carbon content of newly hatched larvae. Based on the smoothing function of the GAM I model, the carbon content at hatching showed a highly significant positive relationship with initial egg biomass (data from Urzúa et al. 2012) and a negative relation with the average incubation temperature during the period of embryonic development (Fig. 5a, b; Table 2). About 60 % of the variation in the carbon content per larva can be explained by effects of seasonal variations in embryonic carbon, while the average incubation temperature contributed 35 % to the variation in the carbon content of newly hatched larvae (Fig. 5a, b).

Effects of environmental conditions prevailing at the time of hatching

The temperature (TH) and phytoplankton biomass (PB) prevailing at the time of hatching are significantly linked with initial larval quality (measured as C content). An additive model of multiple explanatory variables indicated that larval C showed highly significant negative correlations with both TH ($r^2 = 0.57$; $P < 0.001$) and PB ($r^2 = 0.39$; $P < 0.001$) measured at the time of hatching

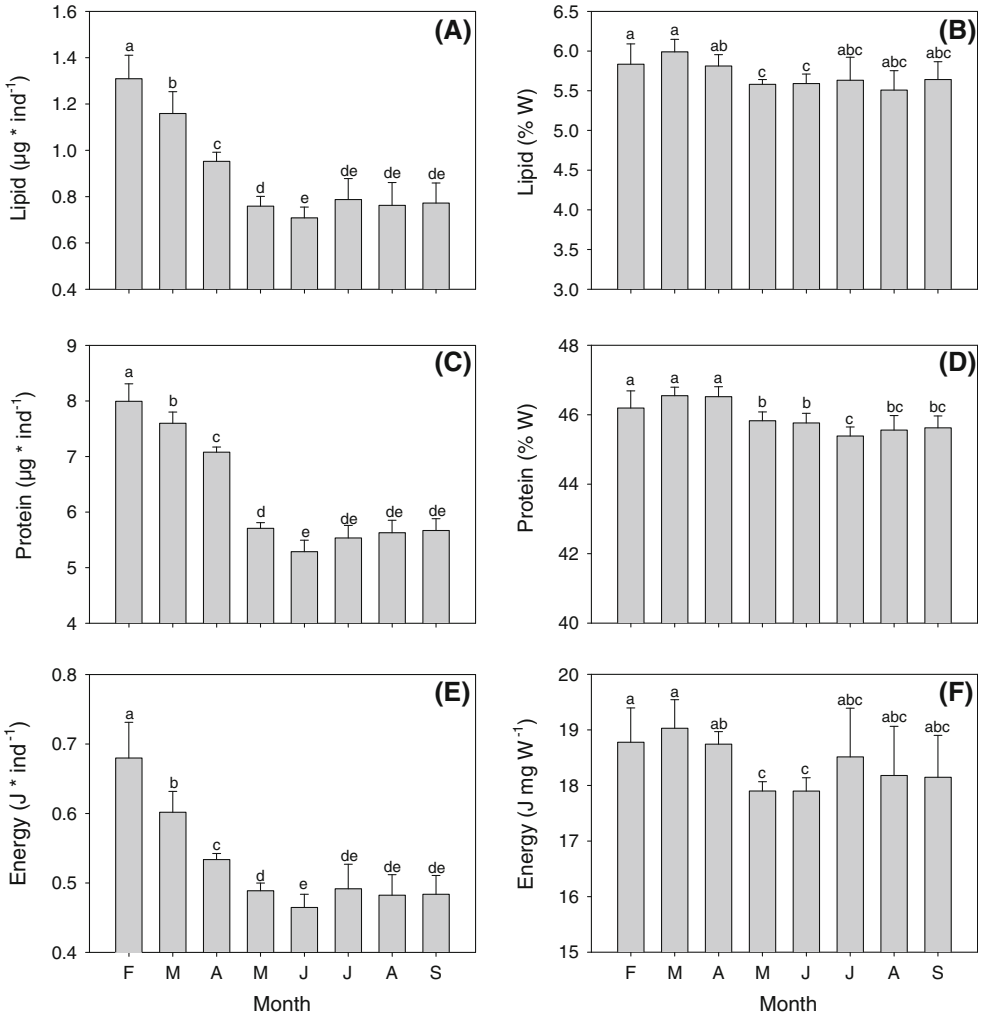

Fig. 3 *Crangon crangon*, newly hatched larvae. Seasonal variations in proximate biochemical composition: **a**, **b** lipid, expressed in µg ind^{-1} and % of *W*, respectively; **c**, **d** protein, expressed in µg ind^{-1} and % of *W*, respectively; **e**, **f** energy content (estimated from biochemical data), expressed in J ind^{-1} and J mg W^{-1}, respectively; mean values ± SD. *Different lower case letters* indicate significant differences among months (after SNK test)

(Table 2). According to the GAM II model, the smoothing function for larval quality showed a maximum level in winter (environmental conditions at the time of hatching: ca. 5 °C and 27 mg C m^{-3}·10; TH and PB, respectively), decreased gradually thereafter, reached a minimum in spring (ca. 10 °C and 38 mg C m^{-3}·10) and increased subsequently again in late summer (ca. 17 °C and 62 mg C m^{-3}·10) (Fig. 5c, d).

Discussion

In marine invertebrates with complex life cycles, the different life-history stages are closely linked to each other (Allen and Marshall 2010; Harrison et al. 2011). In the early stages of the life cycle of *C. crangon*, our results indicate that seasonal variations in egg size are translated to variations in larval biomass. We demonstrate here that larvae hatching from larger "winter eggs" show higher absolute values of elemental (CHN) and biochemical constituents (lipid, protein) than larvae hatching from smaller "summer eggs".

In crustaceans, larval traits at hatching are related to key variables associated with embryonic development (Giménez and Anger 2001; Webb et al. 2007). For example, the average egg incubation temperature exerts significant effects on the development time and bioenergetics of the embryo and influences the initial larval biomass at hatching (Paschke 1998; Fischer et al. 2009). In *C. crangon*, the initial larval biomass was negatively correlated with the incubation temperature during egg development. Compared to larvae hatching from eggs that had been incubated at cold winter temperatures, "summer larvae" showed lower values of biomass. While egg development is

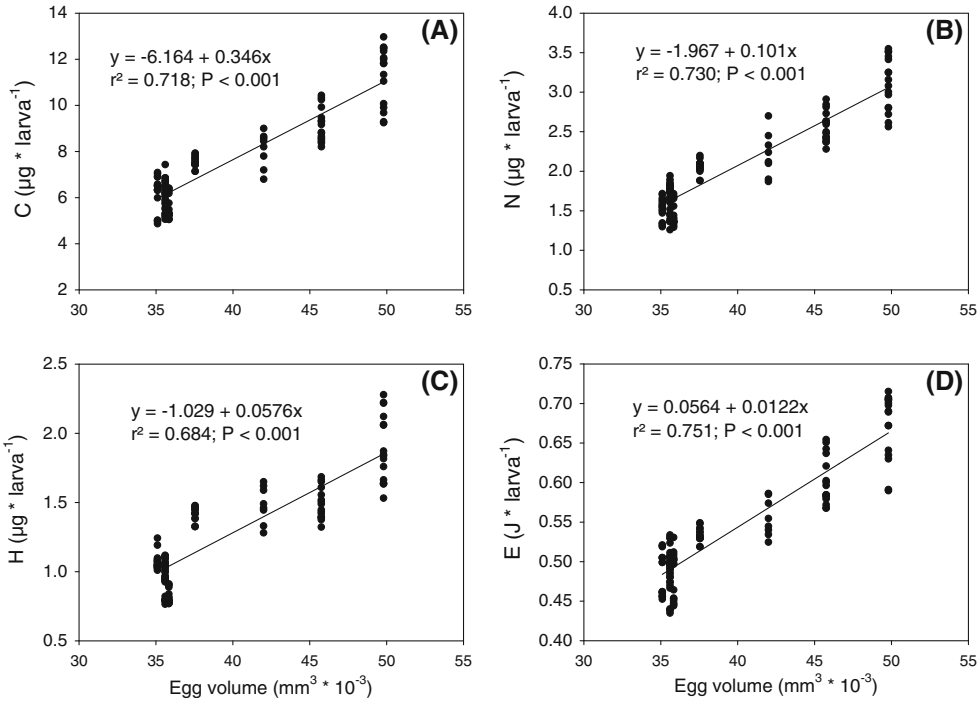

Fig. 4 *Crangon crangon*, newly hatched larvae. Linear regression model evaluating relationship between egg size (volume) and elemental composition of newly hatched larvae: $y =$ **a** carbon, **b** nitrogen, **c** hydrogen and **d** energy content (all expressed in values per larva); a = intercept, b = slope; r^2 = determination coefficient; P = significance level; in all cases $n = 128$

accelerated at higher temperatures, this may be at the cost of changes in metabolic efficiency (Kunisch and Anger 1984), leading to a reduction in initial larval biomass. This has been shown in various caridean shrimp species, for example, *Betaeus emarginatus* and *Pandalus borealis* (Wehrtmann and Lopez 2003; Brillon et al. 2005).

In order to evaluate the adaptive significance of intraspecific variation in egg size, we need to understand how differential traits may be carried over to later life-history stages. Most likely, an enhancement of larval biomass at hatching should improve larval survival under suboptimal food conditions. This may be influenced by genetic or maternal factors that are involved in the determination of larval quality (Palacios et al. 1998; Meidel et al. 1999).

In planktonic organisms living in environments with seasonal variations in productivity (food availability), parental organisms may assess the nutritional conditions that their offspring will likely encounter and adjust the energy investment per offspring accordingly (Fischer et al. 2011). According to the relationship between environmental conditions prevailing at hatching and larval biomass observed in our study, the smoothing function of GAM showed a maximum level in larval biomass during winter, a minimum in the late spring and another increase in late summer. High larval carbon content during late winter may improve the tolerance of fasting, when poor nutritional conditions coincide with prolonged duration of larval development at cold temperatures (Criales and Anger 1986; Paschke et al. 2004; Daewel et al. 2011). Hence, the production of larger "winter larvae" seems to be an adaptive reproductive trait of *C. crangon*, allowing for an extension of the period of reproduction (Siegel et al. 2008; Urzúa et al. 2012), which is in most other decapod crustaceans in temperate regions restricted to late spring and summer (Anger 2001). At higher temperatures and planktonic food concentration, during late spring and summer (Wiltshire et al. 2008), smaller shrimp larvae hatch in the southern North Sea. This match between favorable conditions of food availability and high temperatures with larval peak abundance during spring–summer (Wehrtmann 1989) allows the larvae to exploit rich food resources and reach fast rates of growth and development (Temming and Damm 2002). In this way, our study confirms the hypotheses proposed by Paschke et al. (2004) and provides a chemical basis explaining the previously observed seasonal patterns in larval starvation resistance. Similar seasonal variations in offspring quality and environmental conditions prevailing at hatching have also been described in other aquatic crustacean species, for example in porcelain crab (Gebauer et al. 2010), marine copepods (Acheampong et al. 2011) and limnic cladocerans (Boersma 1997).

While the production of large larvae in winter is probably related mainly to low food availability, larger size could also play a role as a protection from predators (Morgan 1995). Predation is a major cause of larval mortality in *C. crangon* (e.g. Henderson et al. 2006), with various fish species being well-known predators (Tiews 1978). As large "winter larvae" show a prolonged planktonic development time, they are also exposed to a higher risk of predation. By contrast, smaller "summer larvae"

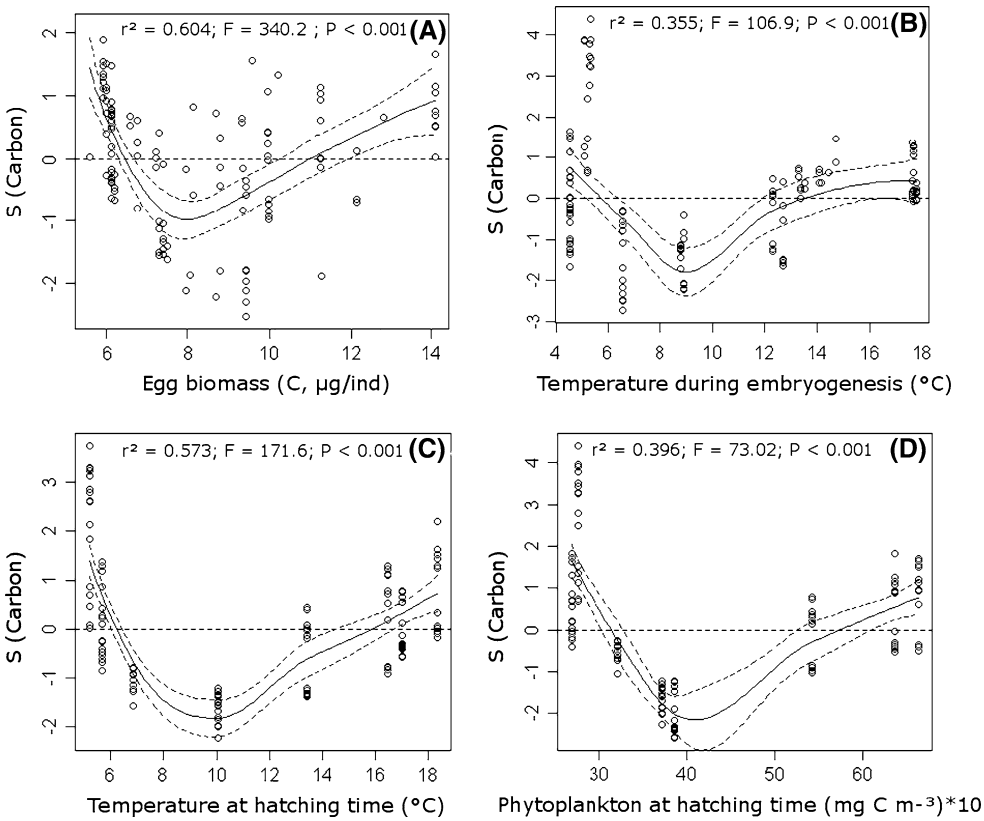

Fig. 5 *Crangon crangon*, newly hatched larvae. Smoothing function (*S*) obtained by generalized additive model (GAM) for larval carbon content (C, µg larva^{-1}) exploring the effects of pre-hatching factors [**a** initial egg biomass and **b** incubation temperature] and the environmental conditions prevailing at the time of hatching [**c** temperature and **d** phytoplankton biomass]. These plots allow evaluating the relationships between explanatory variables (*x*-axis) and adjusted residuals of dependent variable (carbon, *y*-axis). *Solid line* estimated smoothing function; *dotted lines* 95 % confidence intervals. Total variance is quantified by values of r^2, the significance of the smoothing function by values of *F* and *P*; *dots* represent mean values, in all cases $n = 128$. For statistical model and parameters, see Table 2

Table 2 *Crangon crangon*, newly hatched larvae

Parametric coefficients	Estimate	SE	*t* value	r^2	*P* value
Effects of pre-hatching factors					
Intercept	−3.332	0.58	−5.721	–	<0.001
Initial egg biomass	0.402	0.021	17.42	0.60	<0.001
Temperature during embryogenesis	−0.278	0.026	−10.34	0.35	<0.001
Environmental conditions					
Intercept	10.56	0.36	29.31	–	<0.001
Temperature prevailing at hatching time	−0.303	0.023	−13.1	0.57	<0.001
Phytoplankton prevailing at hatching time	−0.081	0.009	−8.545	0.39	<0.001

Additive model of various explanatory variables evaluating the effects of pre-hatching factors and environmental conditions prevailing at the time of hatching on larval condition; evaluated parameters, estimate, SE, *t* values, coefficients of determination (r^2), significance level (*P value*)

show faster rates of development and growth (Linck 1995; Criales and Anger 1986), which reduces the time of exposure to predation and other potential risks in the plankton, enhancing the chance of successful recruitment (Morgan 1995).

Crangon crangon produces fewer but larger "winter eggs" and a higher number of smaller "summer eggs" (Urzúa et al. 2012). In this context, the female energy investment in egg production did not show significant differences between winter and summer. In *C. crangon*, as well as other decapod crustaceans, total energy investment in embryo production is influenced by maternal traits such as female size and abdominal space available for egg incubation (Ouellet and Plante 2004; Moland et al. 2010).

The reproductive traits of *C. crangon* may vary over latitudinal gradients within the large climatic range of distribution of this species (Tiews 1970; Campos and van der Veer 2008). In populations at lower latitudes (e.g. the west coast of Portugal, southwestern Europe), reproduction takes place only from late winter to early summer, with a main spawning and breeding season during spring (Marchand 1981; Viegas et al. 2012). The offspring there is smaller, and no seasonal variations in egg weight have been observed (Viegas et al. 2012). In the warmer and

seasonally less variable Mediterranean Sea, the brown shrimp shows a shorter breeding season, comprising only the coldest months from November to April (Gelin et al. 2000). In conclusion, strong seasonality in plankton production of temperate regions, such as the southern North Sea, may represent a selection factor favouring an evolution of seasonal variability in larval biomass. We suggest that this reproductive trait allows the brown shrimp to extend its reproductive period.

Besides seasonal, also interannual variability has been observed in the biomass of crustacean offspring (e.g. Shirley and Shirley 1989; Giménez 2010; Urzúa et al. 2012). In future investigations, it would thus be interesting, especially in the context of climate change, to further consider variability among year classes of eggs and larvae and their relationships with variations in environmental factors.

Our study shows that seasonal variations in egg biomass of *C. crangon* are propagated to similar patterns of variation in larval biomass at hatching, indicating "carry-over effects" from the embryonic to the larval phase. Future studies of temperate species including *C. crangon* should thus investigate whether seasonal variations in larval biomass are propagated also further into the juvenile phase (see Giménez 2006, 2010; Pechenik 2006). In brown shrimp, comparative studies of the influence of temperature and food conditions on development time, survival and growth of both "winter larvae" and "summer larvae" may reveal implications for the condition of benthic juveniles and adults and thus for the stability and production of an important marine fishery resource.

Acknowledgments We thank the crews of R. V. "Uthörn" for capture and transporting live shrimps to Helgoland; Uwe Nettelmann helped in the maintenance of the animals; Julia Haafke made elemental analyses. We also thank two anonymous reviewers for constructive criticism and helpful suggestions. AU was financially supported by the Deutscher Akademischer Austauschdienst (DAAD, Bonn, Germany) and the Comisión Nacional de Ciencia y Tecnología, CONICYT (Santiago de Chile) funding this study as a part of his doctoral dissertation. The experiments comply with animal manipulation laws in Germany.

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CHAPTER V

Early zoeal development of the shrimp *Hippolyte leptocerus* (Decapoda, Caridea, Hippolytidae)

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Zootaxa 2988: 53–65 (2011)
www.mapress.com/zootaxa/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

Early zoeal development of the shrimp *Hippolyte leptocerus* (Decapoda, Caridea, Hippolytidae)

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Abstract

The morphology of the first three zoeal stages of *Hippolyte leptocerus* (Heller, 1863) are described and illustrated in detail from laboratory-hatched material. The ovigerous females were collected on the Alfacs Bay, Ebro Delta, Spain (Western Mediterranean). The early larval stages (ZI, ZII, ZIII) showed the anterolateral margin of carapace with denticulations, a median tubercle behind rostrum, scaphocerite segmented distally (only ZI and ZII), exopodal seta at the maxillule and pleonite 5 with a pair of dorsolateral spines. The morphology of the first three zoeal stages of *H. leptocerus* is typical of species with an extended larval development. Morphological characteristics of the genus *Hippolyte* are discussed.

Key words: Caridea, *Hippolyte*, zoea, morphology, larval development

Introduction

The genus *Hippolyte* (Leach, 1814) show an wide geographic and ecological distribution, comprising more than 30 species distributed worldwide except in extremely cold waters (d'Udekem d'Acoz, 1996). The taxonomy and systematic of the species of the genus *Hippolyte* is still problematic; the available information reveals a great deal of intraspecific variability in developmental traits, showing a high morphological variability in adults (d'Udekem d'Acoz, 1996; García Raso *et al*. 1998).

Among of the genus *Hippolyte*, 14 species are recently recorded in north-eastern Atlantic and Mediterranean waters (d'Udekem d'Acoz, 1999). *Hyppolyte leptocerus* has been recorded on photophile algae from intertidal zone to 30 m depth along the eastern Atlantic from western Ireland to Mauritania, including the Madeira and Cape Verde Islands and throughout the Mediterranean Sea and Black Sea (d'Udekem d'Acoz, 1996).

Information on larval morphology of genus *Hippolyte* is available for 14 species: *H. acuta* (Stimpson, 1860) (Yokoya, 1957); *H. bifidirostris* (Miers, 1876) (Packer, 1985); *H. clarki* Chace, 1951 (Needler, 1934 as *H. californiensis*); *H. coerulescens* (Fabricius, 1775) (Gurney, 1936 as *H. acuminata*); *H. inermis* Leach, 1815 (Bourdillo-Casanova, 1960; Heegard , 1963; Le Roux, 1963; Zupo and Buttino, 2001); *H. multicolorata* Yaldwyn, 1971 (Packer, 1985); *H. obliquimanus* Dana, 1852 (Terossi *et al.*, 2010), *H. pleuracanthus* (Stimpson, 1871) (Shield, 1978); *H. prideauxiana* Leach, 1817 (Lebour, 1931); *H. sapphica* d'Udekem d'Acoz, 1993 (Ntakis *et al.*, 2010); *H. varians* Leach, 1814 (Sars, 1911; Webb, 1921; Lebour, 1931); *H. ventricosa* H.Milne Edwards, 1837 (as *H. orientalis* Gurney, 1927); *H. williamsi* Schmitt, 1924 (Albornoz & Wehrtmann, 1997) and *H. zostericola* (Smith 1873) (Negreiros-Fransozo *et al.*, 1996). Except for *H. obliquimanus*, *H. pleuracanthus* and *H. sapphica*, the descriptions of their zoeal stages are incomplete or not described in detail. The complete larval development was described only for *H. pleuracanthus* and *H. sapphica*.

The present study aimed to describe in detail the morphology of the three first zoeal stages of *Hyppolyte leptocerus* from a population of western Mediterranean. This represent the first complete description of the early larval stages of genus *Hippolyte* species from eastern Atlantic and western Mediterranean.

Accepted by J. Goy: 7 Jul. 2011; published: 8 Aug. 2011

Material and methods

On June 2010, two ovigerous female (carapace length: 3.3±0.1 mm) of *Hyppolyte leptocerus* were captured by hand net on the Alfacs Bay, Ebro Delta (Western Mediterranean; 40º37' N; 0º36'E), on a sea grass meadows area (*Cimodocea* sp.) at a depth of 0.5 m(Fig. 1A). This constitute the first record of *Hyppolyte leptocerus* in the Ebro Delta. The ovigerous females were transported to the IRTA (Institut de Recerca i Tecnologia Agroalimentàries) in Sant Carles de la Ràpita. In the laboratory, the ovigerous females were placed in an aquarium (63 L) connected to a recirculation unit of sea water (34 psu and 21±1°C) and a natural photoperiod of ca 12h light per day. The newly hatched larvae of one female were transferred to 500 ml beakers. A total of 30 larvae were placed in each beaker. The subsequent larval rearing conditions, (temperature, salinity and photoperiod) were the same as in the maintenance of adult shrimps. The cultures (beakers) were checked every 24 h for moults or mortality, and water and food (rotifer: *Brachyonus plicatilis* and green algae: *Tetraselmis* sp, provided *ad libitum*) were changed daily. Samples of exuviae and specimens obtained of each developmental stage were preserved in 70% ethanol. The newly hatched larvae of the second female were preserved in 70% ethanol.

A minimum of five individuals of zoea I (from two females), three of zoea II and two of zoea III stage were measured and dissected for morphological description. The measurements were made with a Nikon SMZ800 stereo microscope equipped with an image analyzing system (AnalySIS, SIS, Münster, Germany). An Olympus BH-2 microscope was used in the observation of the features of the appendages and drawings were made from photomicrographs taken at different developmental stages.

The following measurements were taken: total length (TL) distance from the tip of the rostrum to the posterior margin of the telson; cephalotorax length (CL) distance from the tip of the rostrum to the posterior margin of the carapace. The descriptions are based on standards procedures proposed by Clark *et al*. (1998). Long aesthetascs of the antennules, the long plumose setae on antenna (scaphocerite) and maxilla (scaphognathite) and the long natatory setae on the distal exopod segments of the maxillipeds and pereiopods are drawn truncated.

The two adult females of the present study were deposited in the Biological Collections of Reference of the Institut de Ciències del Mar (CSIC) in Barcelona.

Results

The comparison of zoeae I from two different hatches allowed to determine morphological and morphometric homogeneity (i.e. no significant differences). All individuals except two third zoeae died in the second zoeal stage. The development time was 4–5 days for ZI and ZII. The first zoeal stage (Fig. 1B) is described in detail, while in subsequent stages only differences and changes are described.

Hyppolyte leptocerus (Heller, 1863)

(Figs 1B–7)

Zoea I. Size. TL= 1.33–1.36 mm; CL = 0.49–0.51 mm.

Carapace (Figs. 2A,B). Eyes sessile, with a single minute median tubercle. Rostrum long, directed slightly downward, exceeding the anterior margin of scaphocerite. Pterygostomial spine present with 2 small spines posteriorly. Supraorbital spines absent.

Antennule (Fig. 2C). Peduncle unsegmented, without setae. Inner flagellum (endopod) represented by a long plumose seta; outer flagellum unsegmented with 4 terminal aesthetascs.

Antenna (Fig. 2D). Birramous, peduncle (protopod) with a distal spine on inner magin. Endopod slender unsegmented, slightly more than ½ length of exopod and with 6 spines on inner side. Exopod (scaphocerite) 3-segmented with 10 (5, 1, 4) plumose setae, plus 1 short simple seta on the distal segment.

Mandible (Fig 2E, F). Asymmetrical; incisor and molar processes differentiated. Mandibular palp absent. Left mandible with lacinia mobilis near incisor process. Rigth mandible with 2 teeth between incisor and molar processes.

Maxillule (Fig. 2G). Coxal endite with 7 setae and basial endite with 7 spinous setae. Endopod unsegmented, with 5 terminal setae. Outer plumose seta (exopodal seta) present.

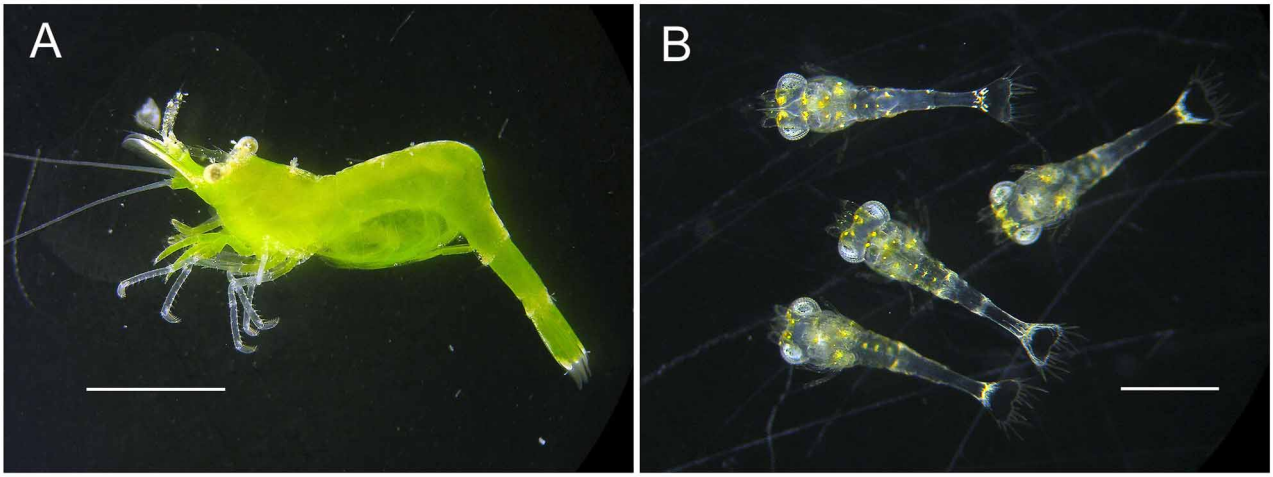

FIGURE 1. *Hippolyte leptocerus*. A, adult female, lateral view; B, zoea I, dorsal view. Scale bar of A = 3 mm; of B = 500 μm.

Maxilla (Fig. 2H). Coxa bilobed, with 7–8 + 3–4 setae. Basial endite bilobed with 5 + 4 setae. Endopod unsegmented and bilobed with 3 + 5 setae. Exopod (scaphognathite) with 5 marginal plumose setae.

First maxilliped (Fig, 3A). Coxa and basis with 5 and 12 setae on inner margin respectively. Endopod 4-segmented, with 3, 1, 2, 4(1 + 3) setae. Exopod incompletely 3-segmented with 4 long plumose natatory setae (one subterminal and 3 terminal).

Second maxilliped (Fig. 3B). Coxa and basis with one and 8 setae on inner margin respectively. Endopod 4-segmented, with 3, 2, 2, 5(1 + 4) setae. Exopod incompletely segmented with 5 long plumose natatory setae (2 subterminal and 3 terminal).

Third maxilliped (Fig. 3C). Protopod with 2 inner setae. Endopod 4-segmented, with 1, 0, 2, 4(1 + 3) seate. Exopod incompletely segmented with 5 long plumose natatory setae (2 subterminal and 3 terminal).

Pereiopods. Absent.

Pleon (Figs. 2A, B). Five somites plus telson, without pleopods and uropods. Pair of posterolateral spines on somite 5. Anal papilla present.

Telson (Figs. 2A,B, 3D). Triangular with a shallow median cleft posteriorly and 7 + 7 setae of different sizes, two outer pairs of setae feathered only on their inner side. Minute spinules present on posterior margin.

Zoea II. Size. TL= 1.55–1.57 mm; CL= 0.55–0.60 μm

Carapace (Figs. 4A, B). Eyes stalked.

Antennule (Fig. 4C). Peduncle incompletely segmented, with 2 distal setae on basal segment. Distal segment with 4 setae.

Antenna (Fig. 4D). Scaphocerite with tendency to lose segmentation. Otherwise unchanged.

Mandible. Unchanged

Maxillule (Fig. 4E). Unchanged.

Maxilla (Fig. 4F). Coxa bilobed, with 8 + 4 setae. Basial endite bilobed with 5 + 5 setae. Exopod (scaphognathite) with 7 plumose marginal setae.

First maxilliped (Fig. 5A). Basis with 14 setae on inner margin. Exopod with one subterminal and 4 terminal long plumose natatory setae.

Second maxilliped (Fig. 5B). Endopod 5-segmented, with 2, 2, 0, 2, 6(1 + 5) setae. Exopod with 2 subterminal and 4 terminal long plumose natatory setae.

Third maxilliped (Fig. 5C). Protopod (basis) with 3 inner setae. Endopod 5-segmented, with 1, 1, 0, 2, 5(1 + 4) seate. Exopod with 2 subterminal and 4 terminal long plumose natatory setae

Pereiopods. First pereiopod present as a bud.

Pleon (Figs. 4A, B). Unchanged.

Telson (Figs. 4A,B, 5D). Posterior margin with 8 + 8 setae of different sizes. Outer pair of setae feathered only on their inner side.

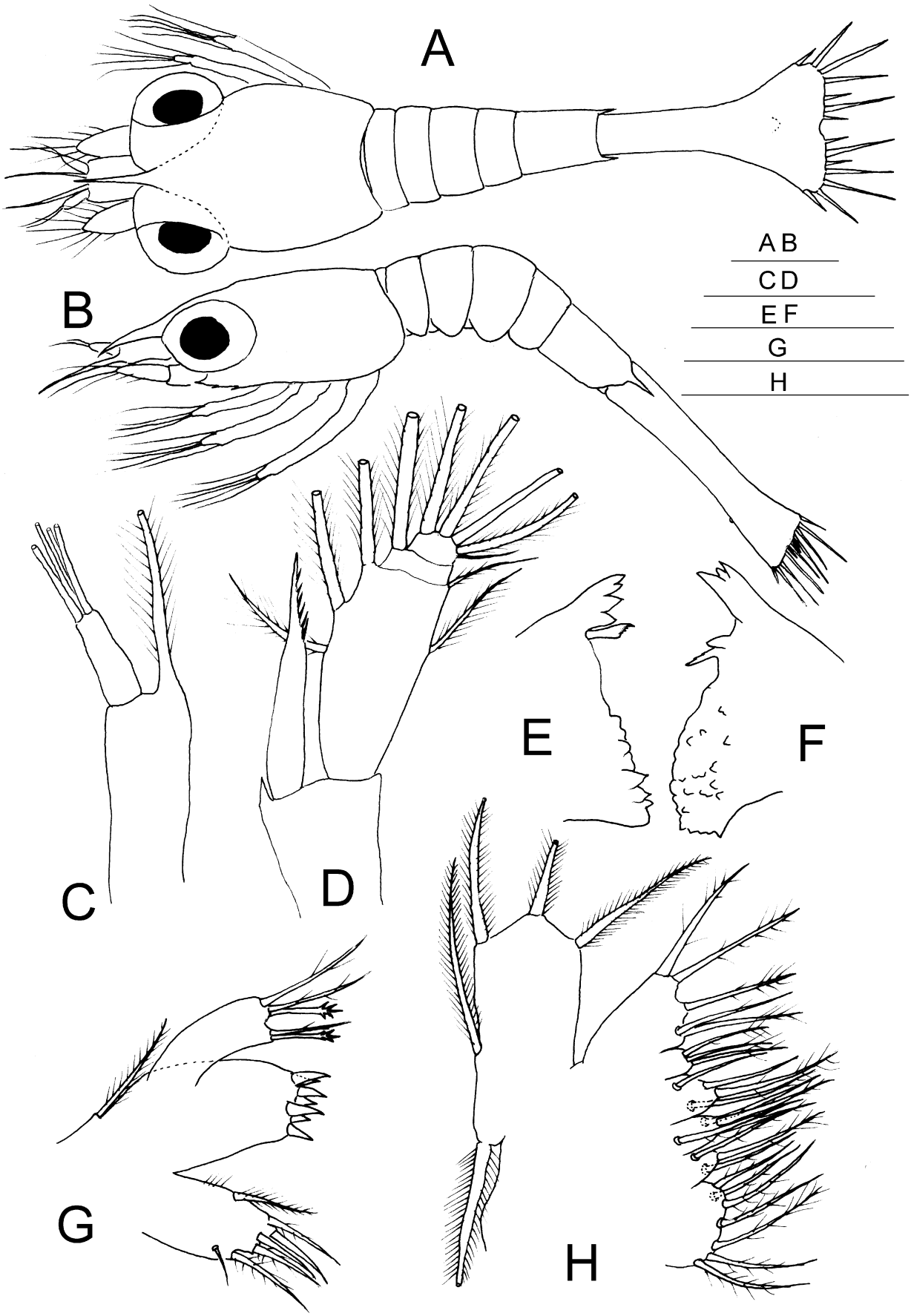

FIGURE 2. *Hippolyte leptocerus*. Zoea I. A, dorsal view; B, lateral view; C, antennule; D, antenna; E, left mandible; F, right mandible; G, maxillule; H, maxilla. Scale bars 200 μm.

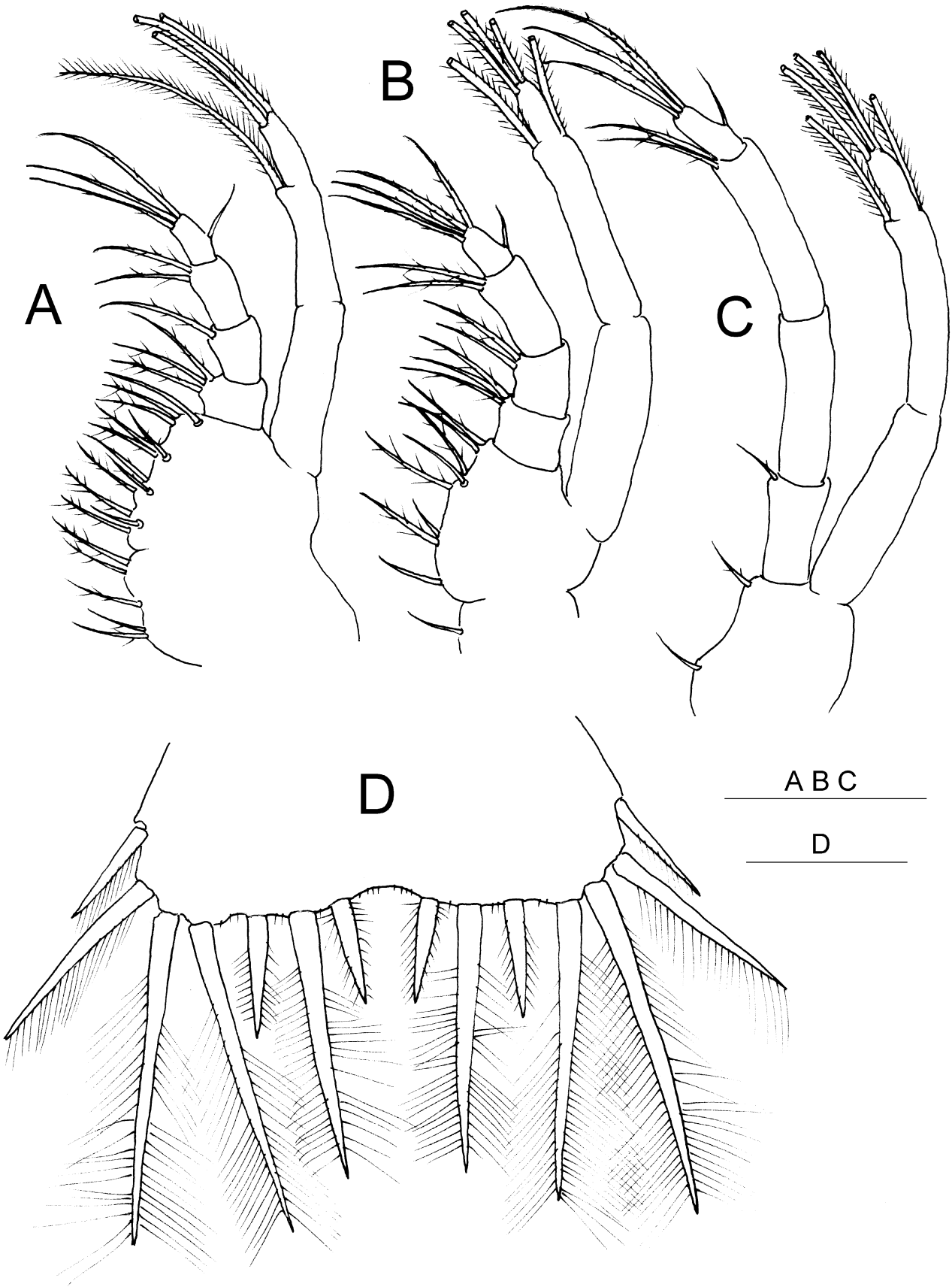

FIGURE 3. *Hippolyte leptocerus*. Zoea I. A, first maxilliped; B, second maxilliped; C, third maxilliped; D, telson. Scale bars 200 μm.

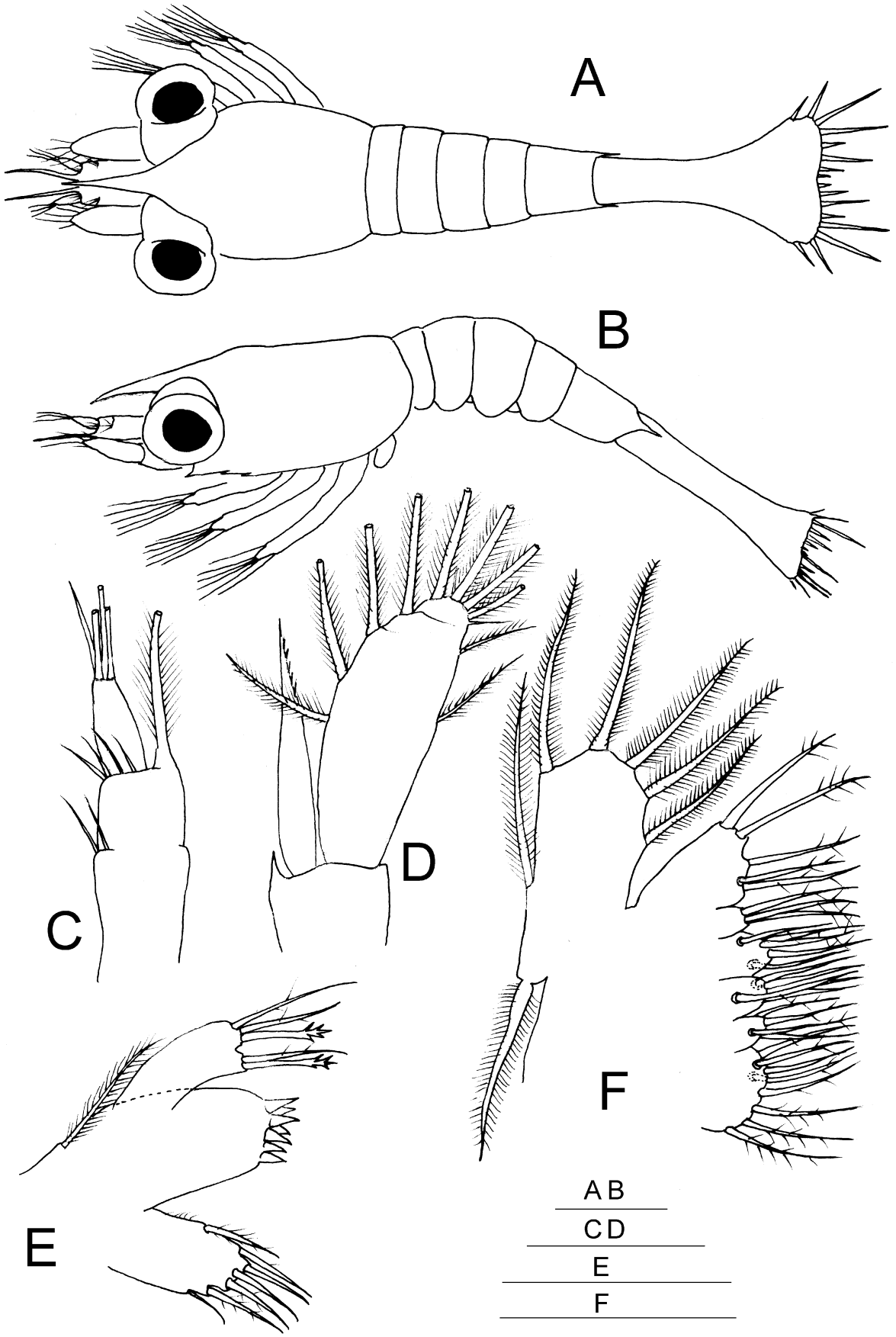

FIGURE 4. *Hippolyte leptocerus.* Zoea II. A, dorsal view; B, lateral view; C, antennule; D, antenna; E, maxillule; F, maxilla. Scale bars 200 µm.

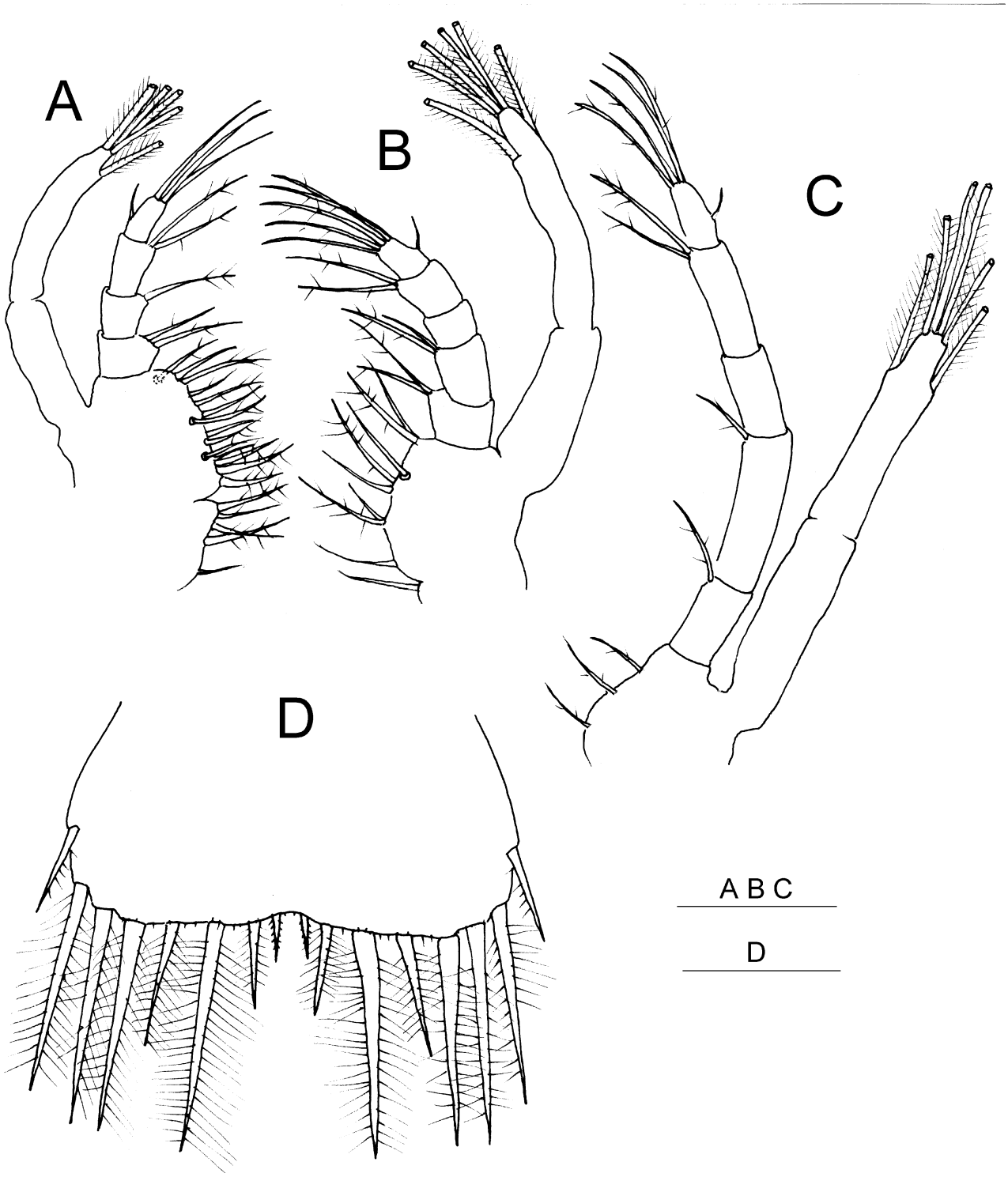

FIGURE 5. *Hippolyte leptocerus.* Zoea II. A, first maxilliped; B, second maxilliped; C, third maxilliped; D, telson. Scale bars 200 μm.

Zoea III. Size. TL= 1.70–2.00 mm; CL = 0.65–0.70 mm.

Carapace (Figs. 6A, B, C). Anteroventral margin with 3 small spines posteriorly to pterygostomial spine.

Antennule (Fig. 6D). Peduncle 3-segmented. Proximal segment bearing a small lateral spine and without setae; second segment with 3 + 2 setae; distal segment with 5 setae. Flagellum with 2 aesthetascs and one seta.

Antenna (Fig. 6E). Exopod (scaphocerite) unsegmented, with a distolateral spine.

Maxillule (Fig. 6F). Basial endites with 8 setae.

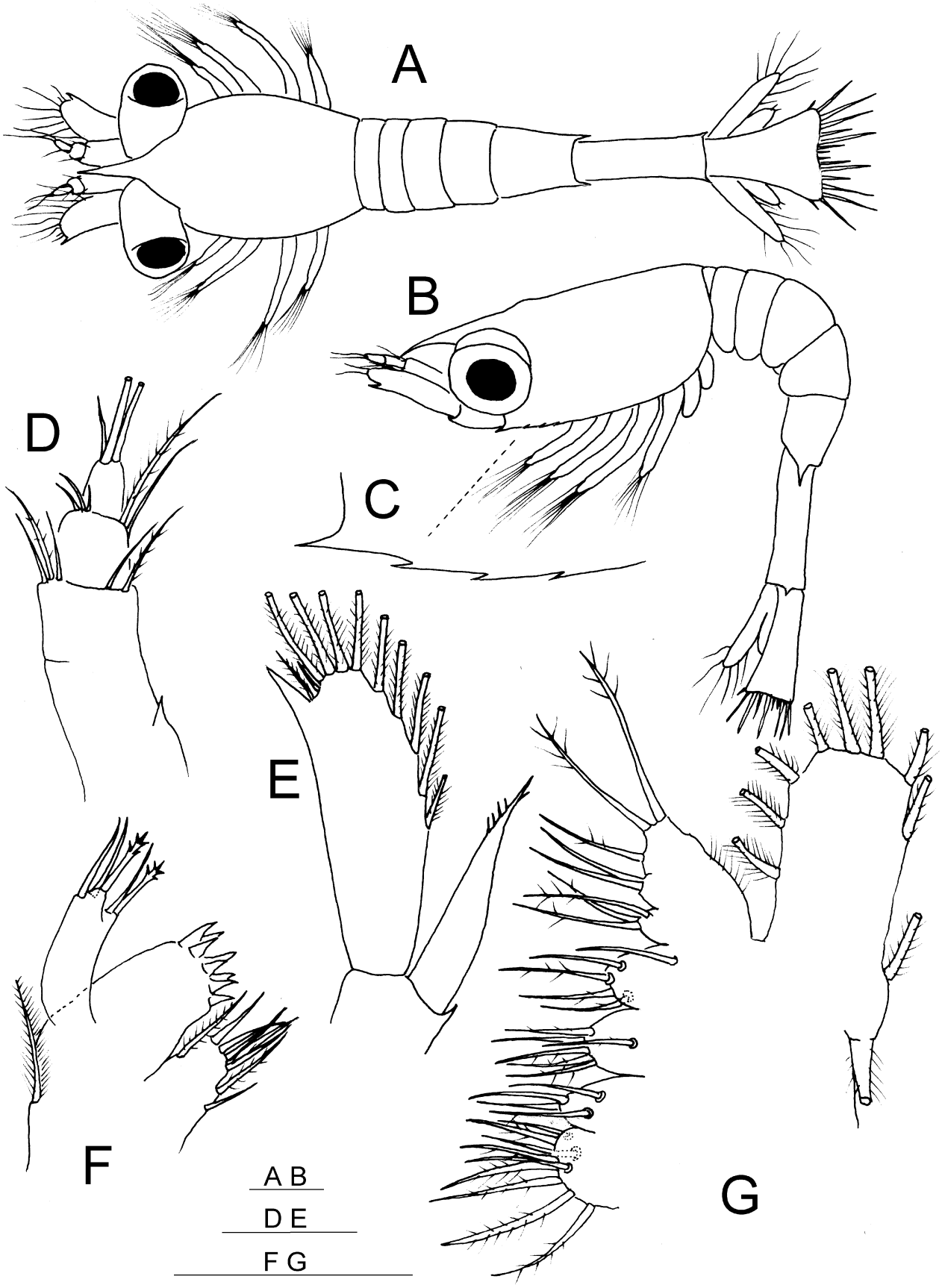

FIGURE 6. *Hippolyte leptocerus.* Zoea III. A, dorsal view; B, lateral view; C, carapace, anteroventral region; D, antennule; E, antenna; F, maxillule; G, maxilla. Scale bars of 200 µm.

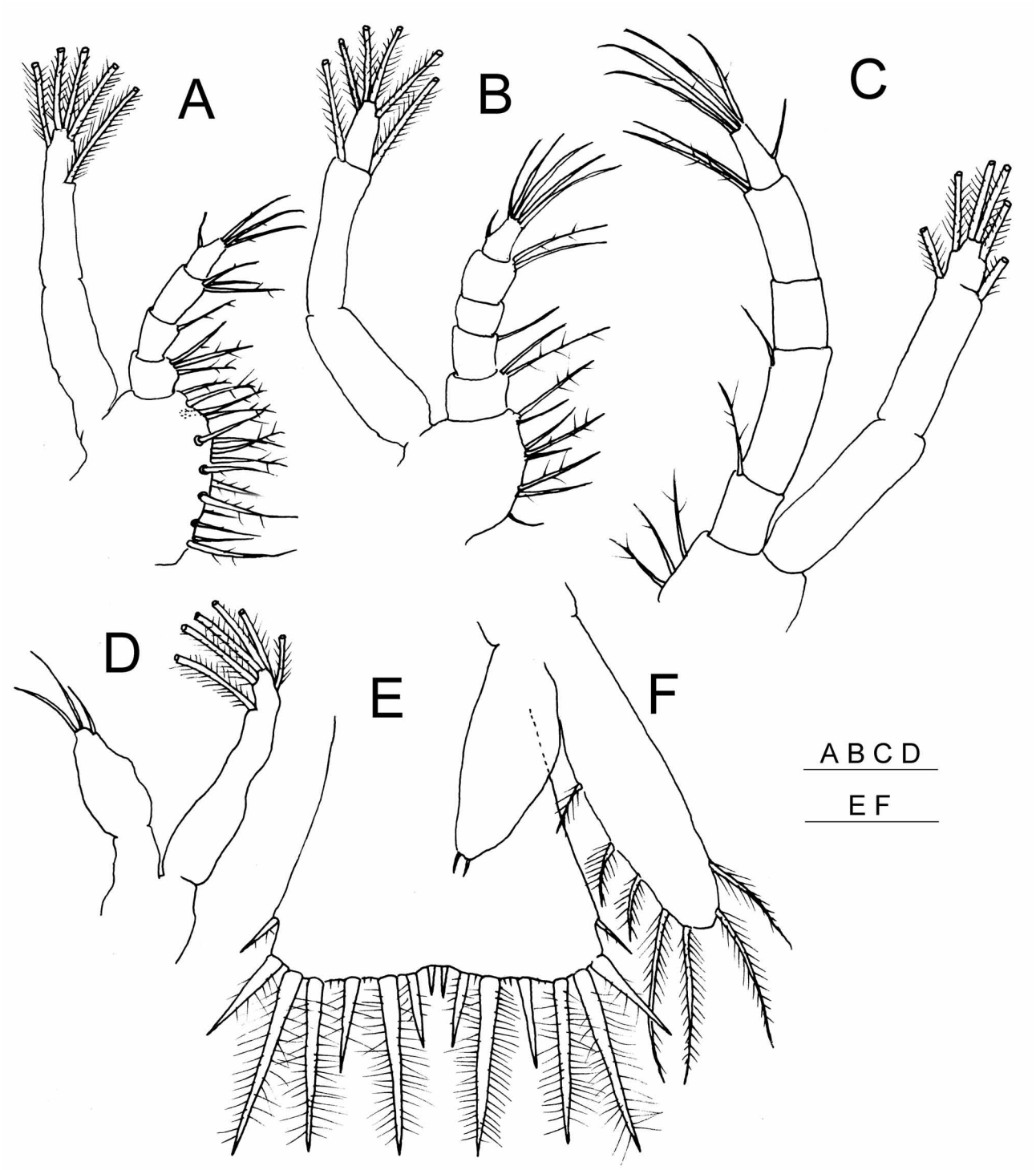

FIGURE 7. *Hippolyte leptocerus.* Zoea III. A, first maxilliped; B, second maxilliped; C, third maxilliped; D, first pereiopod; E, telson; F, uropod. Scale bars 200 μm.

Maxilla (Fig. 6G). Coxa bilobed, with 10 + 4 setae. Basial endite bilobed with 6 + 7 setae. Exopod (scaphognathite) with 10 plumose marginal setae.

First maxilliped (Fig. 7A). Basis with 15 setae on inner margin. Endopod 4-segmented, with 3, 1, 3, 4 setae.

Second maxilliped (Fig. 7B). Endopod 5-segmented, with 2, 1, 0, 2, 6(1 + 5) setae.

Third maxilliped (Fig. 7C). Unchanged.

First pereiopod (Fig. 7D). Birramous. Endopod unsegmented and non-chelated with 3 distal setae. Exopod with 2 subterminal and 4 terminal setae.

Second and third pereiopods. Present as a bud.

Pleon (Figs. 6A, B). 6-segmented. Sixth pleonite with a posterolateral spine.

Telson (Fig. 6A,B, 7E). Separate from pleonite 6.

Uropods (Fig. 7F). Birramous, exopod with 7 plumose setae. Endopod shorter than exopod with 2 distal simple setae.

Discussion

The morphology of three first zoeal stages of *Hippolyte leptocerus* correspond very closely with the characters listed by Lebour (1931) and Gurney (1942) for genus *Hippolyte*: 1) rostrum present; 2) carapace without supraorbital spines; 3) margin anterolateral of carapace with denticulations; 4) median tubercle behind rostrum; 5) without spines on first to fourth pleonite; 6) pleonite 5 with a pair of dorsolateral spines; 7) scaphocerite segmented at the tip in early stages, with tendency to lose segmentation; 8) antennal endopod spine-like; 9) maxillule with outer seta (exopodal seta) on basis and an unsegmented palp; 10) exopods of maxillipeds with 3 apical setae in first zoeal stage; 11) first pereiopods with swimming exopod in the third zoeal stage. In the first zoea of *H. leptocerus* bears an anal papilla, which has been described also for *H. pleuracanthus* (Shield, 1978) and *H. obliquimanus* (Terossi *et al.*, 2010). The absence of papilla was considered such a characteristic of first zoeal stage of the *Hippolyte* (Gurney, 1937; Haynes, 1985; Terossi *et al.*, 2010). The first zoeal stage of nine species of *Hippolyte*, including *H. leptocerus*, the outer setae of telson, in each side (right and left) showed setules only on their inner part. However, Negreiros-Fransozo *et al.* (1996), for ZI of *H. zostericola*, reported plumose setae on both parts (outer and inner), while that in *H. williamsi*, only one seta (outer), showed plumose setae on its inner part (Albornoz & Wehrtmann, 1997)

Table 1 compares the morphology of the first zoea of *H. leptocerus*, *H. obliquimanus*, *H. pleuracanthus* and *H. sapphica*. In general, descriptions of the first zoeal stage of the other *Hippolyte* species are incomplete or, in some cases, very inaccurate. In the present study, the morphological characters of the first zoeal stage of *H. leptocerus* follow the morphological pattern of *H. pleuracanthus* and *H. obliquimanus*, showing only little differences in the setation of the appendages. Notorious differences were observed in comparisons between the species mentioned above and *H. sapphica* (Ntakis *et al*., 2010). For example, the presence of exopodal seta on the maxillule is considered characteristic of the genus *Hippolyte* (Lebour, 1931; Gurney, 1937, 1942; Williamson, 1957). This morphological characteristic is present in *H. leptocerus* and *H. obliquimanus*, but in *H. sapphica* has not been described. This characteristic is not reported in other species of the genus as *H. williamsi* and *H. zostericola*. It is not excluded the possibility that their presence has been omitted because of difficulties in preparing maxillula for microscopical observation. Ntakis *et al.*, (2010) does not describe the anterolateral spines of carapace, nor the spines on protopod of the antennas, which are morphological traits present in the most larval descriptions of the genus *Hippolyte*.

The morphology of early zoeal stages of *H. leptocerus* is typical of species with an extended larval development: 1) antennular peduncle unsegmented, without setae; 2) antenal endopod unsegmented; 3) antenal exopod segmented; 4) absence of pereiopods; 5) absence of pleopods. Gurney (1942) described that the number of larval stages in the genus *Hippolyte* is from 4 to 9. The presence of pereiopods and pleopods in first zoea of *H. sapphica* is unusual in the genus *Hippolyte*. The acceleration in the appearance of characters during ontogeny (peramorphosis) is associated with abbreviated larval development (Clark, 2005; Lai & Shy, 2009). Possibly, the absence of segmentation in the scaphocerite of the first zoea is also a peramorphic character (Table 1). The acceleration development can also be observed in zoeal stages II and III: the presence of spine on the scaphocerite of the second zoeal stage, presence of chelated first pereiopods in second zoeal stage, endopod of the antenna segmented in third zoea, pleopods birramous and segmented from second zoeal stage (Ntakis *et al.*, 2010). However, first zoea of *H. sapphica* not shows other morphological characters present in hippolytid species with abbreviated development: less than 5 zoeal stages (6 zoeal stages in *H. sapphica*), more than 5 setae in the scaphognathite and more than 7 pairs of setae on telson (Haynes, 1985). In contrast, the types of early zoeal development of *H. leptocerus* are very similar to *H. pleuracanthus* (Table 2). Probably, the number of zoeal stages of *H. leptocerus* should be similar to *H. pleuracanthus* (8 zoeal stages). The strong differences in larval morphology between *H. sapphica* and the rest of known Hippolytidae larvae require new studies, including adult morphology and DNA analysis, in order to establish the systematic position of this species. Future research should further investigate in detail the larval morphol-

ogy of more species of genus *Hyppolyte*, including complete larval development in order to establish a consistent set of morphological characters, which are important to enable identification of the larval stages of the genus *Hyppolyte*.

TABLE 1. Comparison of the morphological characters of the first zoea of *Hippolyte leptocerus* (present study), *H. pleuracanthus* (Shield, 1978), *H. sapphica* (Ntakis *et al.* 2010) and *H. obliquimanus* (Terossi *et al.* 2010). Abbreviations: s, seta; sp, spine; ssp, supraorbital spine.

Feature	*H. leptocerus*	*H. pleuracanthus*	*H. sapphica*	*H. obliquimanus*
Zoea I				
Carapace (ssp)	absent	absent	present	absent
Antenna				
Protopod (sp)	present	present	absent	present
Scaphocerite	segmented	segmented	unsegmented	segmented
Scaphocerite (s)	11	9–10	10	11
Maxillule				
Outer seta	present	absent	absent	present
Coxal endite (s)	7	6	6	7
Basial endite (s)	7	5	4	5
Endopod (s)	5	5	10	5
Maxilla				
Coxal endite (s)	8+4	8+3	6+5	9+4
Basial endite (s)	5+4	7+4	4+5	4+4
Endopod (s)	8	8	5	9
Maxilliped 2				
Endopod (s)	3,2,2,5	3,2,2–3,5	2,2,3,4	3,1,2,5
Maxilliped 3				
Endopod (s)	1,0,2,4	1,0,2,4	0,0,2,3	0,0,2,3
Pereiopod 1	absent	absent	present	absent
Pleon				
Somite 5 (sp)	present	present	absent	present
Pleopods	absent	absent	present	absent

TABLE 2. Comparison of the morphological characters of zoeae II and III of *Hippolyte leptocerus* (present study), *H. pleuracanthus* (Shield, 1978) and *H. sapphica* (Ntakis *et al.* 2010). Abbreviations: nd, no data; s, seta; sp, spine.

Feature	*H. leptocerus*	*H. pleuracanthus*	*H. sapphica*
Zoea II			
Antenna			
Scaphocerite (s)	11	11	13
Scaphocerite (sp)	absent	absent	present
Maxillule			
Coxal endite (s)	7	5	6
Basial endite (s)	7	5	8

continued next page

TABLE 2. (continued)

Feature	*H. leptocerus*	*H. pleuracanthus*	*H. sapphica*
Maxilla			
Coxal endite (s)	8+4	9+4	10+4
Basial endite (s)	5+5	4+5	5+7
Endopod (s)	8	8	4
Maxilliped 2			
Endopod (s)	2,2,0,2,6	3,1,3–4,5	3,2,1,4,4
Maxilliped 3			
Endopod (s)	1,1,0,2,5	0,1,0,3,4	2,1,3,4
Pereiopod 1	non chelated	non chelated	chelated
Pleopods	absent	absent	present
Zoea III			
Antenna endopod	unsegmented	unsegmented	9-segmented
Scaphocerite	11	11	13
Maxilla			
Coxal endite (s)	10+4	10+2	6+2
Basial endite (s)	6+7	5+6	8+8
Maxilliped 2			
Endopod (s)	2,1,0,2,6	3,0,0,3,5	3,1,2,4
Maxilliped 3			
Endopod (s)	1,1,0,2,5	2,0,0,3,3	nd
Pleopods	absent	absent	present
Telson (s)	8+8	8+8	7+7

Acknowledgements

The authors thanks to Dr. Enrique García-Raso for the identification of the ovigerous females. This work was supported by the Ministry of Science and Research to GG (post-doctoral fellowship; INIA).

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CHAPTER VI

Nutritional vulnerability of early zoea larvae of the crab *Maja brachydactyla* (Brachyura, Majidae)

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Vol. 16: 253–264, 2012
doi: 10.3354/ab00457

AQUATIC BIOLOGY
Aquat Biol

Published September 5

Nutritional vulnerability of early zoea larvae of the crab *Maja brachydactyla* (Brachyura, Majidae)

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ABSTRACT: We investigated the nutritional vulnerability of Zoeae I of the spider crab *Maja brachydactyla* in experimental treatments with differential periods of starvation. As response variables, the moulting and survival rates, the point-of-reserve-saturation (PRS), the point-of-no-return (PNR), dry mass, elemental composition (carbon, hydrogen, nitrogen; CHN), hepatopancreas lipid vacuoles and activities of digestive enzymes were measured. Average median time spans when 50% of the larvae reached PNR or PRS (PNR_{50} and PRS_{50}) values were 2.8 and 1.9 d, respectively. In PNR treatments, complete mortality occurred only after extended initial starvation periods of ≥5 d, and in PRS treatments, Zoeae I were capable of completing the moulting cycle after a short initial feeding period (1 d). In continuously starved control larvae, the moulting cycle was arrested at the onset of apolysis. Concomitantly, considerable amounts of biomass were lost and the C:N ratio decreased, indicating lipid degradation during starvation. This effect was also microscopically visible as a decline of lipid vacuoles in the hepatopancreas. Feeding after previous starvation periods of <7 d resulted in a re-establishment of the lipid vacuoles, indicating successful capture and ingestion of food, but this did not necessarily allow for completing the moulting cycle. In fed Zoea I larvae, digestive enzyme activities increased during the moulting cycle, while a significant decrease of enzyme activities occurred under starvation conditions. In newly moulted Zoeae II, biomass values and enzyme activities were linearly correlated with the duration of the feeding periods in Zoeae I. This study shows that biomass, elemental composition, the occurrence of lipid vacuoles in the hepatopancreas and activities of digestive enzymes are suitable indicators of the nutritional condition of early zoeal stages of *M. brachydactyla*.

KEY WORDS: Decapoda · Majoidea · Starvation · Point-of-no-return · Point-of-reserve-saturation · CHN · Hepatopancreas · Condition indices

INTRODUCTION

One of the key factors affecting the survival of larvae in the plankton is the patchy and unpredictable nature of food abundance (Yin & Blaxter 1987, Morgan 1995, Pechenik et al. 1996). The capacity of the decapod malacostracan larval stages to tolerate temporary periods of starvation is therefore believed to be essential for their survival in the pelagic environment, particularly in nutritionally unstable habitats (Anger 2001). In newly hatched larvae, fitness is largely determined by initially available yolk resources, which may vary intra- and interspecifically, depending e.g. on seasonal, genetic or maternal factors, and the conditions prevailing during the preceding period of embryonic development (Anger 1995, Giménez et al. 2004, Paschke et al. 2004, Giménez & Anger 2005).

As the initially available yolk reserves may rapidly be catabolized in the absence of food, decapod larvae

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must start feeding soon after hatching to avoid deleterious effects caused by starvation. In previous studies, the effects of food limitation at a scale of 1 or a few days were mostly evaluated by means of 'point-of-no-return (PNR) and 'point-of-reserve-saturation (PRS) experiments, which yield indices of critical points that can be used to compare the degree of nutritional vulnerability in different species or developmental stages (Anger 2001, Gebauer et al. 2010). The PNR is defined as the threshold time at which initially starved larvae have lost the capability to recover from nutritional stress and to moult during subsequent feeding periods, whereas the PRS is the minimum time of initial feeding at which sufficient reserves are accumulated to subsequently complete a larval stage in principle independent of further food supply (Anger & Dawirs 1981).

Knowledge of such critical points in the early larval stages may provide valuable tools for the assessment of the physiological condition of larvae in the field, or for the establishment of successful rearing protocols for aquaculture of commercially valuable decapod crustacean species, helping to enhance larval survival to metamorphosis (Rotllant et al. 2010). Variations in the proportions of various chemical fractions of biomass, e.g. the contents of carbon, nitrogen and hydrogen (collectively referred to as CHN), have frequently been used as indicators of larval condition (e.g. Dawirs 1986, Thatje et al. 2004) or 'quality (Andrés et al. 2010a). Digestive enzyme activities have also recently been applied to evaluate the nutritional condition of larvae (Rotllant et al. 2008, 2010, Andrés et al. 2010b). Furthermore, changes in biomass during individual moult cycles may be used to quantitatively describe and compare the basic patterns of development and growth in decapod larvae (Anger 2001, Rotllant et al. 2012).

The spider crab *Maja brachydactyla* Balss, 1922 (Brachyura, Majoidea) is potentially interesting for aquaculture and has therefore been intensively studied in recent years (e.g. Andrés et al. 2007, Guerao et al. 2008). The larval development of this species comprises 2 zoeal stages and a megalopa, which can successfully be reared under intensive and semi-extensive cultivation conditions, allowing for satisfactory growth and survival (Andrés et al. 2007). The nutritional vulnerability of its larval stages may have practical applications for maximizing feeding efficiency and enhancing rates of growth and survival. Effects of food deprivation were previously studied in mass culture (Rotllant et al. 2010), showing that all 3 larval stages of this species are planktotrophic and that a decrease in food supply causes increased development duration and decreased growth and survival.

The aim of the present study was to quantify the nutritional vulnerability of first-stage zoeae of *Maja brachydactyla* in terms of survival, development time, PNR and PRS indices, changes in dry mass and elemental composition (CHN), effects on the duration of stages within the moulting cycle (Guerao et al. 2010), the appearance of microscopically visible lipid droplets in the digestive gland and activities of digestive enzymes (amylase and protease), testing these response variables as potential indicators of larval condition. Moreover, we considered the variability among individually tested larvae, enhancing the accuracy of all results compared to previous experiments using mass culture techniques.

MATERIALS AND METHODS

Maintenance of broodstock and collection of larvae

Adult *Maja brachydactyla* were captured in January 2010 off the coast of Galicia, Spain, northeastern Atlantic), and transported in cooling containers (ca. 8°C) to the Institut de Recerai Tecnologica Agroalementàries (IRTA; Sant Carles de la Ràpita). Ovigerous females were kept in 1500 l broodstock tanks connected to a recirculation unit maintaining a constant salinity of 36 and a temperature of 18 to 19°C. In September and October 2010, larvae were released from 2 different females. Immediately after hatching, actively swimming Zoea I larvae were collected from the broodstock tanks and transferred to individual experimental beakers with 50 ml seawater kept at a salinity of 36, constant temperature of 18°C and a 12:12 h light:dark photoperiod. A total of 960 Zoeae I were used to study survival and duration of the Zoea I moulting cycle, and 4000 larvae were used for biochemical and histological analyses.

PNR and PRS experiments

PNR experiments included a series of 4 treatments with differential periods of initial starvation followed by continuous feeding (Fig. 1A), as well as a continuously starved (SC) and a continuously fed control (FC) group (80 Zoeae I in each treatment and control). In PRS experiments, there were 4 treatments with differential periods of initial feeding followed by starvation (Fig. 1B). Where applicable, freshly hatched *Artemia franciscana* (Kellog 1906) nauplii

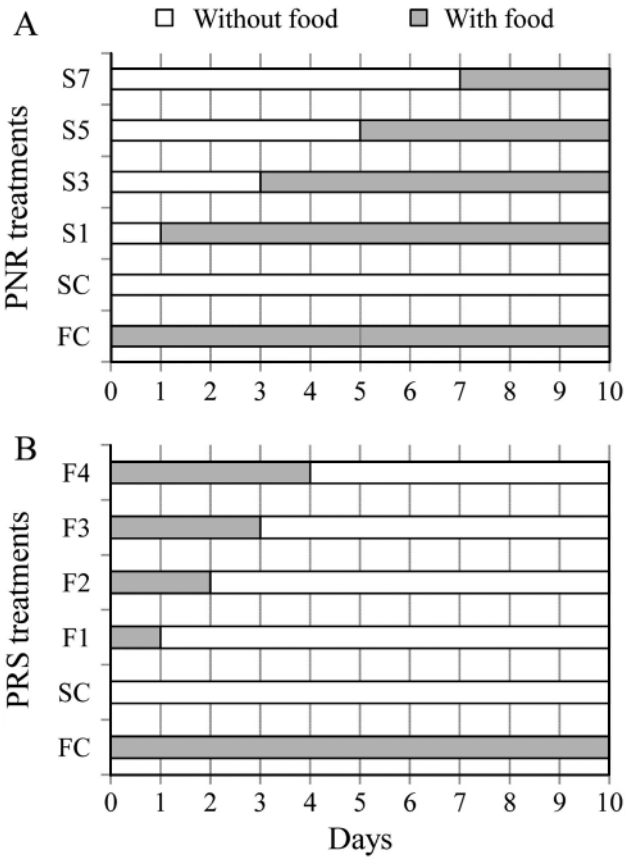

Fig. 1. *Maja brachydactyla*. Experimental design. (A) Point-of-no-return (PNR) experiments, with initial starvation periods followed by feeding (SC = continuously starved control; treatments: S1 = 1 d, S3 = 3 d of starvation, etc.); (B) point-of-reserve-saturation (PRS) treatments, with initial periods of feeding followed by starvation (FC = continuously fed control; treatments: F1 = 1 d of feeding, etc.); time of development (d) through the Zoea I moulting cycle

(Great Salt Lake strain, Utah) were used as food. Nutritional vulnerability was expressed as the median time span when 50% of the larvae reached the PNR or PRS, respectively (PNR_{50}, PRS_{50}), and as a PRS_{50}:PNR_{50} quotient (nutritional vulnerability index, NVI; Gebauer et al. 2010). PNR_{50} and PRS_{50} values were calculated adjusting the sigmoidal Boltzmann model with the least-squares method to the data (Gebauer et al. 2010).

Biochemical analyses

Analyses of dry mass (DM) and CHN were carried out at the Marine Biological Station Helgoland (Alfred Wegener Institute for Polar and Marine Research, BAH/AWI, Germany) following standard techniques (Anger & Harms 1990; for more and updated details, see Andrés et al. 2008). For each mean value, 5 replicate analyses with 3 (Zoea I) or 2 (Zoea II) larvae per replicate measurement were carried out. A fluorometric technique was chosen to measure digestive enzyme activities in individual larvae (total activity, IU ind.$^{-1}$). Individual variability was evaluated from replicates of 15 larvae (for details see Rotllant et al. 2008, 2010, 2012). In n = 5 replicate samples of larvae, larval moulting stages were determined using the same techniques and classification criteria as described by Guerao et al. (2010).

Histological observations

For histological inspection of the digestive gland, 5 larvae from each treatment were fixed in Davison s solution (glacial acetic acid, 100 ml; 95% ethyl alcohol, 300 ml; 10% neutral buffered formalin, 200 ml; distilled water, 300 ml) for 24 h and embedded in paraffin (Humason 1979). Sections of 3 µm were stained with haematoxylin-eosin solution. Measurements of lipid vacuoles in the digestive gland were made using a Leica DM LB microscope equipped with an Olympus DP 70 digital camera and an image analysing system (AnalySIS, SIS). The accumulation of lipids was estimated from histological sections (40×) as a percentage of the total area occupied by vacuoles (vacuole index, VI, in %).

Statistical analyses

Statistical analyses followed standard techniques as described by Sokal & Rohlf (1995). The response variables biomass (DM, CHN) and digestive enzyme activities were analysed using 1-way analysis of variance (ANOVA), considering the number of days of feeding or starvation (PNR, PRS) as experimental factors or explanatory variables, with separate analyses for each zoeal stage. Subsequent to ANOVA, *a posteriori* comparisons were made among treatments using the Student-Newman-Keuls (SNK) test for parametric data or the non-parametric Dunn s test for data sets of unequal size. Homogeneity of variance and normality were checked with Levene and Kolmogorov–Smirnov tests, respectively. When the data did not meet the assumptions, the non-parametric Kruskal–Wallis test was applied. Additionally, relationships between the C:N ratio and digestive enzymatic activities of Zoeae II, and feeding periods of Zoeae I were tested by linear regression analyses using the equation $y = ax + b$.

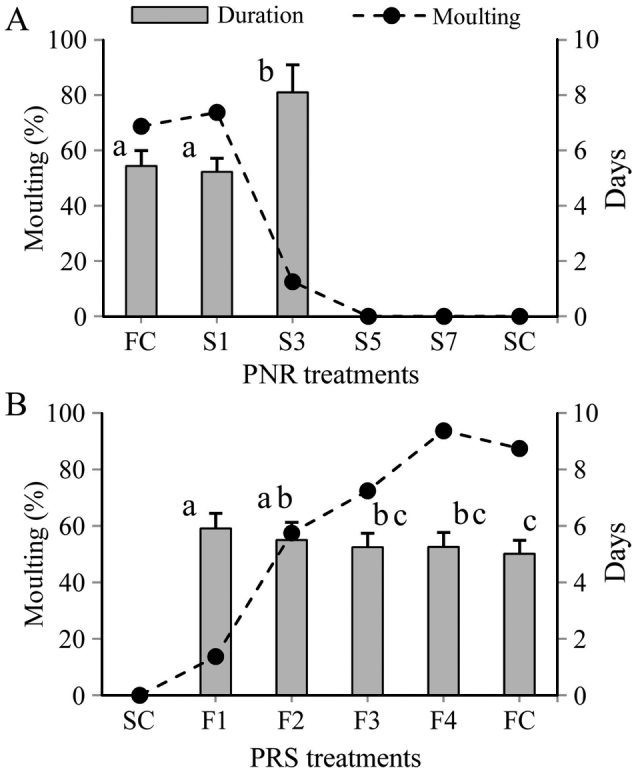

Fig. 2. *Maja brachydactyla*. Survival and developmental duration of the Zoea I stage. (A) point-of-no-return (PNR) treatments; (B) point-of-reserve-saturation (PRS) treatments; different lowercase letters indicate significant differences among days (SNK or Dunn s test)

RESULTS

PNR experiment

The number of days of initial lack of food supply significantly affected larval survival. In all treatments with starvation periods of ≥5 d followed by feeding, no larva was able to recover from initial starvation stress, and complete mortality occurred after a maximum of 10 d and without moulting to Zoeae II (Fig. 2A). PNR_{50} was 2.8 d. Starvation also affected development duration (Fig. 2A), which was significantly longer in Treatment S3 (see Fig. 1 for a complete description of the treatments) compared to S1 and to the FC group (Fig. 2A).

In the FC group, biomass (DM and CHN values per larva) and the C:N ratio increased during the time from hatching to moulting to the Zoea II stage (Fig. 3A, Table 1). In larvae kept under continuous starvation (SC group), by comparison, biomass decreased significantly (Table 1); the C:N ratio decreased initially, but after Day 4 it increased temporarily (Fig. 3A). In Treatments S1, S3 and S5, biomass and C:N values increased after the end of the starvation period, but reached similar values as in the FC group only in Treatment S1 (Table 1, Fig. 3B). A comparison of biomass in newly moulted Zoea II larvae from FC, S1 and S3 showed significant differences, with lower values in S3 than in the other treatments.

The occurrence of successive larval moulting stages is shown in Table 2. In SC larvae, the moulting cycle was consistently arrested at the onset of the apolysis process (beginning of Stage D_0). The delay in moulting described above for the S3 treatment is reflected here in the duration of the individual moulting stages, especially early premoult (D_0). In the S5 treatment, some individuals showed signs of an incipient morphogenesis (Stage D_1), but none of these reached ecdysis.

Food limitation also affected the lipid reserves stored in the digestive gland (Table 3). In the FC group, the lipid content remained high (VI ≥30%) throughout the experiment (Fig. 4B). In SC larvae, by contrast, the lipid content decreased from Day 2

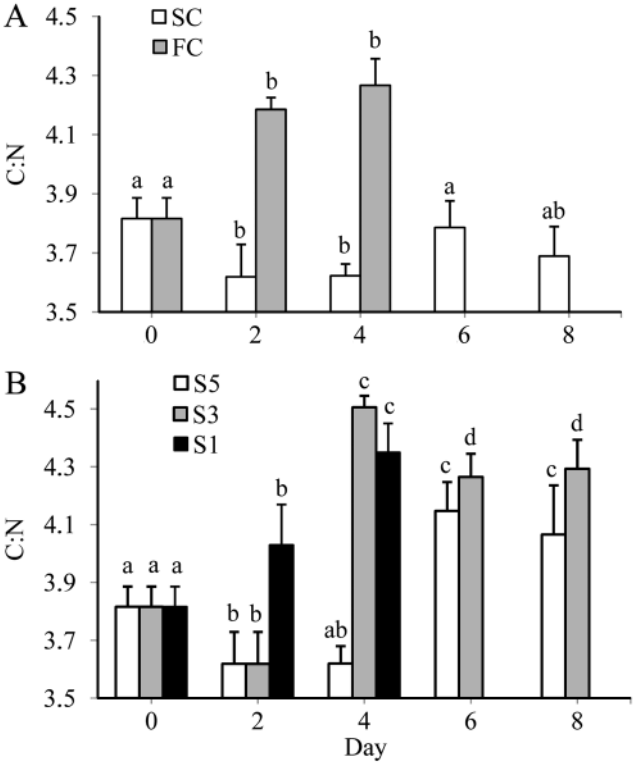

Fig. 3. *Maja brachydactyla*. Point-of-no-return experiment. C:N ratio in (A) continuously starved or continuously fed control groups (SC, FC); (B) Treatments S1, S3, S5 (see Fig. 1); different lowercase letters indicate significant differences among days (SNK or Dunn s test)

Table 1. *Maja brachydactyla*. Point-of-no-return experiment. Dry mass (DM), contents of carbon (C), nitrogen (N) and hydrogen (H) (all in µg ind.$^{-1}$) of Zoeae I and Zoeae II (the latter in **bold**). Treatments as in Fig. 1. Data are ± SE. Different lowercase letters indicate significant differences among days (SNK or Dunn s test). Res. var.: response variable; gaps indicate not applicable

Res. var.	Day	Treatment FC	S1	S3	S5	SC
DM	0	110 ± 15.5^{a}	110 ± 15.5^{a}	110 ± 15.5^{a}	110 ± 15.5^{a}	110 ± 15.5^{a}
	2	129 ± 4.90^{b}	123 ± 6.90^{ab}	99.3 ± 6.15^{a}	99.3 ± 6.15^{a}	99.3 ± 6.15^{a}
	4	142 ± 8.60^{b}	135 ± 8.70^{b}	121 ± 4.40^{b}	96.4 ± 3.43^{a}	96.4 ± 3.43^{a}
	6	**161 ± 7.80^{c}**	**170 ± 20.9^{c}**	115 ± 6.00^{ab}	103 ± 1.02^{a}	91.6 ± 2.11^{b}
	8			119 ± 5.80^{b}	103 ± 4.90^{a}	85.0 ± 2.42^{b}
				138 ± 13.1^{c}		
C	0	35.5 ± 0.85^{a}	35.5 ± 0.85^{a}	35.5 ± 0.85^{a}	35.5 ± 0.85^{a}	35.5 ± 0.85^{a}
	2	46.0 ± 1.50^{ab}	40.6 ± 3.12^{ab}	29.3 ± 1.63^{b}	29.3 ± 1.63^{b}	29.3 ± 1.63^{b}
	4	51.2 ± 7.05^{b}	50.7 ± 3.70^{b}	40.1 ± 1.68^{b}	26.9 ± 0.79^{c}	26.9 ± 0.79^{c}
	6	**58.6 ± 3.88^{b}**	**61.2 ± 9.80^{b}**	38.9 ± 3.04^{b}	29.8 ± 1.24^{b}	24.5 ± 0.47^{d}
	8			39.5 ± 2.88^{b}	30.7 ± 2.64^{b}	21.7 ± 0.39^{d}
				48.3 ± 4.74^{c}		
N	0	9.35 ± 0.22^{a}	9.35 ± 0.22^{a}	9.35 ± 0.22^{a}	9.35 ± 0.22^{a}	9.35 ± 0.22^{a}
	2	11.0 ± 0.30^{ab}	10.0 ± 0.50^{ab}	8.10 ± 0.40^{b}	8.10 ± 0.40^{b}	8.10 ± 0.40^{b}
	4	12.0 ± 1.40^{b}	11.6 ± 0.66^{b}	8.90 ± 0.28^{ab}	7.43 ± 0.18^{c}	7.43 ± 0.18^{c}
	6	**13.3 ± 0.58^{b}**	**13.4 ± 1.50^{b}**	9.12 ± 0.60^{a}	7.20 ± 0.16^{c}	6.47 ± 0.16^{d}
	8			9.20 ± 0.39^{a}	7.57 ± 0.55^{c}	5.88 ± 0.18^{d}
				11.0 ± 0.80^{c}		
H	0	6.33 ± 0.31^{a}	6.33 ± 0.31^{a}	6.33 ± 0.31^{a}	6.33 ± 0.31^{a}	6.33 ± 0.31^{a}
	2	8.0 ± 0.22^{b}	7.20 ± 0.46^{a}	5.65 ± 0.19^{ab}	5.65 ± 0.19^{ab}	5.65 ± 0.19^{ab}
	4	7.05 ± 8.70^{b}	8.90 ± 0.54^{b}	7.30 ± 0.21^{b}	5.38 ± 0.11^{ab}	5.38 ± 0.11^{ab}
	6	**9.96 ± 0.50^{c}**	**10.2 ± 1.35^{c}**	6.90 ± 0.37^{b}	5.78 ± 0.17^{a}	5.12 ± 0.10^{b}
	8			7.00 ± 0.40^{b}	6.38 ± 0.46^{b}	4.69 ± 0.09^{b}
				8.34 ± 0.62^{c}		

Table 2. *Maja brachydactyla*. Moulting stages in the point-of-no-return experiment. Treatments as in Fig. 1. Stage descriptions: A–B: spongy epidermis; C: condensation of the epidermal tissues; D_0: apolysis; D_1: setagenesis; D_2: appearance of new cuticle; E: ecdysis

Day	Treatment FC	S1	S3	S5	S7	SC
0	A–C	A–C	A–C	A–C	A–C	A–C
2	D_0	D_0	D_0	D_0	D_0	D_0
4	D_1–D_2	D_1–D_2	D_0	D_0	D_0	D_0
6	E	E	D_0–D_2	D_0	D_0	D_0
8			D_0–E	D_0	D_0	D_0
10			E	D_1	D_0	D_0

rapidly to VI values <10 %, and to 5 % after Day 4 (Fig. 4A,B). Feeding after periods of initial starvation of 1 to 5 d (S1 to S5) always resulted in a reestablishment of the lipid contents (Fig. 4C,D). In the S7 treatment, the lipid content increased only slightly, without reaching the level of the other treatments (Table 3).

Another effect of food limitation was observed in the activities of digestive enzymes (Table 4). In the FC group, protease and amylase activity increased significantly throughout the Zoea I moult cycle. In larvae kept under SC conditions, by comparison, the enzymatic activities decreased within the first 8 d. A comparison of enzyme activities among Zoeae II obtained from FC, S1 and S3 treatments indicated significant differences with a lower value for S3 compared to the other groups (ANOVA, $p < 0.05$).

PRS experiment

As in the PNR experiment, SC larvae did not moult to Zoeae II. In larvae fed only during the first day, mortality was 86 %, while it was <50 % in larvae that were fed for 2 or more days (Fig. 2B). PRS_{50} was 1.9 d. The number of days with initial food availability also had a significant effect on the duration of the Zoea I moulting cycle (ANOVA, $p < 0.05$; Fig. 2B).

In the FC group, Zoeae I biomass and the C:N ratio increased from hatching to moulting to Zoeae II, similar to the FC in the PNR experiment (Table 5; Fig. 5A). In SC larvae, by contrast, biomass decreased

Table 3. *Maja brachydactyla*. Point-of-no-return experiment. Vacuole index (VI, %) measured in the digestive glands of Zoeae I and Zoeae II (the latter in **bold**). Treatments as in Fig. 1. Data are ± SE. Different lowercase letters indicate significant differences among days (SNK or Dunn s test)

Day	Treatment SC	S7	S5	S3	S1	FC
0	32.90 ± 8.32^{a}	32.90 ± 8.32^{a}	32.90 ± 8.32^{a}	32.90 ± 8.32^{a}	32.90 ± 8.32^{ab}	32.90 ± 8.32^{a}
2	12.10 ± 4.60^{a}	12.10 ± 4.60^{ab}	12.20 ± 4.60^{ab}	9.4 ± 3.60^{b}	23.30 ± 5.61^{a}	33.30 ± 7.61^{a}
4	2.11 ± 2.20^{ab}	2.11 ± 2.20^{b}	2.11 ± 2.20^{b}	34.80 ± 7.25^{a}	45.60 ± 19.20^{ab}	37.30 ± 7.32^{a}
6	2.90 ± 2.30^{ab}	2.90 ± 2.30^{b}	24.50 ± 2.43^{a}	31.10 ± 4.83^{a}	**53.38 ± 5.55^{b}**	**39.80 ± 9.30^{a}**
8	1.20 ± 1.32^{b}	13.20 ± 9.60^{b}	23.40 ± 13.1^{ab}	**39.50 ± 7.51^{a}**		

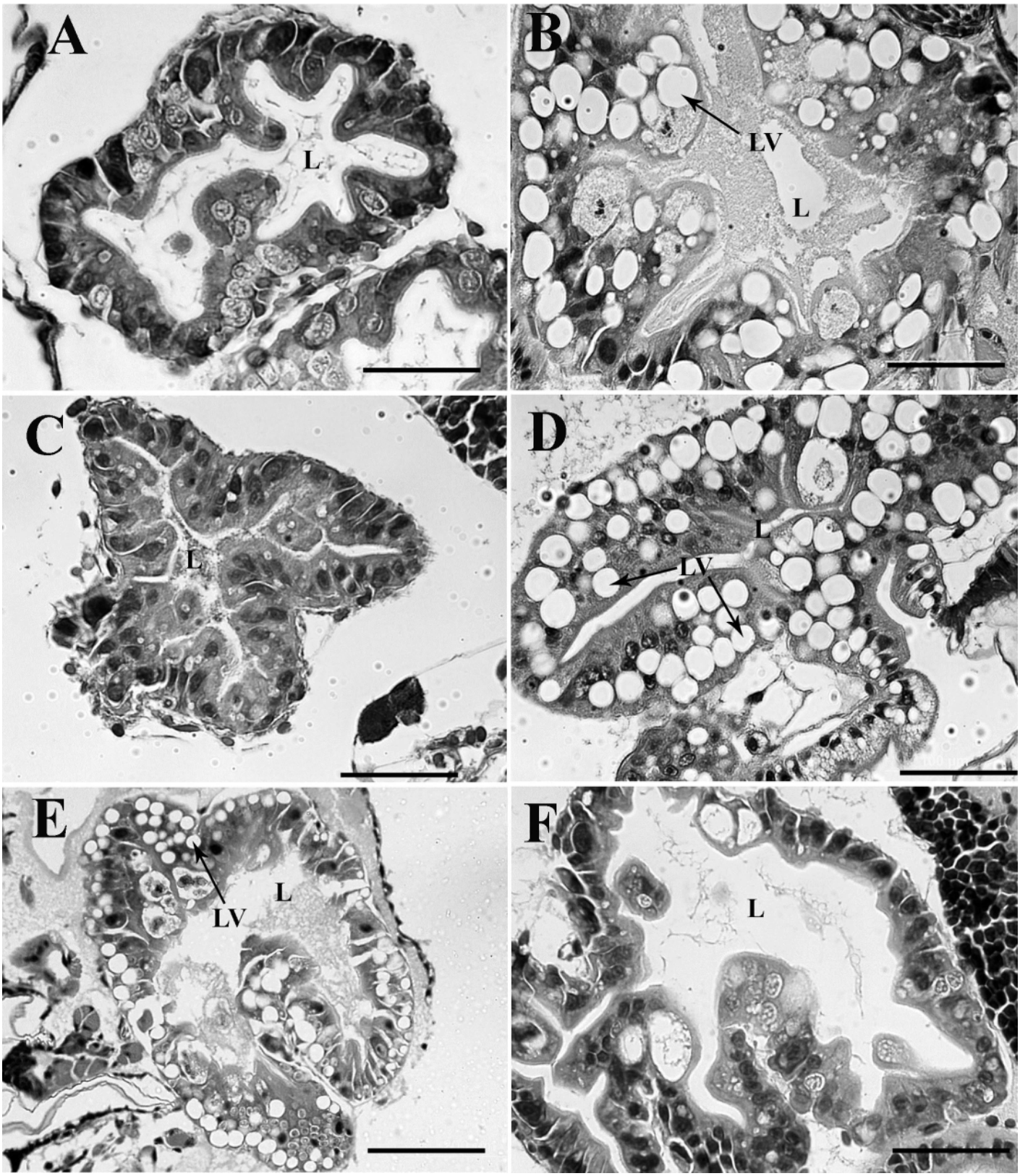

Fig. 4. *Maja brachydactyla*. Histological sections of the digestive gland. (A) Point-of-no-return (PNR) experiment, continuously starved control group (SC), Day 4; (B) PNR experiment; continuously fed control group (FC), Day 4; (C) PNR experiment: Treatment S5 (5 d of starvation), Day 4; (D) PNR experiment: Treatment S5, Day 8; (E) point-of-reserve-saturation (PRS) experiment: Treatment F2 (2 d of feeding), Day 2; (F) PRS experiment: Treatment F2, Day 3. L: lumen; LV: lipid vacuole. Scale bars = 50 µm

Table 4. *Maja brachydactyla*. Total amylase and protease activities (IU ind.$^{-1}$) in different treatments (point-of-no-return experiment) in Zoeae I and Zoeae II (the latter in **bold**). Treatments as in Fig. 1. Data are ± SE. Different lowercase letters indicate significant differences among days (SNK or Dunn s test)

	Day	Treatment				
		SC	S5	S3	S1	FC
Amylase	0	3611 ± 1263^{a}	3611 ± 1263^{a}	3611 ± 1263^{a}	3611 ± 1263^{a}	3611 ± 1263^{a}
	2	2664 ± 1040^{ab}	2664 ± 1040^{ab}	2664 ± 1040^{a}	7548 ± 2113^{b}	12324 ± 9038^{b}
	4	4016 ± 4136^{a}	4016 ± 4136^{a}	5214 ± 1936^{b}	8343 ± 3900^{bc}	6459 ± 4509^{ab}
	6	1628 ± 1548^{b}	1224 ± 986^{bc}	4195 ± 2241^{ab}	**11443 ± 7156^{c}**	**11467 ± 3990^{b}**
	8	446 ± 257^{c}	920 ± 723^{c}	1812 ± 1132^{a}		
				2617 ± 1751^{a}		
Proteases	0	3008 ± 1208^{a}	3008 ± 1208^{a}	3008 ± 1208^{a}	3008 ± 1208^{a}	3008 ± 1208^{a}
	2	2760 ± 983^{ab}	2760 ± 983^{ab}	2760 ± 983^{ab}	2796 ± 712^{a}	5136 ± 832^{b}
	4	1614 ± 372^{b}	1614 ± 372^{b}	1689 ± 560^{b}	4127 ± 1365^{b}	3896 ± 1774^{ab}
	6	516 ± 354^{b}	314 ± 282^{c}	11509 ± 770^{b}	**3630 ± 1993^{ab}**	**4927 ± 1570^{b}**
	8	80.2 ± 20.5^{c}	156 ± 53.9^{c}	1243 ± 845^{b}		
				1659 ± 1867		

Table 5. *Maja brachydactyla*. Point-of-reserve-saturation experiment. Dry mass (DM), contents of carbon (C), nitrogen (N) and hydrogen (H) (all in µg ind.$^{-1}$) of Zoeae I and Zoeae II (the latter in **bold**). Treatments as in Fig. 1. Data are ± SE. Different lowercase letters indicate significant differences among days (after SNK or Dunn s test); (–): missing data; gaps: not applicable

	Day	Treatment				
		FC	F3	F2	F1	SC
DM	0	108 ± 2.50^{ab}	108 ± 2.50^{ab}	108 ± 2.50^{ab}	108 ± 2.50^{ab}	108 ± 2.50^{ab}
	1	129 ± 8.20^{b}	129 ± 8.20^{b}	129 ± 8.20^{b}	129 ± 8.20^{b}	115±3.50^{b}
	2	141 ± 6.90^{abc}	141 ± 6.90^{abc}	141 ± 6.90^{abc}	111 ± 2.7^{c}	109 ± 5.60^{ab}
	3	149 ± 5.30^{bc}	149 ± 5.30^{bc}	122 ± 8.02^{ab}	114 ± 4.30^{c}	106 ± 2.00^{a}
	4	151 ± 7.4^{bc}	137 ± 4.13^{abc}	113 ± 7.20^{ab}	111 ± 1.90^{c}	101 ± 1.50^{a}
	5	**192 ± 9.70^{c}**	**136 ± 8.64^{abc}**	107 ± 2.90^{b}	102 ± 0.62^{c}	93.6 ± 3.90^{c}
				108 ± 5.80^{b}		
	6				92.4 ± 3.70^{d}	92.9 ± 0.98^{c}
					86.3 ± 5.46^{d}	
C	0	37.2 ± 0.41^{a}	37.2 ± 0.41^{a}	37.2 ± 0.41^{a}	37.2 ± 0.41^{a}	37.2 ± 0.41^{a}
	1	44.9 ± 2.80^{b}	44.9 ± 2.80^{b}	44.9 ± 2.80^{b}	44.9 ± 2.80^{b}	35.7 ± 0.54^{b}
	2	50.7 ± 3.50^{c}	50.7 ± 3.50^{c}	50.7 ± 3.50^{c}	35.8 ± 1.40^{a}	33.4 ± 1.15^{c}
	3	54.1 ± 1.35^{c}	54.1 ± 1.35^{c}	38.6 ± 3.80^{a}	36.1 ± 1.60^{a}	31.1 ± 0.59^{d}
	4	–	–	–	–	–
	5	**71.5 ± 1.13^{d}**	–	–	–	27.4 ± 0.90^{e}
	6				28.3 ± 1.13^{c}	26.6 ± 0.35^{e}
					28.6 ± 0.56^{c}	
N	0	9.5 ± 0.10^{a}	9.5 ± 0.10^{a}	9.5 ± 0.10^{a}	9.5 ± 0.10^{a}	9.5 ± 0.10^{a}
	1	11.0 ± 0.50^{b}	11.0 ± 0.50^{b}	11.0 ± 0.50^{b}	11.0 ± 0.50^{b}	9.26 ± 0.14^{a}
	2	12.1 ± 0.54^{c}	12.1 ± 0.54^{c}	12.1 ± 0.54^{c}	9.30 ± 0.34^{a}	8.68 ± 0.25^{b}
	3	12.9 ± 0.20^{c}	12.9 ± 0.20^{c}	12.9 ± 0.20	9.37 ± 0.35^{a}	8.00 ± 0.16^{c}
	4	–	–	–	–	–
	5	**15.3 ± 0.06^{d}**	–	–	–	6.97 ± 0.22^{d}
	6				6.79 ± 0.19^{c}	5.17 ± 0.08^{b}
					7.70 ± 0.18^{c}	
H	0	6.10 ± 0.08^{a}	6.10 ± 0.08^{a}	6.10 ± 0.08^{a}	6.10 ± 0.08^{a}	6.10 ± 0.08^{a}
	1	7.13 ± 0.40^{b}	7.13 ± 0.40^{b}	7.13 ± 0.40^{b}	7.13 ± 0.40^{b}	5.89 ± 0.15^{a}
	2	7.91 ± 0.52^{c}	7.91 ± 0.52^{c}	7.91 ± 0.52^{c}	5.7 0± 0.19^{a}	5.47 ± 0.27^{a}
	3	8.34 ± 0.22^{c}	8.34 ± 0.22^{c}	6.16 ± 0.53^{a}	5.75 ± 0.19^{a}	5.1 ± 0.13^{b}
	4	–	–	–	–	–
	5	**12.7 ± 0.48^{d}**	–	–	–	5.50 ± 0.24^{b}
	6				**5.38 ± 0.19^{a}**	5.17 ± 0.08^{b}

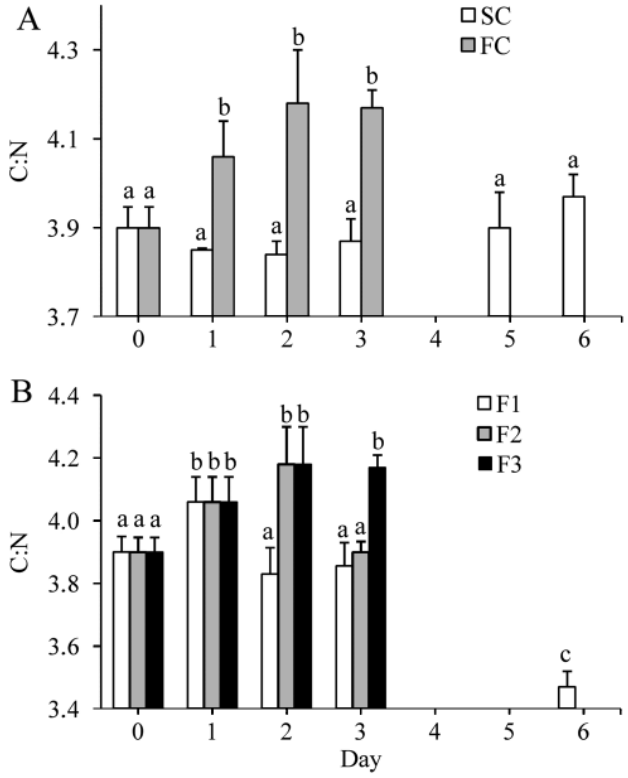

Fig. 5. *Maja brachydactyla*. Point-of-reserve-saturation experiment. C:N ratio in (A) continuously starved or continuously fed control groups (SC, FC); (B) Treatments F1 to F3 (see Fig. 1); different lowercase letters indicate significant differences among days (SNK or Dunn s test)

continuously. The C:N mass ratio varied only slightly, following a similar pattern to that described for the PNR experiment (Fig. 5A). In the F1 and F2 treatments, biomass and the C:N ratio decreased during the final period of starvation (Table 5, Fig. 5B). A comparison of biomass and C:N in Zoeae II obtained from different treatments indicated significant differences, with lower values in F1 and F2 compared to FC treatments. The DM of newly moulted Zoeae II was linearly correlated with the duration of the feeding periods in the preceding Zoea I moulting cycle (Fig. 6A), indicating that differential growth in Zoeae I translated to different biomass in the subsequent larval stage.

The occurrence of the successive moulting stages is shown in Table 6. Under SC conditions, the moulting cycle was again arrested at the beginning of apolysis (stage D_0), while the larvae in all other treatments were able to reach Stage E (ecdysis to Zoea II). In Treatments F1 and F2, the duration of the moulting stages increased, especially in the premoult stages, D (Table 6). The patterns in the FC and SC control groups were similar to those obtained in the PNR experiment.

Under FC conditions, the lipid content in the hepatopancreas remained high (VI ≥30%), whereas it declined rapidly in starved larvae (Table 7, Fig. 4E,F). The PRS experiment also confirms the effects of food limitation on digestive enzyme activities as shown above (Tables 4 & 8), with enzymatic activities decreasing during starvation periods. Like the DM values per larva, amylase and protease activities measured in newly moulted Zoeae II were also linearly correlated with the duration of the feeding periods in Zoeae I (Table 8, Fig. 6B,C), again showing carry-over effects of larval condition in the previous stage.

DISCUSSION

Using an experimental approach with gradational periods of addition or withdrawal of food (PNR and PRS experiments; Anger & Dawirs 1981) in combination with high-resolution measurements of chemical

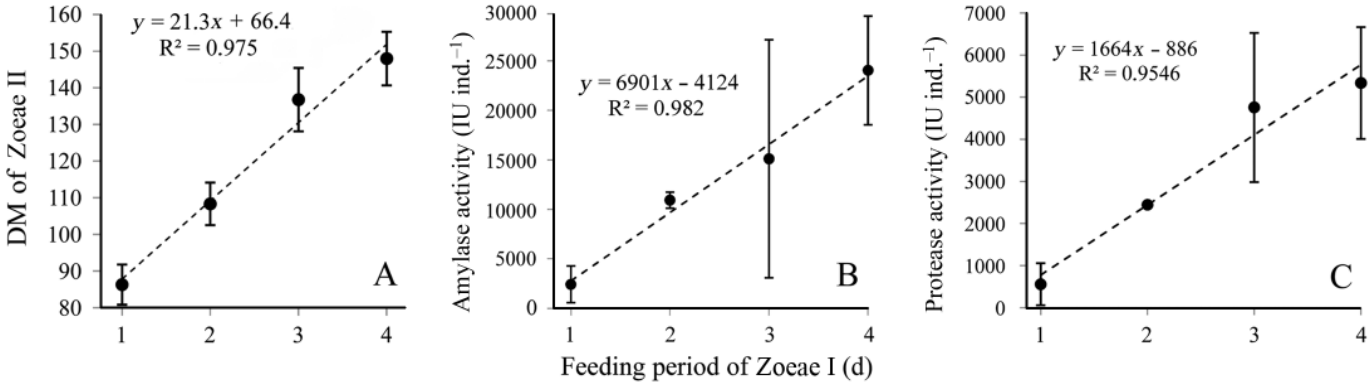

Fig. 6. *Maja brachydactyla*. Point-of-reserve-saturation experiment. Relationships between the number of days of initial feeding in the Zoea I stage and (A) dry mass (DM) of newly moulted Zoea II larvae, (B) amylase activity in Zoeae II, (C) protease activity in Zoeae II

Table 6. *Maja brachydactyla.* Moulting stages in the point-of-reserve-saturation experiment. Treatments as in Fig. 1. Stage descriptions as in Table 2

Day	Treatment					
	FC	F4	F3	F2	F1	SC
0	A–C	A–C	A–C	A–C	A–C	A–C
2	D_0	D_0	D_0	D_0	D_0	D_0
4	D_1–D_2	D_1–D_2	D_1–D_2	D_1–D_2	D_1–D_2	D_0
6	E	E	D_2–E	D_2	D_0 –D_2	D_0
8			E	D_2–E	D_2–E	D_0
10					E	D_0

and physiological parameters, our study shows conspicuous larval response patterns within the moulting cycle of the first zoeal stage of the commercially exploited spider crab *Maja brachydactyla*. Similar to closely related species such as the majid *Hyas araneus* (Anger & Dawirs 1981, 1982, Anger et al. 1989), and consistent with recent observations in *M. brachydactyla* (Andrés et al. 2008, Rotllant et al. 2010), the first-stage larvae are obligatorily planktotrophic, requiring food in order to successfully reach the second zoeal stage. Continuously fed zoeae showed significant growth in total DM and organically bound elements (CHN) per larva, an increase of the C:N ratio (indicating lipid accumulation), as well as an accumulation of fat vacuoles in the hepatopancreas, and an increase in the activities of major digestive enzymes (total protease and amylase). Continuously starved larvae, by contrast, showed opposite trends in all of these parameters, and they eventually died (after a maximum of 11 d), without moulting to

Table 7. *Maja brachydactyla*. Point-of-reserve-saturation experiment. Vacuole index (VI, %) measured in the digestive glands of Zoeae I and Zoeae II (the latter in **bold**). Treatments as in Fig. 1. Data are ± SE. Different lowercase letters indicate significant differences among days (SNK or Dunn s test)

Day	Treatment				
	FC	F3	F2	F1	SC
0	12.2 ± 5.80^{a}	12.2 ± 5.80^{a}	12.2 ± 5.80^{a}	12.2 ± 5.80^{ab}	12.2 ± 5.80^{a}
1	28.1 ± 8.31^{b}	28.1 ± 8.31^{b}	28.1 ± 8.31^{b}	28.1 ± 8.31^{a}	8.41 ± 10.2^{a}
2	24.4 ± 5.11^{ab}	24.4 ± 5.11^{ab}	24.4 ± 5.11^{b}	12.2 ± 2.12^{ab}	4.72 ± 2.70^{a}
3	28.5 ± 6.01^{b}	24.6 ± 2.80^{b}	6.30 ± 1.30^{a}	8.02 ± 3.80^{ab}	2.04 ± 0.10^{a}
4	31.5 ± 7.43^{b}	19.1 ± 7.30^{ab}	4.50 ± 2.50^{a}	6.01 ± 2.55^{ab}	3.70 ± 3.21^{a}
5	**27.0 ± 7.80^{ab}**	**25.3 ± 6.35^{ab}**	3.30 ± 3.10^{a}	1.10 ± 1.23^{b}	1.83 ± 1.50^{a}
6			**7.2 ± 2.82^{a}**	3.01 ± 1.40^{b}	1.25 ± 1.10^{a}
				1.40 ± 1.71^{b}	
7				1.60 ± 1.52^{b}	1.37 ± 1.10^{a}
8					0.50 ± 2.20^{a}

Table 8. *Maja brachydactyla*. Total amylase and proteases activities (IU ind.$^{-1}$) in different treatments (point-of-reserve-saturation experiment) in Zoeae I and Zoeae II (the latter in **bold**). Treatments as in Fig. 1. Data are ± SE. Different lowercase letters indicate significant differences among days (after SNK test or Dunn s test)

	Day	Treatment				
		FC	F3	F2	F1	SC
Amylase	0	5627 ± 3378^{a}	5627 ± 3378^{a}	5627 ± 3378^{a}	5627 ± 3378^{a}	5627 ± 3378^{a}
	1	16917 ± 9097^{b}	16917 ± 9097^{b}	16917 ± 9097^{b}	12737 ± 6236^{b}	4719 ± 2740^{a}
	2	29297 ± 14385^{c}	29297 ± 14385^{c}	29297 ± 14385^{c}	10237 ± 6618^{bc}	6564 ± 5486^{a}
	3	26396 ± 13976^{bc}	26396 ± 13976^{bc}	12922 ± 8354^{bd}	11494 ± 9209^{ac}	3635 ± 2481^{ab}
	4	29031 ± 15178^{c}	13469 ± 4526^{b}	15262 ± 10395^{bd}	5677 ± 4714^{a}	3922 ± 3833^{ab}
	5	**24044 ± 15486^{bc}**	**15114 ± 12023^{b}**	8114 ± 3038^{ad}	2293 ± 1789^{a}	3378 ± 3216^{b}
				10406 ± 8807^{ad}		
	6				**2433 ± 1857^{a}**	1828 ± 1083^{b}
Proteases	0	3551 ± 1147^{a}	3551 ± 1147^{a}	3551 ± 1147^{a}	3551 ± 1147^{a}	3551 ± 1147^{a}
	1	3386 ± 1412^{a}	3386 ± 1412^{a}	3386 ± 1412^{a}	3386 ± 1412^{a}	3949 ± 1651^{a}
	2	5123 ± 1182^{bc}	5123 ± 1182^{b}	5123 ± 1182^{b}	3167 ± 1598^{a}	2741 ± 1208^{a}
	3	5175 ± 1483^{bc}	5175 ± 1483^{b}	3020 ± 1662^{ac}	3239 ± 1377^{a}	1389 ± 819^{b}
	4	4224 ± 1993^{ab}	4909 ± 1736^{b}	3473 ± 1406^{ac}	3083 ± 1253^{a}	1308 ± 636^{b}
	5	**5338 ± 1328^{c}**	**4757 ± 1773^{b}**	2446 ± 1134^{c}	1177 ± 1171^{b}	1239 ± 778^{b}
				3519 ± 1700^{a}		
	6				**559 ± 601^{b}**	826 ± 571^{b}

the Zoea II stage (cf. Rotllant et al. 2010). These general patterns of response to feeding and starvation, respectively, provide various quantitative and qualitative criteria for an evaluation of the physiological condition of larvae developing under intermediate or unknown nutritional situations.

Temporary or local occurrence of poor nutritional conditions is considered one of the key factors determining larval survival in the plankton (Morgan 1995, Giménez & Anger 2005). While experimental conditions of both ad libitum feeding or complete absence of food represent extreme scenarios that may have little if any ecological relevance in natural habitats, they define the end points (maximum and minimum, respectively) of a relative scale of larval survival, development, growth and physiological responses to food limitation. In this context, PNR and PRS experiments are useful tools for the study of the nutritional needs or vulnerability of decapod crustacean larvae.

Congruent with the results of numerous previous studies with larvae of various other decapod groups (e.g. Dawirs 1984, Staton & Sulkin 1991, Paschke et al. 2004, Gebauer et al. 2010), our experiments demonstrate that initial periods of food limitation beginning soon after hatching are much more critical than a later occurrence of famine. Besides the timing, the duration of such periods is also very important. While a single day of initial starvation and subsequent feeding did not cause irreversible damage affecting larval survival or development in comparison to a continuously fed control group (cf. S1 versus FC; Fig. 2A), an initial lack of food for 3 d (corresponding to >50% of the moult-cycle duration of fed larvae) caused high mortality, similar to that in a continuously starved control group. This PNR effect reflects irreversible structural or functional damage, most probably in the R-cells of the hepatopancreas (Storch & Anger 1983, Anger et al. 1985), which may be considered a 'monitor organ for assessments of the nutritional condition of crustaceans (see Vogt et al. 1985 and references therein). Although the present study showed that a successful capture of prey, digestion of food, and eventually, even a restoration of previously degraded lipid reserves in the hepatopancreas may still be possible after extended periods of starvation, some metabolic disorder seems to occur, preventing an efficient utilization of those energy reserves accumulated after the PNR.

In PRS experiments, only 2 d of initial feeding (<30% of the moult-cycle duration), followed by complete lack of food for the remaining >70% of the moult cycle, may allow for similar rates of survival and development to the Zoea II stage as in a continuously fed control group (F2 versus FC; Fig. 2B). This again shows the importance of the timing (early versus late) of starvation periods. Microscopic examination of the larval moulting cycle in our study demonstrated that the PRS was reached at the transition between stages C and D_0, when the apolysis of epidermal tissues from the old cuticle begins. This confirms the 'D_0 threshold (Anger 1987) as a critical point, after which a decapod larva becomes in principle independent from food for the rest of the moulting cycle.

When the biomass accumulated during an early period of larval feeding and growth is considered at the time of the PRS_{50}, one can see that Zoeae I of *Maja brachydactyla* have reached about 70 to 80% of the maximum DM that continuously fed sibling larvae would show at their moult to the Zoea II stage, although the developmental period from hatching to the PRS corresponds to only about 50% of total moult-cycle duration (present study; cf. Rotllant et al. 2010). This apparent discrepancy between a relatively short time span and high biomass growth is normally due to maximum instantaneous growth rates during the postmoult and intermoult stages, which are followed by lower accumulation rates in premoult (for review of growth patterns, see Anger 2001). As CHN and biochemical data have consistently shown, the steep initial growth phase is typically associated with a particularly fast accumulation of lipids (reflected by increasing C:N values and fat droplets in the hepatopancreas; cf. Figs. 3 & 5, Tables 3 & 7), which can later be utilised as an endogenous energy source during periods of food limitation.

Hence, the rapid initial accumulation of biomass and energy allows for subsequent food-independent development in complete absence of food after the PRS, so that larvae may successfully moult to zoeae II, although they have 40 to 45% less biomass and significantly lower C:N values than continuously fed control larvae. As a consequence, the biomass of newly moulted Zoeae II of *Maja brachydactyla* in our study was linearly correlated with the duration of initial feeding periods in the preceding Zoea I stage. This range of biomass at ecdysis is similar to values observed in preliminary experiments with the same species using mass-rearing techniques (Rotllant et al. 2010). Very similar relative figures were also obtained in experiments with another spider crab, *Hyas araneus* (Anger & Dawirs 1982, Anger & Spindler 1987), and in the portunid crab *Carcinus maenas* (Dawirs 1986). Interestingly, a newly moulted Zoea II originating from a PRS experiment may contain less

biomass than a newly hatched Zoea I, still remaining viable and partially compensating the previous losses through a lengthening of the Zoea II moulting cycle (Anger & Spindler 1987).

In conclusion, the present study corroborates previously proposed hypotheses on critical points and mechanisms of starvation effects within individual larval moulting cycles in majid crabs and other decapod crustaceans. For comparisons among different species or larval stages, however, PNR_{50} or PRS_{50} must be considered in relation to the moult-cycle duration of fed larvae reared under otherwise similar conditions, as environmental factors such as temperature and salinity may influence larval condition and development time. As another comparative index, Gebauer et al. (2010) recently proposed the NVI, defined as the quotient of PRS_{50}:PNR_{50}, which increases with increasing nutritional vulnerability and decreases with decreasing larval dependence on food availability. In majid spider crabs including *Maja brachydactyla* (present study) and *Hyas araneus* (Anger & Dawirs 1981), low PRS and high PNR values, and consequently, NVI indices well below 1, indicated a relatively high level of early larval independence from food (for comparison of literature data, see Gebauer et al. 2010).

The activities of digestive enzymes (protease, amylase) that have been measured in larvae of *Maja brachydactyla* (Rotllant et al. 2008, 2012, Andrés et al. 2010b) reflect a strong capacity to digest food immediately from hatching. Under ad libitum conditions of feeding, the enzyme activities increased throughout the moulting cycle of Zoeae I, while continuously starved sibling larvae showed decreasing values. Also in successive larval stages of *Hyas araneus*, Hirche & Anger (1987) and Harms et al. (1991) showed that trypsin and amylase activities increased during development, similar to the present study. The early appearance and developmental increase in protease activity of fed larvae indicates an immediate and continuously increasing need for protein degradation, which yields amino acids required for growth, morphogenesis and, probably to a lesser extent, as a metabolic energy source (Rotllant et al. 2010). However, a high level of variability among measurements of protease and amylase activities in otherwise similar larvae suggests that enzyme activities may be less suitable indicators of the nutritional condition of spider crab larvae. In conclusion, this study of the nutritional vulnerability of first-stage larval *M. brachydactyla* has shown that data on survival and development as well as those from determinations of DM, CHN, histological observations in the digestive gland and, to a lesser degree, those on digestive enzyme activities can be used as indicators of larval dependence on food and of physiological responses to transitory or extended period of food limitation. Although these individual variables differ in their respective explanatory power, a combined study of various criteria, in particular when these are considered in relation to the moulting cycle (Guerao et al. 2010), should allow for reliable assessments of the nutritional condition of early decapod larvae.

Acknowledgements. Bench fees were funded by INIA (RTA2008-00003-00-00 project) to G.R. Financial support was provided by the Ministry of Science and Research to G.G. (post-doctoral fellowship; INIA). We thank Glòria Macià for support as the hatchery and laboratory technician at IRTA, Sant Carles de la Ràpita.

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Editorial responsibility: Christine Paetzold, Oldendorf/Luhe, Germany

Submitted: April 11, 2012; Accepted: June 26, 2012
Proofs received from author(s): August 17, 2012

GENERAL DISCUSSION

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One of the most drastic and hence, biologically most fascinating adaptive processes is the evolutionary transition of marine species to freshwater or terrestrial environments. Those transitions constitute dramatic shifts between "adaptive zones", which have initiated radiations on various times scales (Lee and Bell 1999). For instance, introduced species can colonize a new recipient region within a few years or decades (e.g. European shore crab, Thresher et al. 2003), whereas other species (e.g. king crab, Thatje et al. 2005) may take decades or centuries. While such "invasions" (mostly man-made) are short-term events, evolutionary transitions require numerous generations and geologic time scales (e.g. palaemonid shrimps; Anger 2013).

Among the decapod crustaceans, caridean shrimps have been particularly successful invaders of brackish and freshwater habitats (Bauer 2004). They are therefore considered as models for the evaluation of evolutionary transitions from marine to freshwater habitats (see Chapters I-II). In *Macrobrachium amazonicum* (Chapter I), for example, palaeogeographic evidence of marine incursions during the middle Miocene (Lovejoy et al. 1998, 2006), suggest that its ancestors migrated from the Caribbean coast into western Amazonian inland waters, where brackish and freshwater conditions prevailed. The invaders subsequently spread over an expanding, interconnected, and increasingly limnic subandine lake system that was formed due to the Andean orogenesis (Räsänen et al. 1990; Hoorn et al. 1995; Wesselingh et al. 2002), where they gradually adapted to freshwater. Eventually, the southernmost populations were separated from those remaining in the Amazon and Orinoco basins, when the formation of the modern South American drainage system took place and a continental divide became effective between the Amazon and La Plata basins, i.e. during the late Miocene through the Pliocene (Campbell 1990; Lundberg et al. 1998). This paleogeographic event (Andean orogenesis) must have caused a segregation of populations and subsequent speciation (Albert et al. 2006; Hubert and Renno 2006; Anger 2013).

Phylogenetic divergence in reproductive and developmental traits is generally considered as a crucial step in allopatric speciation within monophyletic groups. Our comparative biochemical study (Chapter I) shows that phylogenetically relevant life-history traits of the South American freshwater shrimp *Macrobrachium amazonicum* vary significantly between two geographically isolated populations (Amazon delta vs. Pantanal). Newly hatched larvae

produced by shrimps from the Amazon delta were significantly smaller and showed lower values of dry weight (W), carbon (C), hydrogen (H), nitrogen (N), protein, and unsaturated fatty acid compared to those from the Pantanal. On the other hand, they contained significantly higher quantities of total lipid and saturated fatty acids and, in consequence, higher ratios of lipid:protein, C:N, and saturated:unsaturated fatty acids. All these differences in biomass and chemical composition suggest that the larvae of the Amazon population are energetically better adapted to planktonic food limitation, which likely occurs during riverine downstream transport towards coastal marine waters. This also explains previous observations of much stronger initial starvation tolerance in larvae from the Amazon versus those from the Pantanal. The latter develop in highly productive lentic inland waters, where large body size and a strong musculature (indicated by high protein content) should facilitate their role as planktonic predators and allow for fast growth. An initial independence of food, lecithotrophy in the zoea I stage (Anger and Hayd 2010), as well as a preference for oligohaline rather than fully limnic conditions observed in the Pantanal larvae (Charmantier and Anger 2011) are interpreted as traits that have persisted from an ancestral estuarine clade. Altogether, consistent ontogenetic differences between shrimps originating from inland (Pantanal) and estuarine waters (Amazon), respectively, are hardly compatible with the population concept, suggesting that *M. amazonicum* represents a complex of closely related, but separate species.

As an additional model, I evaluated reproductive traits in the shrimp *Palaemonetes zariquieyi*, which appear to be adaptive in liminic environments (Chapter II). Our data may explain how some decapod crustaceans evolved during one of the most dramatic episodes of oceanic change, the Messinian salinity crisis (Krijgsman et al. 1999; García et al. 2011). Isolation from the Atlantic Ocean was established between 5.59 and 5.33 million years ago, causing a large fall in Mediterranean water level, followed by erosion and deposition of non-marine sediments in a large basin, the `Lago Mare' (Pedley et al. 2007; García et al. 2011). During this paleogeographic event, con-specific Mediterranean and Atlantic populations were separated and probably evolved to separate species, *Palaemonetes zariquieyi* and *Palaemonetes varians*, respectively. Under a scenario of larval retention within parental habitats, the early life-history stages of *P. zariquieyi* may have developed adaptations to non-marine conditions (low salinities, food limitation, including an abbreviated mode of larval development, full lecithotrophy, and changes in the chemical composition of their offspring.

The chemical composition of *Palaemonetes zariquieyi* (Chapter II) shows significant variations through ontogeny. For instance, absolute values of dry weight (W), carbon (C) and hydrogen (H) per individual decreased significantly throughout larval development, whereas nitrogen (N) values per larva did not show significant differences during ontogeny. Similarly, the proportions of C and H (in % of W) decreased from the zoea I (ZI) to the first juvenile stage (J), while the percentage of nitrogen remained stable. The total lipid content, both in absolute and relative terms, decreased gradually during the course of non-feeding larval development. This was in *P. zariquieyi*, similarly as reported also for lecithotrophic larvae of other decapod crustaceans (Nates and McKenney 2000; Kattner et al. 2003), due to mainly a utilization of neutral lipids (NLs) as an energy source. The fraction of polar lipids (PLs), which are important structural components of membranes, remained stable, so that their percentage within the total lipid content increased. These patterns of reserve utilization are similar to those previously observed in other palaemonid shrimps and other groups of decapod crustaceans with abbreviated and lecithotrophic mode of larval development, suggesting multiple convergent evolution of bioenergetic traits allowing for reproduction in aquatic environments with planktonic food limitation.

In pelagic environments, crustacean larvae are also exposed to seasonal variations in environmental conditions. Combined effects of key variables such as food and temperature can thus influence their growth and development, and consequently, also affect later phases of the life cycle (carry-over effects or cascade effects; Giménez 2006; Giménez 2010). Such effects were studied in the North Sea shrimp, *Crangon crangon* (Chapters III-IV). Its reproductive pattern is characterized by a continuous spawning season from mid winter throughout spring and late summer (Siegel et al. 2008; Chapter III). During this long period, this species shows seasonal variations in egg size and biomass (Boddeke 1982; Chapter III), which translate to differential larval size and biomass at hatching (Chapter IV). Compared with larvae originating from summer eggs, those hatching from winter eggs show reduced nutritional vulnerability (Paschke et al. 2004), tend to be larger (Chapter IV), and require fewer stages to reach the first juvenile stage (Linck 1995). This indicates carry-over effects persisting from embryogenesis through the larval phase to the onset of the benthic phase.

In aquatic organisms such as *C. crangon*, parental organisms may assess seasonal variations in the nutritional conditions that their offspring will likely encounter and adjust the energy

investment per offspring accordingly (Fischer et al. 2011). Considering the relationship between environmental conditions prevailing at hatching and larval biomass observed in our study (Chapters IV), the smoothing function of generalized additive models (GAM) showed a maximum level in larval biomass during winter, a minimum in the late spring, and another increase in late summer. High larval C content (as a proxy of the energy content) during late winter may improve the tolerance of fasting, when poor nutritional conditions coincide with prolonged duration of larval development at cold temperatures (Paschke 1998; Daewel et al. 2011). Therefore, the production of larger ‘‘winter larvae’’ seems to be an adaptive reproductive characteristic of *Crangon crangon*, allowing for an extension of the reproductive period in the southern North Sea (Chapters III-IV), which is in most other decapod crustaceans in temperate regions restricted to late spring and summer (Anger 2001). In late spring and summer, by contrast, smaller larvae with smaller energy reserves hatch at higher temperatures and higher planktonic food concentrations (Wiltshire et al. 2008). This match between optimal conditions of food availability and high temperatures with larval peak abundance during spring–summer (Wehrtmann 1989) allows the larvae to exploit rich food resources and reach fast rates of growth and development (Temming and Damm 2002). Similar seasonal variations in offspring biomass and environmental conditions prevailing at the time of hatching have also been described in other aquatic crustacean species, for instance in crabs (Bas et al. 2007; Gebauer et al. 2010), marine copepods (Acheampong et al. 2011), and limnic cladocerans (Boersma 1997).

In *Crangon crangon* populations living in temperate regions such as the southern North Sea, pronounced seasonality of food availability may have selected for the evolution of a recurring seasonal pattern of intraspecific variation in the reproductive energy investment per offspring. Additionally, due to the large range of distribution of *C. crangon*, reproductive traits may vary over a latitudinal gradient (Tiews 1970; Campos and van der Veer 2008). In contrast to populations living at relatively high latitudes (e.g. in the North Sea), those at lower latitudes (e.g. on the west coast of Portugal), reproduce only from late winter to early summer, with a main spawning and breeding season during spring (Marchand 1981; Viegas et al. 2012). The offspring there is smaller, and no seasonal variations in egg size and weight have been observed (Viegas et al. 2012). In the warmer and seasonally still less variable Mediterranean Sea, *C. crangon* shows an even shorter breeding season, comprising only the coldest months from November to April (Gelin et al. 2000).

Biological invasions have recently become a major concern, in particular in the context of climate change (Ruiz et al. 2000; Garcia-Berthou et al. 2005). The success of invasive species in new habitats is commonly based on pre-adaptations evolved in the original environment, and/or on variability in life-history traits (Paglianti and Gherardi 2004; Roth and Kitchell 2005). In species with complex life cycles, the larval phase is therefore crucial not only for the establishment and persistence of new populations, but also for range extensions within recipient regions. This has been suggested, for instance, for the Chinese mitten crab, *Eriocheir sinensis*, in Europe (Clark et al. 1998; Herborg et al. 2003) and along the west coast of the USA (Rudnick et al. 2005), for the European shore crab, *Carcinus maenas*, in Australia (Thresher et al. 2003) and on both the Atlantic and Pacific coasts of North America (Carlton and Cohen 2003; Behrens Yamada et al. 2005; Cameron and Metaxas 2005), as well as for the Asian shore crab, *Hemigrapsus sanguineus*, which is spreading along the northwestern and northeastern Atlantic coasts (Park et al. 2005). Similarly, the shrimp *Hippolyte leptocerus*, which originates from the eastern Atlantic (Mauritania), is considered as an invasive species in the Mediterranean and the Black Sea (D'Udekem D'Acoz 1996). It shows an extended larval development (Chapter V), which should facilitate a continued range extension.

For sustainable fisheries and economically feasible aquaculture of commercially valuable crustacean species (FAO 2010), the knowledge of larval biology is highly important (Anger 2001). In this context, the spider crab *Maja brachydactyla* is considered as an interesting model species. It has been overexploited by fisheries, and artificial cultivation is currently under development (e.g. Andrés et al. 2007, Guerao et al. 2010). In Chapter VI, we revealed that biomass, elemental composition, the occurrence of lipid vacuoles in the hepatopancreas, and activities of digestive enzymes are suitable indicators of the nutritional condition of early zoeal stages. This information may thus have practical implications for maximizing larval feeding efficiency and enhancing rates of growth and survival.

In summary, the life–history traits of decapod crustaceans studied for this thesis have implications for evolutionary biology, population dynamics, community ecology, biogeography, and assessments of the invasiveness of introduced species. The study of evolutionary adaptations to non-marine conditions (e.g. larval tolerance of low or unstable salinities; reduced dependence on planktonic food production) can significantly contribute to the understanding of speciation in limnic and terrestrial environments. In commercially

exploited crustaceans, basic information from larval biology is highly important for the development of economically feasible aquaculture techniques and for sustainable fisheries management. Also, the duration of larval development in the plankton in combination with ecophysiological characteristics (e.g. larval tolerance of variations in temperature, salinity, or food availability) determines the dispersal capacity of a species on ecological and biogeographical scales. Hence, information on life-history traits is critical for the evaluation of distribution patterns across climatic gradients and of the invasiveness of introduced species. Finally, variability in reproductive traits is related to variations in environmental key factors such as temperature and food availability. Future studies should therefore investigate how life history traits, population dynamics, and distribution patterns of decapod crustacean species may be affected by climate change.

SUMMARY

SUMMARY

In the present thesis, various decapod crustacean species were used as model organisms to identify evolutionary adaptations in life-history traits to different environmental conditions, especially in larval development and growth.

A comparative biochemical study (Chapter I) showed that biologically relevant larval traits of a tropical shrimp, *Macrobrachium amazonicum*, vary significantly between two geographically isolated populations (Amazon Delta vs. Pantanal). Newly hatched larvae from the Amazon delta are significantly smaller and show lower dry weight (W) and lower contents of C, H, N, protein, and unsaturated fatty acids per individual than larvae from the Pantanal population. On the other hand, they contained significantly higher quantities of total lipid and saturated fatty acids. These differences in biomass and chemical composition suggest that the larvae from the Amazon Delta are, compared to those produced in the Pantanal, energetically better adapted to planktonic food limitation. Altogether, ontogenetic differences in shrimps from the Pantanal (inland waters) and Amazon (estuary) suggest the existence of different species.

In *Palaemonetes zariquieyi*, a shrimp with an abbreviated and lecithotrophic larval development, I quantified ontogenetic changes in larval biomass (W, C, H, N) and lipid composition (total lipids, lipid classes, fatty acids) (Chapter II). Absolute values of W, C and H (per individual) decreased significantly during larval development, while N values per larva did not show significant changes. Similarly, also the proportions of C and H (in % of W) decreased, while the percentage of N remained constant. The total lipid content, both in absolute and relative terms, decreased during larval development. Our results suggest that the lecithotrophy of *P. zariquieyi* is primarily fuelled by the utilization of neutral lipids (especially triacyglycerides), whereas polar lipids are preserved as structurally indispensable components. Similar patterns of reserve utilization have been observed also in other groups of decapod crustaceans with an abbreviated and lecithotrophic mode of larval development. This indicates a multiple convergent evolution of bioenergetic adaptations to food-limited conditions in aquatic environments.

In *Crangon crangon*, a species of shrimp that is important for coastal fisheries and as a key species in the benthic food web, I studied reproductive adaptations to the pronounced seasonality of the North Sea ecosystem (Chapters III-IV). The reproductive pattern of this species is characterized by continuous spawning from winter to late summer. During this

extended period, *C. crangon* shows seasonal variations in egg size and biomass, which are interpreted in the context of changes in environmental conditions, e.g. day length, temperature and food availability. Seasonal variation in egg size translates to differential larval size and biomass at hatching. Compared with summer larvae, those hatching from winter eggs show reduced nutritional vulnerability, tend to be larger, and require fewer stages to reach the first juvenile stage. This indicates carry-over effects persisting from embryogenesis through the larval phase to the onset of the benthic phase. Intraspecific variability in larval biomass and alternative pathways of development are interpreted as flexible adaptive strategies compensating for strong seasonality in plankton production and periods with food limitation.

The extended larval development of the "invasive" shrimp *Hippolyte leptocerus* suggests that, in invasive species with complex life cycles, the planktonic larval phase is crucial for the establishment of new populations and the extension of the range of distribution (Chapter V).

A profound knowledge of larval biology is highly important also for sustainable fisheries as well as for crustacean aquaculture. In this context, biomass, chemical composition and activities of digestive enzymes proved to be suitable indicators of the nutritional condition of early larval stages of the crab *Maja brachydactyla* (Chapter VI). Possible applications include the optimization of feeding efficiency in order to increase growth and survival rates.

Altogether, the aspects of life-histories of decapod crustaceans studied in this thesis have important implications for evolutionary biology, population dynamics, community ecology, biogeography, fisheries and aquaculture. Intraspecific variability in reproductive traits of decapod crustaceans is most likely related to environmental key factors such as temperature and food availability. Future studies should therefore investigate how the life-history traits, population dynamics, and distribution patterns of decapod species may be affected by global climate change.

ZUSAMMENFASSUNG

ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurden verschiedene dekapode Crustaceen-Arten als Modellorganismen verwendet, um in deren Lebenszyklus evolutive Anpassungen an unterschiedliche Umgebungsbedingungen zu identifizieren, insbesondere in der Larvalentwicklung und im Larvalwachstum.

Eine vergleichende biochemische Untersuchung (Kap. I) zeigte, dass biologisch relevante Larvalmerkmale einer tropischen Garnele, *Macrobrachium amazonicum*, sich bei zwei geographisch isolierten Populationen (Amazonas-Delta bzw. Pantanal) signifikant voneinander unterscheiden. Frisch geschlüpfte Larven aus dem Amazonas-Delta sind signifikant kleiner und weisen ein geringeres Trockengewicht (W) sowie geringere Gehalte an C, H, N, Protein sowie ungesättigten Fettsäuren pro Individuum auf als die Larven der Population aus dem Pantanal. Andererseits besaßen sie signifikant höhere Gehalte an Gesamtlipid und gesättigten Fettsäuren. Diese Unterschiede in der Biomasse und der chemischen Zusammensetzung legen den Schluss nahe, dass die Larven aus dem Amazonas-Delta im Vergleich zu den im Pantanal produzierten energetisch besser an ein limitiertes Plankton-Angebot angepasst sind. Insgesamt weisen die ontogenetischen Unterschiede der Garnelen aus dem Pantanal (Binnengewässer) und dem Amazonas Delta (Ästuar) auf die Existenz unterschiedlicher Spezies hin.

Bei *Palaemonetes zariquieyi*, einer Garnele mit verkürzter und lecithotropher Larvalentwicklung, quantifizierte ich ontogenetische Veränderungen in der larvalen Biomasse (W, C, H, N) und Lipidzusammensetzung (Gesamtlipid, Lipidklassen, Fettsäuren) (Kap. II). Die absoluten W-, C- und H-Werte (pro Individuum) nahmen während der Larvalentwicklung signifikant ab, während die N-Werte keine signifikanten Veränderungen zeigten. In ähnlicher Weise nahmen auch die relativen C- und H-Anteile (in % von W) ab, während der Prozentsatz an N konstant blieb. Der Gesamt-Lipidgehalt nahm während der Larvalentwicklung sowohl absolut als auch relativ ab. Unsere Ergebnisse legen nahe, dass die Lecithotrophie von *P. zariquieyi* primär durch den Umsatz von Neutrallipiden (besonders Triglyzeriden) gewährleistet wird, während polare Lipide als strukturell unerlässliche Komponenten erhalten bleiben. Ähnliche Muster der Reservemobilisierung wurden auch bei anderen dekapoden Crustaceeen mit verkürzter lecithotropher Larvalentwicklung beobachtet. Dies deutet auf eine multiple konvergente Evolution bioenergetischer Anpassungen an nahrungslimitierte Bedingungen in aquatischen Lebensräumen hin.

Bei der Garnele *Crangon crangon*, die für die küstennahe Krabbenfischerei sowie als Schlüsselart im benthischen Nahrungsnetz wichtig ist, wurden reproduktive Anpassungen an

die ausgeprägte Saisonalität des Nordsee-Ökosystems untersucht (Kap. III-IV). Das Reproduktionsmuster dieser Spezies ist durch ein kontinuierliches Laichen vom Winter bis zum Spätsommer charakterisiert. Während dieses ausgedehnten Zeitraumes zeigt *C. crangon* saisonale Variationen der Eigröße und -Biomasse, die im Zusammenhang mit Veränderungen in den Umweltbedingungen (z. B. Tageslänge, Temperatur, Nahrungsverfügbarkeit) interpretiert werden. Die saisonal variierende Eigröße bedingt eine unterschiedliche Körpergröße und -Biomasse beim Larvenschlupf. Verglichen mit Sommerlarven zeigen die aus Wintereiern hervorgehenden Larven eine reduzierte Verwundbarkeit gegenüber Nahrungslimitierung. Beim Schlüpfen sind sie durchschnittlich größer, und sie benötigen weniger Larvalstadien bis zum Erreichen des 1. Juvenilstadiums. Dies bedeutet, dass es Übertragungseffekte gibt, die von der Embryogenese über die Larvalphase bis hin zum Beginn der benthischen Lebensphase erhalten bleiben. Die intraspezifische Variabilität in der Biomasse der Larven und die alternativen Entwicklungswege werden als flexible Anpassungsstrategien an starke Saisonalität der Planktonproduktion mit Phasen von Futterknappheit interpretiert.

Die ausgedehnte Larvalentwicklung der “invasiven” Garnele *Hippolyte leptocerus* deutet darauf hin, dass bei eingeschleppten Arten mit komplexem Lebenszyklus die planktische Larvalphase für die Gründung neuer Populationen und die Erweiterung des Verbreitungsgebietes entscheidend ist (Kap. V). Eine fundierte Kenntnis der Larvalbiologie ist auch für eine nachhaltige Fischerei sowie für die Aquakultur von Crustaceen äußerst wichtig. Bei der Krabbe *Maja brachydactyla* erwiesen sich in letztgenanntem Zusammenhang die Biomasse, die chemische Zusammensetzung und die Aktivität von Verdauungsenzymen als geeignete Indikatoren für die Beurteilung des Ernährungszustandes früher Larvalstadien (Kap. VI). Mögliche Anwendungen liegen in einer Optimierung der Fütterungseffizienz zwecks Steigerung von Wachstums- und Überlebensraten.

Insgesamt konnten aus den in der vorliegenden Arbeit untersuchten Aspekten der Lebenszyklen dekapoder Krebse wichtige Erkenntnisse bezüglich Evolutionsbiologie, Populationsdynamik, Gemeinschaftsökologie, Biogeographie, Fischerei und Aquakultur gewonnen werden. Intraspezifische Variabilität in Reproduktionsmerkmalen dekapoder Crustaceen ist höchstwahrscheinlich verbunden mit variablen Schlüsselfaktoren, z.B. Temperatur und Nahrungsverfügbarkeit. Zukünftige Untersuchungen sollten deshalb analysieren, wie Merkmale des Lebenszyklus, der Populationsdynamik und der Verteilungsmuster dekapoder Krebse durch globale Klimaveränderungen beeinflusst werden.

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DANKSAGUNG

DANKSAGUNG

Meine Arbeit wurde von der Comision Nacional de Ciencia y Tecnología, CONICYT (Santiago de Chile) und dem Deutschen Akademischen Austauschdienst, DAAD (Bonn) finanziert.

Ein besonderes Dankeschön an Herrn Dr. Klaus Anger für seine unermüdliche Unterstützung und wertvollen Ratschläge.

Ich möchte mich auch ganz herzlich bei Frau Prof. Dr. Karen Wiltshire (AWI-Helgoland) und Herrn Prof. Dr. Maarten Boersma (AWI-Helgoland) für die vielfältige Unterstützung bedanken.

Mein Dank gilt weiterhin Herrn Dr. Kurt Paschke (Chile-LECOFIC, UACh), Frau Dr. Paulina Gebauer (Chile-IMAR, ULA), Herrn Dr. Juan Cancino (Chile-UCSC), Frau Dr. Romina Ituarte (Argentina-MDP), Frau Dr. Liliam Hayd (Brasil-UEMS), Frau Dr. Guiomar Rottlant (Spain-IRTA), Herrn Dr. Guillermo Guerao (Spain-IRTA), Herrn Dr. Ricardo Calado (Portugal-CESAM), Herrn Dr. Luis Giménez (U.K.-Bangor University), Frau Dr. Gabriela Torres (U.K.-Bangor University), Frau Dr. Karin Boos (Germany-Bremen), Herrn Dr. Reinhard Saborowski (Germany-Bremerhaven), Herrn Dr. Florian Hantzsche (Germany-Ingolstadt), Frau Evamaria Krause (Germany-Helgoland), Herrn Uwe Nettelmann (Germany-Helgoland), Herrn Charlie Lührs (Germany-Bremerhaven), Herrn Jan Beermann (Germany-Helgoland), Frau Bettina Oppermann (Germany-Hamburg), Frau Julia Haafke (Germany-Plön), Frau Judith Lucas (Germany-Helgoland) und Herrn César Cáceres (Germany-Hamburg).

Meinen Kollegen danke ich für die tägliche Unterstützung und die gemeinsame Arbeit: Achim, Carlos, Detlef, Helgo, Heino, Silvia, Claudia, Gunda, Birgit, Michael, Ute, Ulrich, Helgo, Kathrin, Isabel, Jan, Jasmin, Cédric, Nicole, Katharina, Arne, Martin, Nicole, Sonja, Florian, Rebecca, Thomas, Evamaria, Rebekka, Rene, Anabel, Judith, Felix, Lena, Sopha, Roman, Jinpeng, Wenjin, Mathias, Stephanie, Christoph, Marco, Markus, Suse, Hilke, Dieter, Karl-Walter, Christine, Kristine, Christiane, Dr. Gunnar Gerdts, Dr. Antje Wichels, Prof. Dr. Fischer und Prof. Dr. Franke.

Und nicht zuletzt möchte ich meiner Familie danken, ohne die diese Arbeit nicht möglich gewesen wäre.

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Publikationen

Urzúa, Á., Paschke, K., Gebauer, P. and Anger, K. (2012) Seasonal and interannual variations in size, biomass and chemical composition of the eggs of North Sea shrimp, *Crangon crangon* (Decapoda: Caridea), Marine Biology 159: 583-599

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Urzúa, Á., Boersma, M. and Anger, K. (2012) Intraspecific variability in reproductive traits of North Sea shrimp, *Crangon crangon*. Journal of Crustacean Biology (In Preparation)

Hayd L., **Urzúa, Á.** and Anger, K. (2012) Larval growth and chemical composition of two populations of *M. amazonicum* (Pantanal vs. Amazonas) under different salinity rearing conditions. Aquaculture (In Preparation)

Tagungsbeiträge

Urzúa, Á. and Anger, K. (2012) Seasonal variations in offspring biomass of brown shrimp, *Crangon crangon* (Decapoda, Caridae). *The Crustacean Society Summer Meeting and the 10th Colloquium Crustacea Decapoda Mediterranea, 3-7 June. 2012, Athens, Greece* (Poster)

Urzúa, Á., Paschke, K., Gebauer, P. and Anger, K. (2011) Seasonal and inter-annual variations in size, biomass and chemical composition of the eggs of North Sea shrimp, *Crangon crangon* (Decapoda: Caridae). *15. Deutschsprachige Crustaceologen-Tagung, 07-10 April. 2011, Regensburg, Germany* (Vortrag)

Boos, K., Cook, E. J., **Urzúa, Á.,** Gutow, L. and Saborowski, R. (2011) Effects of ocean acidification on reproductive traits in the caprellid amphipod *Caprella mutica* Schurin, 1935, *15. Deutschsprachige Crustaceologen-Tagung, 07-10 April, Regensburg, Germany* (Vortrag)

Anger, K., **Urzúa, Á**. and Hayd, L. (2010) Patterns of early larval feeding, biochemical composition and growth in a population of *Macrobrachium amazonicum* from the Pantanal, southwestern Brazil: comparison with a coastal population from the Amazon estuary. *Seventh International Crustacean Congress, 20-25 June. 2010, Qingdao, China* (Vortrag)

Anger, K., Hayd, L., dos Santos, A., Charmantier, G., **Urzúa, Á**., Schubert, R., Knott, J., Remetin, M. and Nettelmann, U. (2009) Differential life-history traits in geographically isolated populations of the Amazon River prawn, *Macrobrachium amazonicum*, suggest speciation, Vortrag. *The Crustacean Society Summer Meeting & 47th Annual Meeting of Carcinological Society of Japan, 20-24 Sept. 2009, Tokyo, Japan* (Vortrag)

Techniken

Haltung und Aufzucht von aquatischen Invertebraten und Fischen

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Biochemische Analytik (Proteine, Lipide, Fettsäuren), Enzymkinetik (Proteasen, Amylasen, Lipasen), Molekularbiologische Methoden [Elementaranalysen (CHN), DNA:RNA, PCR], Bioassays

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Sprachen: Spanisch, Deutsch und Englisch

Helgoland, 31.10.2012

ERKLÄRUNG

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Hiermit erkläre ich, dass die vorliegende Dissertation, abgesehen von der Beratung meiner Betreuer, selbstständig von mir angefertigt wurde und dass sie nach Form und Inhalt meine eigene Arbeit ist. Sie wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Dies ist mein einziges und bisher erstes Promotionsverfahren. Diese Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden. Des Weiteren erkläre ich, dass ich Zuhörer bei der Disputation zulasse.

Helgoland, den 31.10. 2012 Ángel Urzúa