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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Ciências do Mar e do Ambiente, realizada sob a orientação científica do Doutor Ricardo Jorge Guerra Calado, Investigador Principal no Departamento de Biologia da Universidade de Aveiro e do Professor Doutor Henrique José de Barros Brito Queiroga, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro

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Dedico esta tese a Miguel, Carolina e Leo.

La inspiración existe, pero tiene que encontrarte trabajando

Pablo Picasso

o júri

presidente

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palavras-chave

Caranguejos braquiúros, aprovisionamento maternal, desenvolvimento embrionário, ciclos de vida complexos, desempenho dos juvenis pós-assentamento, plascidade fenotípica, efeitos latentes, lipidómica

resumo

A maioria dos invertebrados bentónicos marinhos apresentam ciclos de vida complexos, com características contrastantes entre os diferentes estádios. Estes organismos normalmente exibem uma forma larval pelágica (ou várias), a qual sofre um evento dramático que muda a sua morfologia e comportamento – a metamorfose. Este evento dá origem ao estádio juvenil, o qual também experimenta mudanças na sua morfologia, comportamento e fisiologia, até atingir o estádio adulto e ser sexualmente maduro. O percurso entre a eclosão e o assentamento é um desafio que só uma pequena fracção de indivíduos consegue superar. Revelar os processos que condicionam as estratégias do ciclo de vida e o êxito no assentamento dos invertebrados bentónicos marinhos é essencial para compreender a dinâmica das populações adultas, assim como a sua trajetória a nível ecológico e evolutivo. Neste sentido, esta tese analisa importantes estádios no ciclo de vida de uma espécie modelo de invertebrados bentónicos marinhos: o desenvolvimento embrionário, o assentamento larval e o desempenho dos primeiros estádios da vida bentónica do caranguejo *Carcinus maenas*.

A primeira parte desta tese analisa os efeitos maternais, como fonte de variabilidade na qualidade da descendência, usando ferramentas bioquímicas. Explora a existência hipotética da variabilidade embrionária, como consequência da posição dos embriões dentro da câmara de incubação no momento da extrusão e durante a embriogênese. O perfil dos ácidos gordos dos embriões, na fase inicial (estádio 1) e fase tardia (estádio 3) da embriogênese, foram usados como estimativa para analisar o investimento maternal e a qualidade das larvas no momento da eclosão, respectivamente. Através de uma abordagem lipidómica, é comparada a embriogênese de *C. maenas* e *Necora puber*, dois caranguejos braquiúros estreitamente relacionados que ocorrem em simpatria na área de estudo. A dinâmica dos lípidos polares é revelada pela primeira vez nestas espécies de interesse ecológico e comercial. Para finalizar, duas escalas temporais diferentes, anual e sazonal, são utilizadas para investigar a existência de variabilidade temporal na qualidade da descendência, incluindo o papel do tamanho maternal como outra fonte de variabilidade.

A segunda parte da tese foca-se no início da vida bentónica dos juvenis de *C. maenas*. Megalopas selvagens recolhidas na Ria de Aveiro, são usadas para entender como as experiências da vida pelágica antes de se tornar competentes (recetivas aos sinais de assentamento) podem condicionar a sua vida bentónica. As larvas competentes são submetidas a condições tróficas e abióticas extremas para compreender como a sua plasticidade fenotípica e os potenciais efeitos latentes influenciam as fases subsequentes do ciclo de vida.

O estudo dos efeitos maternais revelou que *C. maenas* não apresenta variabilidade dentro da massa de embriões recém extrudidos (estádio 1), nem nos que estão para eclodir (estádio 3). Portanto, o investimento maternal e as condições durante a incubação foram homogéneas em toda a massa de embriões. No entanto, uma análise de alta resolução mostrou que a embriogênese é um processo dinâmico, sugerindo uma especialização das funções dos lípidos durante o desenvolvimento embrionário. Por outro lado, o investimento maternal e as condições de incubação não foram influenciadas pelo tamanho maternal. Contudo, a composição lipídica dos ovos foi condicionada pelo período no qual a maturação do ovário ocorreu, enquanto as condições ambientais existentes durante a incubação modificaram o perfil de ácidos gordos dos embriões prontos para eclodir.

resumo (cont.)

Após a fase planctônica, as larvas competentes exibiram diferentes níveis de rendimento pós-assentamento, reflectindo claramente as condições pelágicas experimentadas durante a vida larval. Condições ótimas de crescimento não apagam a pegada da vida larval, sendo transferidas as consequências para o início da vida bentónica e condicionando o fenotipo dos caranguejos juvenis. A vida larval desempenha um papel tão importante na história de vida dos invertebrados bentónicos marinhos que, inclusivamente, a exposição a condições tróficas e abióticas adversas durante poucos dias influenciam o desempenho após o assentamento.

Globalmente, a metamorfose não é, definitivamente, um novo começo para *C. maenas*. Na verdade, o desempenho pós-metamorfose no início da vida bentónica deste caranguejo braquiúra pode ser fortemente condicionada pelos efeitos latentes que são transferidos desde a vida larval pelágica até os estádios bentónicos, juvenil e/ou adulto.

keywords

Brachyuran crabs, maternal provisioning, embryonic development, complex life cycles, post-settlement performance, phenotypic plasticity, carry-over effects, lipidomics

abstract

The majority of benthic marine invertebrates display complex life cycles characterized by life stages with contrasting features. These organisms commonly exhibit a dispersive pelagic larval form(s) that experiences a dramatic event that shifts its morphology and behaviour – the metamorphosis. This event gives origin to a benthic juvenile stage that may also experience additional changes in its morphology, behaviour and physiology until reaching the adult stage and becoming sexually mature. The path between hatching and settlement is a challenging one that only a minute fraction of individuals overcomes. Unravelling the processes that condition the life history strategies and settlement success of benthic marine invertebrates is essential to understand the shaping of adult populations, as well as ecological and evolutionary pathways. In this regard, the present thesis analyses important stages in the life history of a model benthic marine invertebrate with a complex life cycle: the embryonic development, larval settlement and early benthic performance of the green crab *Carcinus maenas*.

The first part of this thesis analyses maternal effects as a source of variability in offspring quality, using biochemical tools. It explores the hypothetical existence of embryonic variability as a consequence of their positioning within the brooding chamber at oviposition and during embryogenesis. Fatty acid profiles of embryos in early (stage 1) and late (stage 3) stages are used as proxies to analyse maternal provisioning and larval quality at hatching, respectively. Through a lipidomic approach, the embryogenesis of *C. maenas* and *Necora puber*, two closely related brachyuran crabs that occur in sympatry in the study site, is compared. Polar lipid dynamics is unravelled for the first time in these ecologically and economically important species. Finally, two different time scales, annual and seasonal, are employed to investigate the existence of temporal variability in offspring quality, with the role that maternal size may play as another source of variability also being investigated.

The second part of this thesis focus in the early benthic life of juvenile *C. maenas*. Wild megalopae collected in Ria de Aveiro are used to understand how the pelagic life experienced by developing larvae before becoming competent (receptive to settlement cues) can condition their early benthic life. Competent larvae are exposed to extreme abiotic and trophic conditions to comprehend how their phenotypic plasticity and potential carry-over effects influence consecutive life stages.

The part of the study addressing maternal effects revealed that *C. maenas* did not present any significant within brood variability in newly extruded (stage 1) and ready to hatch (stage 3) embryos. Therefore, maternal provisioning and the incubation environment within the brooding chamber were homogeneous across the whole brood. Nevertheless, a high-resolution lipidomic analysis showed that embryogenesis is a dynamic process, suggesting a specialization of lipid functions along the embryonic development. Moreover, maternal provisioning and incubation environment were not influenced by maternal size. Nonetheless, the lipid composition of the yolk was conditioned by the period in which the ovarian maturation occurred. While the environmental conditions prevailing during the incubation altered the fatty acid profile of the ready to hatch embryos.

After experiencing a planktonic life, competent larvae displayed different post-settlement performance, clearly reflecting the pelagic conditions that they have experienced during their larval life. Optimal grow-out conditions did not delete these larval fingerprints, with their consequences being carried-over to early benthic life and conditioning the phenotype of juvenile crabs. Larval life plays such an important role in the life history of benthic marine invertebrates that even exposure during a few days to extreme abiotic and trophic conditions can influence their post-settlement performance.

Overall, metamorphosis is definitely not a new beginning for *C. maenas*. In fact, the early post-metamorphosis performance of this brachyuran crab can be strongly conditioned by trait mediated effects that are carried over from its embryonic and/or pelagic larval life to its benthic juvenile and/or adult stages.

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PART I

GENERAL INTRODUCTION

1. 1 OVERVIEW

1.1.1 BENTHIC MARINE INVERTEBRATES AND THEIR COMPLEX LIFE CYCLE

Most marine benthic invertebrates develop through complex life cycles (Roughgarden et al. 1988) using two reproductive strategies. The most ancestral one is external fertilization in which parental organism release their gametes into the water column and resulting embryos remain on the benthos or plankton. The other reproductive strategy is internal fertilization where the embryos are brooded by parents in more or less specialized structures, or laid in the benthos in protective embryonic capsules or embryonic masses. Newly hatched larvae dwell in the plankton for a few minutes to several months, depending on the species. A small fraction of those newly hatched larvae returns to adult or nursery habitats to metamorphose and settle for adult life. Only a small fraction of settlers successfully reach the adult stage, where they attain reproductive maturity and truly recruit to the population (Fig. 1.1).

Given the dispersive potential of pelagic larvae (Queiroga & Blanton 2005, Thorrold 2006), these life stages play a key role in the structuring and dynamics of populations (Cowen & Sponaugle 2009). Despite their poor swimming ability and offshore transport originated by oceanographic currents, larvae may regulate their transport in the pelagic environment by exploiting circulation patterns (Warner & Cowen 2002, Cowen et al. 2006) and thus balance dispersion and connectivity (avoiding dispersing to unsuitable habitats for benthic life). Siegel et al. (2008) showed that larval connectivity is not constant during settlement and varies as a function of different biological and physical processes. In the same way, a study in a region of strong upwelling (Morgan et al. 2009) showed that crustacean larvae of most species remain close to the shore during their development. Therefore, connectivity among populations depends on oceanographic processes (Siegel et al. 2008) and biological factors, such as larval duration (Shanks et al. 2003) and dispersal cost (Pineda et al. 2007). Although longer pelagic development periods can increase the dispersal distance (Shanks et al. 2003), faster-developing larvae commonly experience higher survival in the plankton (Morgan 1995a) and thus are more likely to select a suitable

settlement habitat (Moksnes & Heck 2006). In the same way, larvae which have developed more rapidly in the plankton usually settle under better fitness conditions, this increasing the likelihood of success as juveniles (Pechenik 2006, Giménez 2010). Therefore, a higher dispersal potential comes at the cost of a lower larval survival and a potentially poorer post-settlement performance (Pechenik 2006), with the quality of early benthic stages conditioning the potential for population connectivity.

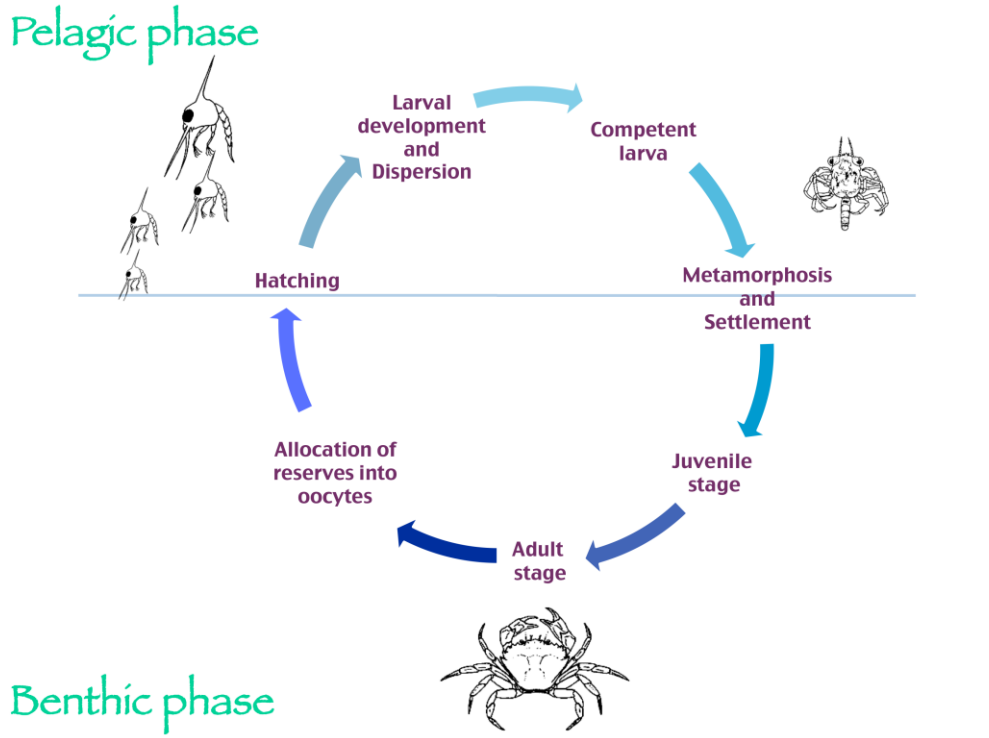

Figure 1.1 Schematic representation of a bi-phasic life cycle of a benthic marine invertebrate. Larvae or gametes are released to the water column and undergo a pelagic lifestyle in the planktonic. Competent larvae (those already receptive to settlement cues) select a suitable habitat to undergo metamorphosis and settle to eventually grow to sexual maturity and recruit to the adult population. The illustrations refer to the life cycle of the green crab *Carcinus maenas*, the model species addressed in the present thesis. Illustration by the author.

Complex life cycles have profound implications on population dynamics, community structure and evolution (Jablonski & Lutz 1983, Kirby et al. 2007, Marshall & Morgan 2011). Each life history stage of a benthic marine organism has to overcome a number of challenges to successfully advance in its life cycle. Environmental and oceanographic

conditions influence pelagic life history (Giménez 2011), while competence influences settlement and recruitment processes (Moksnes 2004, Moksnes & Heck 2006). Moreover, carry-over effects (latent effects originated during embryonic or larval development that manifest during next stages of the life cycle, see the section 1.1.1.3) and benthic conditions influence reproductive success (Pechenik 2006). On the other hand, trophic ecology of benthic marine invertebrates plays a crucial role in each stage of their life cycle, conditioning the population dynamics (Calado and Leal, 2015). For that reason, adult populations can be regulated during each phase of the life cycle. In other words, as different life phases are interlinked, the history of a previous phase can condition the performance of the following. Conditions that favour a successful larval development can at times promote an overcrowding of settlement sites, thus resulting in intense post-settlement interactions with conspecifics and even high mortality of newly settlers (Underwood & Keough 2001). In this regard, a recent work has shown that variation in larval phenotype can be more important than variation in larval supply in the shaping of marine populations (Burgess & Marshall 2011). Overall, despite their contrasting differences in size, morphology, behavior and physiology, pelagic and benthic life stages must be studied simultaneously, as the transition from pelagic to benthic life (through metamorphosis) does not reset larval history (Eckman 1996, Pechenik 2006).

1.1.1.1 LARVAL NUTRITION

According to the "growth-mortality hypotheses" (Anderson 1988), larvae able to successfully metamorphose would be those that are: larger at a given age (the "bigger is better" hypothesis (Miller et al. 1988)), grow faster (the "growth- rate" hypothesis (Bailey & Houde 1989)) and/or spend less time in early larval life (the "stage-duration" hypothesis (Anderson 1988)) (for a review on this topic see Pineda et al. 2007). In this regard, it is legitimate to affirm that larval development is essentially dependent on environmental factors and available nutrients to fuel morphogenesis and growth (Boidron-Métairon 1995, O'Connor et al. 2007). Marine larvae present two different development modes: planktotrophy (with planktonic food being required to advance in development) and lecithotrophy (were development depends on the degradation of internal reserves

provided through maternal provisioning) (Thorson 1950). However, recent studies suggest the existence of intermediate development modes with facultative feeding larvae (for review see Allen & Pernet 2007). Planktotrophs commonly spend more time in the plankton (Olson & Olson 1989), thus increasing their chances for geographic dispersion (Shanks et al. 2003). Nonetheless, this potential usually comes at cost, usually translated in an increase in mortality rates (Morgan 1995a).

Food quality and quantity play a strong influence in larval survival and growth (Rumrill 1990). In the pelagic environment, food quality shows day-to-day shifts. Although larvae are able to feed on an extensive array of particle sizes and food types (e.g., bacteria, detritus, dissolved organic matter, phytoplankton, zooplankton), their nutritional value differs among and within dietary items (Harms et al. 1994, Boidron-Métairon 1995, Perez & Sulkin 2005). Additionally, nutritional requirements change over larval development, thus increasing the chances of larvae facing suboptimal trophic scenarios (Harms et al. 1994). Low food quality, restricted access to food or extended starvation periods generate nutritional stress, increasing larval development time and mortality (Giménez & Anger 2005, D'Urban Jackson et al. 2014). In some species, larvae show developmental plasticity in response to food limitation, increasing the number of larval instars necessary to reach metamorphosis (González-Ortegón & Giménez 2014).

Most marine larvae develop on the continental shelf. Although shelf areas are commonly rich in planktonic food it is commonly distributed in a patchy pattern as a consequence of oceanographic phenomena (Gallager et al. 2004, Genin et al. 2005, Gómez-Gutiérrez et al. 2007). A larval strategy to balance predation risk and food intake is diel vertical migration (Liu et al. 2003). In patchy environments, species that perform diel vertical migrations undergo restricted periods of food availability (dos Santos et al. 2008), as they can only feed during short periods of time (Sulkin et al. 1998). Therefore, larvae can change their behavior to maximize food intake during brief encounters with prey (e.g., reduction in swimming activities or increasing feeding rates) (Calado et al. 2008, Pochelon et al. 2009).

In conclusion, although nutritional stress may not lead directly to the death of the organism, it can induce physiological stress. This stress may extend the duration of larval development and consequently increase the risk of planktonic larvae to be predated (Morgan 1995a) and/or impacting the performance of subsequent phases of their life cycle (see the section 1.1.1.3).

1.1.1.2 PHENOTYPIC PLASTICITY

Phenotypic plasticity is the ability of a single genotype to alter its phenotype in response to temporal or spatial shifts in the environment (DeWitt & Scheiner 2003, West-Eberhard 2005). In complex life cycles, individuals occupy distinct niches at different stages of their life history, which commonly implies the occurrence of dramatic ontogenetic changes in locomotion and feeding modes. Therefore, phenotypes that carry a selective advantage in one life history stage can also suffer a selective disadvantage in a subsequent one (Giménez & Anger 2003). Despite this apparent constraint, the partitioning of life cycles into distinct stages may display some benefits to the species, once larvae and adults perform specialized functions (e.g., dispersal, growth or reproduction) and they can use different resources available in each environment (Rowe & Ludwig 1991, Moran 1994). Nevertheless, life history stages share the same genome, the same tissues and body (although with different forms and plans), which must somehow constraint natural selection (Wray 1995, Aguirre et al. 2014).

Organisms' features are shaped by diverse factors throughout their life cycle. Larvae are highly vulnerable to changes in environmental conditions and, therefore, environmental factors prevailing during pelagic life (e.g., temperature, salinity, food supply) are a source of larval trait variability. Specifically, temperature and food availability play a crucial role in developmental rates at all life stages (Giménez 2010, Giménez 2011, González-Ortegón & Giménez 2014, D'Urban Jackson et al. 2014). Moreover, offspring size also defines a number of those traits, influencing developmental time, survival in the plankton and success at metamorphosis (Marshall & Keough 2006, Moran & McAlister 2009). Since the larval history of a given individual is not completely deleted (if deleted at all) at

metamorphosis (Pechenik et al. 1998, Pechenik 2006), the larval phenotype at settlement should ultimately reflect the pool of effects promoted by larval nutrition, larval duration and maternal provisioning (Giménez & Anger 2003, Marshall & Keough 2009, Giménez 2010). Consequently, phenology affects inter-annual variability in recruitment, as not all larvae settle at an identical physiological condition (Giménez 2010). Furthermore, survival of new settlers is also related with predation and interspecific competition, and this may shift along variable time frames (Rius et al. 2014, Tapia-Lewin & Pardo 2014). Therefore, it may be legitimate to assume that under constant post-settlement conditions the best larval phenotype will give origin to the best post-metamorphic performance and, consequently, to more competitive juveniles (Giménez 2010).

On the other hand, as was explained above, larvae can be a source of connectivity among populations (Shanks et al. 2003). During the planktonic life of pelagic larvae they are exposed to different environmental conditions that can lead to variation in larval phenotypes (Marshall et al. 2010). Consequently, the phenotype of a population is not only the product of the local environment it experiences, it is also the result of larval experience during its dispersal phase (Giménez 2004, Gebauer et al. 2013). Additionally, the phenotype of a given population also reflects the genetic influence of other populations that arrive through larval dispersion (Shanks et al. 2003, Jenkins et al. 2009, Pineda et al. 2009). Larval dispersal from one population to another bears a signature phenotype that is a product of its natal environment and pelagic experience, which has implications in ecological and evolutionary pathways (Marshall & Morgan 2011).

1.1.1.3 CARRY-OVER EFFECTS

Pechenik et al. (1998) changed the way marine ecologists addressed research on complex life cycles when they stated that *"metamorphosis is not a new beginning"* (Pechenik et al. 1998). In this type of marine organisms metamorphosis should be mainly regarded as a transition between pelagic and benthic lifestyles and not as a reset point to previous environmental and trophic experiences. A similar rational may be employed for larval hatching, as the conditions experienced by embryos during incubation may also influence

the fitness of newly hatched and developing planktonic larvae, with larval life also influencing their post-metamorphic performance (Pechenik et al. 1998, Giménez et al. 2004, Pechenik 2006, Giménez 2010). Stressful environments in any life stage can generate phenotypic traits that are only exhibited in later in life. When a trait mediated effect has its origin on embryonic or larval experiences and it is expressed later in juvenile or adult stages it is referred to as a "carry-over" or "latent" effect (Pechenik 2006).

The phenotypic plasticity of the organisms under unfavourable conditions (e.g., extreme environments, nutritional stress or delayed metamorphosis) is a bridge to the next phase and/or a drawback when negative carry-over effects are persistent and/or magnified. The cost of phenotypic plasticity may cause great impact on the success of following life stages. Although internal processes (i.e., physiological and molecular mechanisms) through which carry-over effects are mediated are still poorly studied, their consequences in larval and juvenile life have been addressed by several studies in the latest years (Urzúa & Anger 2012, Smith et al. 2013, Oliphant & Thatje 2013, Allen & Marshall 2013). As carry-over effects influence survival, development and growth rates, the performance of a given phase of a complex life cycle depends not only on present environmental and trophic conditions experienced by that life stage, but also on prevailing conditions experienced during the preceding phases.

Carry-over effects have important consequences at both levels: individual performance and population dynamics (Marshall & Keough 2006, Allen & Marshall 2010, Smith et al. 2013). Survival is affected by environmental conditions experienced during embryonic and larval development (González-Ortegón & Giménez 2014, D'Urban Jackson et al. 2014). Moreover, juveniles displaying a small body size resulting from a prolonged pre-metamorphic life exhibit a weaker competitive ability for space, refuge or food (Hines 1986, Pechenik et al. 1993, Gebauer et al. 1999). Consequently, reduced body mass or size can be carried over through juvenile stages affecting fitness and post-settlement survival (Giménez et al. 2004, Giménez 2010). Furthermore, new settlers are usually susceptible to high predation and their survival can be related to their condition at settlement, as small

juveniles have limited capacity of movement between habitats with different feeding conditions and are more prone to cannibalism (Moksnes et al. 1997, 1998, Luppi et al. 2001, Moksnes 2004). Therefore, the variability in the traits of settlers, lead to variations in settlement success and post-settlement growth and survival (Jarret 2003), and consequently influence population dynamics and community structure.

1.1.2 MATERNAL EFFECTS

Maternal effects have been the subject of intense research in terrestrial ecosystems (Mousseau & Fox 1998). Nevertheless, it has not received the same interest concerning the marine environment (Marshall et al. 2008a). Maternal investment is probably one of the most important sources of variability of offspring phenotypes in marine organisms. The consequences of maternal investment in the life history of marine organisms (Bernardo 1996) and their population dynamics (Saenz-Agudelo et al. 2015) are certainly not negligible. Females are the link between transgenerational effects on phenotypes (Marshall et al. 2008a). The influence of maternal effects can start to take place even before reproduction (e.g., since sexual maturity) and continue in latter adult life (e.g., female nutritional status) (Mousseau & Fox 1998). However, the complex life cycle of marine invertebrates makes it difficult to decouple maternal effects from environmental and trophic effects experienced during larval and young benthic life (Calado and Leal, 2015).

According to Lacey (1998), maternal effects can be described as "*any influence on offspring phenotype that cannot be attributed solely to offspring genotype, to the direct action of the components of the offspring's environment, or to their combination*". This definition of maternal effects will be the one considered in the following sections of the present thesis. Due to the key role played by females in reproduction, namely at oogenesis, maternal effects have been considered as an obvious source of phenotypic variations (Mousseau & Fox 1998, Uller 2008). Nevertheless, despite of father's contribution being usually only genetic, recent studies have demonstrated that males can also play an important role in offspring variability (Ritchie & Marshall 2013, Kindsvater & Alonzo 2014).

1.1.2.1 MATERNAL SIZE AND NUTRITIONAL STATUS

Bernardo (1996) proposed the introduction of maternal phenotypes in the study of offspring fitness, as maternal features are prone to different types of selection. Consequently, maternal effects may exert influence on the offspring even before the beginning of the reproductive process. Certain aspects, such as female size, availability of food or environmental conditions experienced, may affect future offspring. In benthic marine invertebrates, there is still no consensus on the relationship between the status of the female and the fitness of its offspring. This lack of consensus commonly occurs when the link between female size/age and offspring fitness is analysed. For decapod crustaceans, several authors have suggested that old females produce a larger offspring of better quality, once smaller females are forced to partition energy between oocyte production and more frequent moults (Moland et al. 2010, Sato and Suzuki 2010, Wieland and Siegstad 2012). However, some studies did not find a relationship between female size and offspring quality (Koopman & Siders 2013, Swiney et al. 2013). On the other hand, Gardner (1997) showed a decline in the fecundity of larger crabs due to an increase in embryonic size.

In the same way, the nutritional status of the female may exert diverse effects on offspring. While some studies showed that nutritional stress experienced in the maternal environment can reduce offspring fitness (Bayne et al. 1975; Guisande and Harris 1995; Steer et al. 2004; Allen et al. 2009), other studies suggested an opposite trend. Offspring fitness can increasing in response to a decline in the availability of maternal dietary items (Guisande et al. 1996, Allen et al. 2008). In another way, Bertram and Strathmann (1998) suggested that in benthic marine invertebrates with feeding larvae, maternal nutrition is a weak cue to nutrition of offspring, as larvae disperse away from the maternal environment. Therefore, larval intra-specific competition for food is rare in the plankton (Strathmann 1996) and thus high fecundities associated with a good maternal diet is unlike to result in density-dependent competition for food (Bertram & Strathmann 1998).

1.1.2.2 OFFSPRING PROVISIONING AND MATERNAL BEHAVIOUR

Reproduction is the parental contribution to the future generation and one of the biological processes with greater energy expense. Benthic marine invertebrates must allocate sufficient energetic resources during reproduction to ensure that some larvae will survive and successfully metamorphose to in a suitable habitat for juvenile growth, attain sexual maturity and recruit to the adult population (Thorson 1950, Strathmann 1990, 1993). For free-spawning invertebrates, maternal investment for the subsequent generation is mostly provided through oocytes. A high portion of the lipid metabolism of the female is geared to the provision of oocytes with lipids that may fuel embryonic development. In decapod crustaceans, females may transfer as much as 60% of their lipid reserves to oocytes during the oogenesis (Herring 1973). The nutritive material of the oocytes is composed by proteins, lipids and carbohydrates, that will be utilized differently by developing embryos (Jaeckle 1995). Female lipid reserves depend on their diet and, consequently, the lipid profile exhibited by developing embryos are affected by the nutritional status of the mother (Racotta et al. 2003, Smith et al. 2004, Calado et al. 2010). Besides, maternal provisioning can be affected by environmental factors that can act as sources of offspring variability (Moran & McAlister 2009, Urzúa et al. 2011, Rotllant et al. 2014). Juveniles with identical genetic backgrounds can differ dramatically in their chances of survival and reproduction due to differences in the amount of resources that they first received from their mothers at oogenesis (Marshall et al. 2008a). Similarly, the quality of maternal investment in their oocytes, as well as variations in environmental conditions during embryonic development, can affect larval development and survival (Giménez & Anger 2003).

Even after hatching, maternal investment can affect larval survival. Lack of pigmentation has been considered a morphological adaptation to pelagic predation pressure (Anger 2001). Furthermore, larval pigmentation can hide the larvae within their surroundings. During oogenesis, females transfer stored carotenoids from their diet to developing oocytes (Ghidalia 1985). Therefore, an herbivorous diet, rich in carotenoids, can result in

showy larvae (red larvae), more vulnerable to planktivorous organisms, while a predatory diet would produce more cryptic larvae (yellow-green larvae) (Christy 2011).

Furthermore, many benthic marine invertebrates brood their offspring in more or less specialized structures. Brooding species display a form of parental care, which includes the protection of embryos from predators and the generation of local environmental conditions that favour embryonic development. The high level of packing exhibited in some broods can limit oxygen availability in the centre of the mass of embryos (Fernández et al. 2000, 2003, Baeza & Fernández 2002), thus shifting embryonic development and negatively affecting the metabolic pathways that take place during embryogenesis (Chaffee & Strathmann 1984, Lardies & Fernández 2002). In brachyuran crabs, females show a specific behaviour to provide oxygen to developing embryos (e.g., abdominal flapping), which benefits their development and survival (Fernández et al. 2000, Baeza & Fernández 2002, Fernández & Brante 2003). Ovigerous females are capable of modifying their behaviour during the brooding period, namely by increasing the frequency of abdominal flapping throughout embryonic development, which matches an increase in oxygen demand during late stages of embryogenesis (Naylor et al. 1999, Baeza & Fernández 2002). Nevertheless, parental care also involves an energetic cost for brooding females (Fernández et al. 2000, Fernández & Brante 2003). In this case selection should favour females that find a balance between maternal investment and offspring fitness.

1.1.2.3 DISPERSAL ENVIRONMENT AND OFFSPRING PHENOTYPE

Females' environment can condition the phenotypic plasticity of their offspring. Marshall and Uller (2007) suggested that mothers can modify the phenotype of their offspring when maternal environment is a good predictor of offspring environment. In this regard, female environment can play a key role on the tolerance displayed by embryos of some benthic marine invertebrates to certain unfavourable abiotic conditions (e.g., salinity shifts) (Renborg et al. 2014). Moreover, female environment may influence the dispersal ability of their offspring. Krug (1998) showed that mothers in poor-quality environments produce more dispersive offspring than mothers in higher-quality environments.

Certain females may determine the environment in which their embryos develop (e.g., active selection of location for oviposition) and/or the environment in which their larvae are released, thus reducing the negative effects that suboptimal abiotic conditions may have in their offspring. For these reasons larval hatching does not occur at a random time or place. This process is known to be synchronized seasonally and annually with environmental cycles (e.g., light-dark, tidal and lunar cycles), which enhance the chance of larval survival (Morgan & Christy 1994, Morgan 1995b, Zeng & Naylor 1997, Morgan et al. 2011). Most estuarine decapod species follow an export strategy, i.e., their larvae are transported out of the adult environment, develop in coastal marine areas, and reinvade the adult habitat only in the last larval phase (Strathmann 1982). Larvae can perform active vertical migrations in the water column and use tidal currents for early larval seaward transport and later re-immigration (Queiroga et al. 1997, 2006, Anger 2001).

In decapod crustaceans most larvae are planktotrophic (Anger 2001). From a maternal investment perspective, larvae with this mode of development are energetically less expensive to produce, as they must concentrate and clear particles from the plankton in order to complete their development and metamorphosis (Jaeckle 1995). On the other hand, planktotrophs spend more time in the "perilous" plankton relatively to lecithotrophic larvae (Thorson 1950, Vaughn & Allen 2010) which develop by mostly (or even solely) using resources provided by maternal investment (e.g., through egg yolk reserves) (Boidron-Métairon 1995). Although the nutritional status of planktotrophic larvae does not depend directly on the mother, the size of offspring at hatching is strongly determined by maternal investment (Smith & Fretwell 1974). Size conditions the survival and the access to food of newly hatched larvae, with smaller specimens commonly being more vulnerable to starvation (Anger 1987, Strathmann 1987), in contrast to larger larvae (Morgan 1995a, Giménez & Anger 2001, Marshall et al. 2003). In this regard, Marshall et al. (2008b) advocate that under an unpredictable environment selection may favour mothers that produce a range of offspring phenotypes, while under stable environments the mother can bet in a specific phenotype. Particularly, in benthic marine invertebrates with bi-phasic life cycles, mothers may not be able to accurately predict offspring

environment (Marshall & Keough 2006). Therefore, if mothers cannot 'predict' the habitat of their offspring, natural selection should favour mothers that produce a range of offspring phenotypes (e.g., different larval sizes) (McGinley et al. 1987).

1.1.3 EMBRYOGENESIS

The oocyte is, perhaps, the singular most influential cell in the life history of any marine invertebrate (Jaeckle 1995). It provides half of the genetic complement in sexually reproducing species. Moreover, oocyte serves as source of nutritional and structural materials necessary for the embryonic development and to sustain early ontogeny of free-spawning species, until feeding begins. Lipids present in the yolk nourish the embryos, providing the metabolic energy required for their development. Generally, the amount of lipids present in the yolk is correlated with the egg size and the time between spawning and hatching or larval first feeding (Rainuzzo et al. 1997, Moran & McAlister 2009). Therefore, the volume of yolk (egg size) at oviposition is employed as a potential indicator of developmental mode for free-spawning invertebrates. In general, small eggs develop into planktotrophic larvae with a long larval period, while larger eggs commonly develop into lecithotrophic larvae with a short larval duration (Thorson 1950, Thatje et al. 2004, 2005). Nonetheless, larger egg sizes may not always be a good predictor of larval development type. As an example, the clawed lobsters of genus *Homarus* display some of the largest embryos in decapod crustaceans, but their larvae are planktotrophic and not lecithotrophic (Sasaki et al. 1986, Rosa et al. 2007).

The following sections are focused on decapod crustacean embryos, namely on the brachyuran crab *Carcinus maenas*, the model species selected in this thesis.

1.1.3.1 YOLK COMPOSITION

Lipovitellin is the major component of the oocyte yolk of most marine invertebrates (Lee 1991). Although lipovitellin is a lipoprotein with approximately equal amounts of proteins and lipids, the last group represents the most important energy source for developing

crustacean embryos (Herring 1974, Amsler & George 1984). In decapod crustaceans, the reproductive process starts with vitellogenesis, when the female transfers lipids stored in the ovaries to the oocytes. The origin of lipids in the ovary is still not fully known. Lipids may be mobilized from maternal reserves stored in the hepatopancreas (Harrison 1990), directly from the digestion of ingested prey (Clarke 1982) or both. After fertilization, embryos rely on lipovitellins and lipid droplets stored in the yolk, once lipid metabolism independent of egg provision is minimal (Rosa & Nunes 2003). In general, lipovitellin is composed of lipids (48%), proteins (50%) and carbohydrates (2%) (Jaeckle 1995). The major lipid classes are phospholipids (PL) (mostly phosphatidylcholine), along with lesser amounts of triacylglycerols (TAG), cholesterol, and carotenoids (Kerr 1969, Lee 1991).

During embryogenesis the yolk volume decreases while embryonic volume increases. This is due to the increase in permeabilization of the embryonic membrane before hatching, which allows a major water uptake from the environment (Charmantier & Chamantier-Daures 2001). Additionally, this increase in water content may also be driven by the accumulation of metabolic water, a product of respiration. Simultaneously, lipid and protein components are degraded to nourish the embryo and to generate energy, membranes and hormones (Lee & Walker 1995). Shortly before the hatching, residual yolk is limited to bilateral masses in the cephalic region, which is commonly metabolized when newly hatched larvae enter an active feeding state (Walker et al. 2006).

1.1.3.2 LIPID DYNAMICS DURING EMBRYOGENESIS

In decapod crustaceans developing embryos can be staged using a number of scales, such as the one proposed by Rosa et al. (2007): stage 1 – uniform yolk and no embryonic development visible (Fig. 1.2.a); stage 2 - eyes clearly visible with half of the yolk consumed (Fig. 1.2.b); stage 3 – almost no yolk present and embryo fully developed (Fig. 1.2.c). During the transition between newly extruded and ready to hatch embryos, a number of more or less complex biochemical pathways take place, with some lipid species being selectively catabolized or retained (e.g., Sasaki et al. 1986, Rosa et al. 2007). While the lipid profile of newly laid embryos can be used as a proxy for maternal investment,

lipid profiles of embryos about to hatch can be used to somehow predict larval quality and fitness (Wickins et al. 1995, Sulkin & McKeen 1999, Nates & McKenney Jr 2000).

The catabolism of high-energy content of lipids (*ca.* 39 kJ g^{-1}) offers an energetic advantage over proteins and carbohydrates (both *ca.* 17 to 18 kJ g^{-1}) through embryogenesis, thus explaining the key role that these molecules play during this life stage of marine invertebrates. As referred above, the consumption of certain yolk lipids by the embryo promotes more or less dramatic shifts in their biochemical composition (Rosa et al. 2007), which may ultimately impact larval quality and survival (Anger 2001). PL are key components of structural membranes (Kontara et al. 1997, González-Félix et al. 2002) and due to their polarity they are also important emulsifying agents in biological systems and play an active role in lipid transport in developing embryos (Fraser et al. 1988). TAG are considered the most important energy reserves during embryogenesis and early larval development (Palacios et al. 1999). The free fatty acids released during the utilization of TAG can be incorporated into PL and used for morphogenesis and somatic growth.

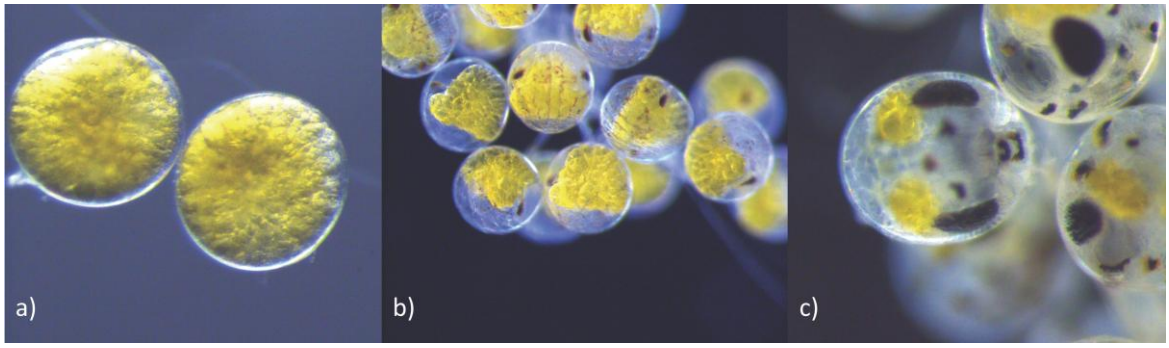

Figure 1.2 Images of *Carcinus maenas* embryos. a) Stage 1 – uniform yolk and no embryonic development visible. b) Stage 2 – visible eyes and half of the yolk consumed. c) Stage 3 – embryos full developed and residual yolk in the cephalic region. Images by the author.

Endogenous lipid reserves are catabolized through embryogenesis in accordance with the genetic program of each species and, therefore, lipid dynamics in decapod crustacean should be summarized in general terms. During embryogenesis, unsaturated fatty acids (UFA) are more readily catabolized than saturated fatty acids (SFA). Within the UFA,

monounsaturated fatty acids (MUFA) are more consumed than polyunsaturated fatty acids (PUFA) (Rosa et al. 2007). Although the catabolism of SFA is an important energy source, its proportion does not change so much during embryogenesis, as SFA are non-essential and can be synthesized *de novo* or obtained by desaturation of MUFA and PUFA. Therefore, SFA can either be selectively retained or used and replaced through *de novo* synthesis (Rosa et al. 2007). Moreover, PUFA play an important role in embryonic development, contributing to the functional maturation of the central nervous system (Bell & Dick 1990). Within the PUFA, there is a special group of fatty acids, highly-unsaturated fatty acid (HUFA), which includes essential fatty acids (fatty acids that cannot be synthesized *de novo* or are biosynthesized but not with an efficiency sufficient to meet the embryos needs (Parrish 2009)). While SFA are expected to be catabolized as energy source, essential fatty acids are retained for growth and development (Wehrtmann & Graeve 1998). Crustaceans have a limited ability to biosynthesize essential fatty acids *de novo* (Cavalli et al. 1999, Suprayudi et al. 2004), therefore their presence in the embryos depends on their intake by the female through dietary items (e.g., 20:4*n*-6 (arachidonic acid, ARA) derivative from the precursor 18:2*n*-6 (linoleic acid) and 22:6*n*-3 (docosahexaenoic acid, DHA) formed from the α-linolenic acid, 18:3*n-3*) (Fig. 1.3). Essential fatty acids as 20:5*n*-3 (eicosapentaenoic acid, EPA) and DHA are required for cell differentiation and membrane formation during embryogenesis (Fischer et al. 2009). Moreover, these fatty acids determine the development of neural system (Beltz et al. 2007), hatchability (Xu et al. 1994) and early larval resistance under adverse environmental conditions (Anger 2001).

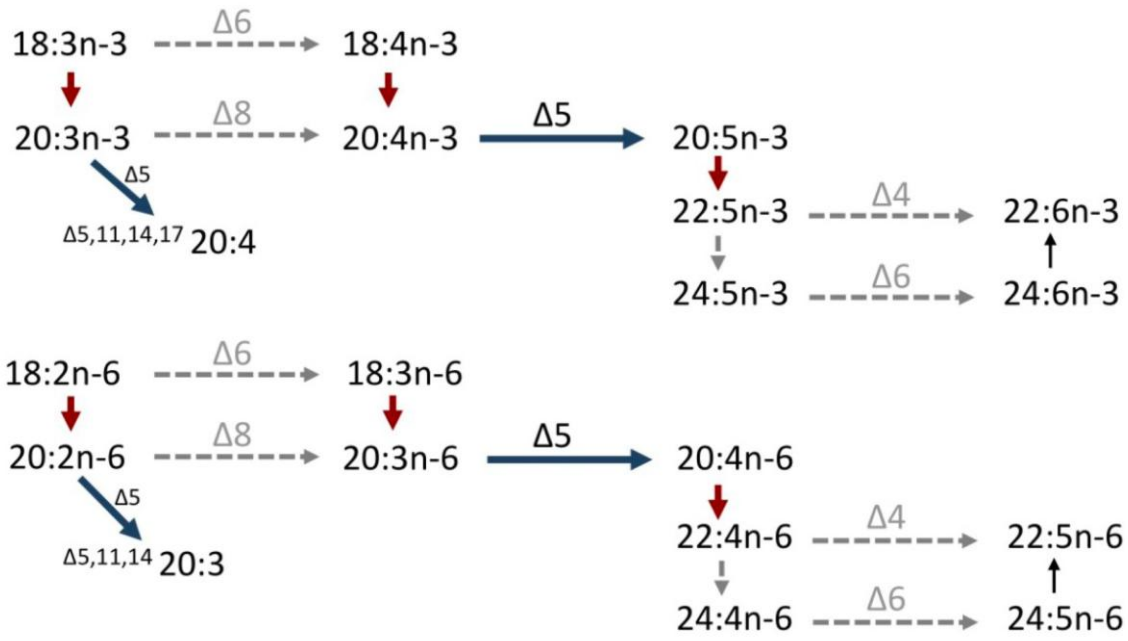

Figure 1.3 Synthetic representations of PUFA biosynthetic pathways in marine invertebrates from C18 PUFA, α-linoleic and linoleic acids. Solid arrows indicate demonstrated activities, whereas dashed arrows show vertebrate-based activities not determined in most of marine invertebrates. Horizontal dark blue arrows are desaturation reactions and red arrows are elongation reactions. Source: Monroig et al. 2013

1.1.3.3 BROODING CONDITIONS

Females of decapod crustaceans extrude their embryos and carry them under the abdomen until hatching (with the exception of the Dendrobranchiata). In family Portunidea, embryos are attached to the female abdomen through funiculus, which wrap around the setae of pleopods (Goudeau 1983) (Fig. 1.4). Embryos of *Carcinus maenas* are surrounded by a trichromatic membrane (Cheung 1966). The superficial layer corresponds to the vitelline envelope, which is already detectable at the ovarian level in oocytes, while the internal part is deposited at embryogenesis (Goudeau & Lachaise 1980).

During the incubation process, the environmental conditions within the brooding chamber can affect embryonic quality (as well as that of newly hatched larvae) (Moran & McAlister

2009). Embryonic metabolic rates increase directly with temperature, decreasing the duration of embryonic development (Wear 1974) and enhancing the utilization rates of endogenous reserves (García-Guerrero et al. 2003). The prevailing salinity in the maternal habitat during incubation is another source of variability that may condition embryonic metabolism during embryogenesis (Giménez & Anger 2003). Differences in salinity experienced by inner and outer embryos of broods can generate differences in osmolarity. Therefore, at lower salinities embryos use more resources during their development to compensate for such osmotic effect, which increases the duration of embryonic development (Giménez & Anger 2001). Moreover, low salinities during the pre-hatching period reduces the concentration of osmotically active organic molecules inside the embryos, generating newly hatched larvae with a lower dry weigh (Laughlin & French 1989).

Furthermore, positioning of extruded embryos at oviposition along the abdominal pleopods in females brooding chamber can also be a source of offspring variability (Pochelon et al. 2011, Leal et al. 2013). Embryos in the center of the brooding mass are commonly exposed to lower concentrations of oxygen than those in the periphery. Contrasting oxygen levels can cause variations in embryonic metabolic rates (Brante et al. 2003), which can lead to asynchronous larval hatching (Fernández et al. 2003).

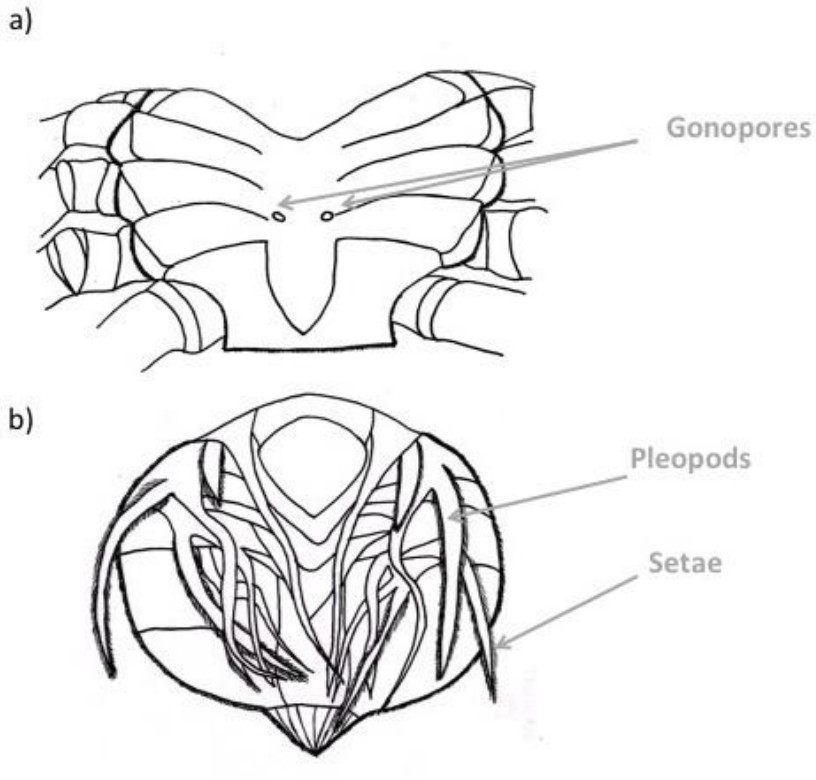

Figure 1.4 Embryo attachment system in brachyuran crabs. Diagram of the brooding chamber of a female: a) thorax, b) abdomen. After release from the gonopores, the embryos attach to the long setae of the pleopods where they are ventilated by rhythmic movements of the abdomen and the pleopods; the frequency of these movements increases along embryogenesis. Illustrations by the author.

1.1.4 THE GREEN CRAB CARCINUS MAENAS *AS A MODEL SPECIES*

The green crab *Carcinus maenas* (L.) is the most common intertidal decapod crustacean of European estuaries and coastal waters (from shore pools up to a depth of 60 m). Its native range extends from 70° N in Norway to 22° N in Mauritania, including Iceland, the Faroe Islands, the British Islands and the western part of the Baltic Sea (Crothers 1968, D'Udekem d'Acoz 1999). The wide environmental tolerance, high fecundity and long larval development make this species one of the most successful coastal marine invaders in the word (Yamada 2001, Darling et al. 2008). Currently, it is present on the temperate shores of every continent (e.g., Argentina, Australia, Canada, Japan, South Africa, United States)

("Global Invasive Species Database" 2009). The dispersal rate of *C. maenas* ranges from 63 to 173 km/yr along the east and west coasts, respectively, of North America (Grosholz & Ruiz 1995, 1996, Miller 1996, Chew 1998). It has been reported to be responsible for significant impacts on molluscs and other crustaceans, through predation, competition and burrowing activities (Bravo et al. 2007).

Reproduction in *C. maenas* starts after the moulting event (the shedding of the old exoskeleton – the exuviae) of females. The mating involves the formation of pairs of soft-shelled females with hard-shelled males (Hayden et al. 2007). Males reach sexual maturity at a carapace width of 25-30 mm and females at 15-31 mm (Crothers 1967). It is an iteroparous species (species that reproduces more than once in a lifetime) with internal fecundation. Ovarian development occurs biannually, with two well-marked breeding seasons, one in spring and the second in the autumn (Broekhuysen 1936, Lyons et al. 2012). During each spawning event, a female may produce up to 200 000 oocytes per brood (Broekhuysen 1936), which, after fertilization, are carried attached to the pleopods for a variable period of time (being directly correlated with environmental temperature of the maternal habitat). At temperate regions, the average duration may range from 6 to 11 weeks (Wear 1974, Hartnoll & Paul 1982). Larval hatching occurs asynchronously, as ovigerous females of *C. maenas* release their larvae during two or more consecutive events (over a period of up to 72 h) (Zeng and Naylor 1997). Larvae hatch inside estuaries during night ebbing tides and are quickly flushed to the shelf (Queiroga et al. 2002), where larval development takes place. The larval life cycle includes four planktotrophic zoeae and one megalopa (Rice & Ingle 1975). The larvae spend four to six weeks on an oceanic habitat, depending on water temperature (Dawirs 1985, Mohamedeen & Hartnoll 1989, Nagaraj 1993), before returning to the estuary to metamorphose and settle, shifting from a pelagic to a benthic lifestyle.

Several reasons led to selection of *C. maenas* as the biological model to perform the present thesis: i) high local abundance, thus being easy to collect from the wild; ii) suitability for laboratory studies; and iii) significant scientific information already available

on its life cycle, behaviour and larval ecology. Its extended reproductive period (Queiroga 1995), high fecundity and in depth knowledge of its reproductive system (Goudeau 1982a, b, 1983, Lyons et al. 2012) allowed the use of *C. maenas* as model species to study maternal investment of a benthic marine invertebrate with a bi-phasic life cycle. On the other hand, previous studies of *C. maenas* in Ria de Aveiro (Queiroga et al. 2006, Domingues et al. 2011) showed that during the settlement period sufficient numbers of larvae can be collected from the plankton during supply events to perform laboratory experiments using wild larvae, thus allowing a more in depth knowledge of its larval ecology and the consequences of embryonic and larval life on its post-metamorphic performance. Despite being a well-studied species several aspects of its life history still remain unclear, including lipids dynamics during embryogenesis, variability in maternal investment and how variable oceanographic conditions may affect larval quality at metamorphosis and during early benthic life.

1.1.5 STUDY AREA: RIA DE AVEIRO

Ria de Aveiro (Fig. 1.5) is a shallow coastal lagoon in the western margin of mainland Portugal (40° 37' 17'' N, 8° 44' 56'' W). The estuarine system is formed by four main channels (Mira, 25 km; Ílhavo, 15 km; Espinheiro, 17 km and São Jacinto, 29 km long) and several branches and interconnections, which increases the complexity in this system (Dias et al. 1999). The coastal lagoon is very shallow, with an average depth of only 1 m (Rodrigues et al. 2009) and maximum depths of about 20 m near the inlet. An artifical channel in the sandbar of about 1.3 km connects the lagoon to the sea (Fig. 1.4). The lagoon circulation is mainly influenced by tides, with a semidiurnal tidal regime of *ca.* 12.42h (Pereira et al. 2000) and a tidal range of 2 m at the mouth (minimum: 0.6 m in neap tides; maximum: 3.2 m in spring tides) (Dias et al. 2000). Besides tides, river flows also influence the physical dynamics of Ria de Aveiro, affecting salinity and water temperature (Dias et al. 2000). Freshwater contributions come essentially from two rivers: Vouga and Antuã (Dias et al. 1999, Dias & Lopes 2006).

Ria de Aveiro, as well as the north-western coast of the Iberian Peninsula, is affected by seasonal upwelling (Fiuza et al. 1998). In this area, upwelling events are commonly explained through the action of northerly winds along the shelf that generate an Ekman drift directed offshore. Consequently, cooler and nutrient-rich water from depth emerges to the surface and exerts a fertilization effect on coastal waters, thus promoting primary production and fuelling marine food chains (Guisande et al. 2001, Huthnance et al. 2002, Santos et al. 2004). Upwelling events are frequent phenomena during spring-summer months, overlapping in time with the breeding season of many marine organisms. This nutrient-rich water mass, along with dissolved CO_2 and solar energy, promotes the occurrence of high levels of primary production which supports a high biological diversity in this geographic region (Santos et al. 2004, Otero et al. 2009, Alvarez et al. 2013).

Figure 1.5 Study area Ria de Aveiro, NW Portugal. Image courtesy of Fernando Ricardo.

1.2 GENERAL OBJECTIVES AND THESIS OUTLINE

The general aims of this thesis were to better understand the maternal effects and post-settlement performance of marine benthic invertebrates with a complex life cycle, using *Carcinus maenas* as a model. Maternal investment, embryogenesis, megalopa competence, larval quality, early benthic performance and carry-over effect were investigated. More specifically, the objectives of the present thesis were to:

- Investigate the existence of within-brood variation in the fatty acid profiles of *C. maenas* during embryonic development.
- Describe the interannual variability in maternal provisioning and embryogenesis in *C. maenas*.
- Describe the variability in maternal provisioning and embryonic catabolism during the reproductive season of *C. maenas*.
- Document the effect of female size in maternal provisioning and embryogenesis in *C. maenas*.
- Unravel the polar lipid dynamics during the incubation period of embryos of two sympatric brachyuran crabs: *C. maenas* and *Necora puber*.
- Evaluate larval quality, megalopa competence and juvenile performance of *C. maenas* during consecutive settlement events.
- Compare the juvenile performance of *C. maenas* juveniles originating from megalopa exposed to suboptimal abiotic conditions, namely low salinity.

This thesis is divided in four parts: I) General Introduction, II) Maternal Provisioning, III) Settlement Dynamics and Post-settlement Performance and IV) Concluding Remarks. Parts II and III are composed of different chapters with specific and well defined objectives, organized in individual sections (Abstract, Introduction, Materials and Methods, Results, Discussion and References).

Part I General Introduction

See above for the contents of this Part of the thesis.

Part II Maternal Provisioning – A Biochemical Perspective

This Part explores the consequences of maternal effects from two complementary points of view: maternal investment in offspring and resource partitioning during the embryogenesis of *C. maenas* (and also *N. puber*, Chapter 2.4). Embryos were analysed employing a lipidomic approach. This Part is divided into four chapters, each one corresponding to a study with specific objectives.

Chapter 2.1 analyses the fatty acid profile of different areas of the brooding chamber during the embryogenesis of *C. maenas*. The embryonic mass was divided in two sides (right and left) and each side in two regions (external and internal). A total of four areas were analysed to evaluate if embryos position within the brooding chamber can act as a source of offspring variability. Two embryonic stages were analysed: stage 1, as a proxy for maternal investment and stage 3, as a proxy for the quality of newly hatched larvae. This chapter corresponds to a manuscript submitted to the journal Biological Bulletin.

√ Felisa Rey, Ana S. P. Moreira, Fernando Ricardo, Manuel A. Coimbra, M. Rosário M. Domingues, Pedro Domingues, Rui Rosa, Henrique Queiroga, Ricardo Calado. *Fatty acids of highly-packed embryos of* Carcinus maenas *reveal homogenous maternal provisioning and no within-brood variation at hatching.*

Chapter 2.2 investigates the interannual variability in maternal provisioning and incubation process of *C. maenas*. Maternal environmental conditions and fatty acid profiles of embryos from two consecutive years were analysed to better understand offspring variability. This chapter corresponds to a manuscript in preparation.

√ Felisa Rey, M. Rosário M. Domingues, Pedro Domingues, Rui Rosa, María D. M. Orgaz, Henrique Queiroga, Ricardo Calado (in preparation). *Biochemical profile of* Carcinus maenas *embryos is unaffected by female size but varies significantly over consecutive years in a coastal lagoon.*

Chapter 2.3 investigates maternal effects on offspring of *C. maenas* by examining how maternal size and available nutritional resources during the reproductive season can influence its offspring. The fatty acid profiles of embryos from small and large females were analysed at the beginning and at the end of the reproductive season, in two important stages of embryogenesis: stage 1 (newly extruded embryos) and stage 3 (embryos ready to hatch). This chapter corresponds to a manuscript in preparation.

√ Felisa Rey, M. Rosário M. Domingues, Pedro Domingues, Rui Rosa, Henrique Queiroga, Ricardo Calado (in preparation). *No matter if you are big or small, fatty acid profiles reveal that late breeding females of* Carcinus maenas *display a higher maternal provisioning.*

Chapter 2.4 describes the embryonic development of two phylogenetically close (both are members of family Portunidae) and sympatric brachyuran crab species, *C. maenas* and *N. puber*, using a lipidomic approach. This study unravels the polar lipid dynamics during embryogenesis at a resolution level never reached before and shows how two phylogenetically close species that have access to similar dietary items display contrasting lipid dynamics during embryogenesis. This chapter corresponds to a manuscript already published in *Scientific Reports*.

√ Felisa Rey, Eliana Alves, Tânia Melo, Pedro Domingues, Henrique Queiroga, Rui Rosa, M. Rosário M. Domingues, Ricardo Calado (2015). *Unravelling polar lipids dynamics during embryonic development of two sympatric brachyuran crabs (*Carcinus maenas *and* Necora puber*) using lipidomics*. *Scientific Reports* 5:14549 doi: 10.1038/srep14549

Part III Settlement Dynamics and Post-settlement Performance

This Part explores the early benthic performance of *C. maenas*. Wild megalopae collected in Ria de Aveiro were used in laboratory experiments to understand how their pelagic life

history affects their early benthic life. This Part is divided into two chapters, each one corresponding to a study with specific objectives.

Chapter 3.1 evaluates larval competence and juvenile performance of *C. maenas* during consecutive settlement events. Following larval collection in Ria de Aveiro and metamorphosis of megalopa in the laboratory, the first juvenile crab instars experienced different feeding scenarios to evaluate their performance. This chapter corresponds to a manuscript accepted to publish in Marine Ecology Progress Series.

√ Felisa Rey, Gina M. Silva Neto, Cláudio Brandão, Daniela Ramos, Bruna Silva, Rui Rosa, Henrique Queiroga, Ricardo Calado. *Contrasting benthic performance over consecutive larval supply events of a marine invertebrate with bi-phasic life. Marine Ecology Progress Series* doi: 10.3354/meps11629

Chapter 3.2 examines the post-settlement performance of *C. maenas* juveniles after exposure of megalopa to low salinities. The laboratory experiment was performed with wild megalopae collected from Ria de Aveiro. This study corresponds to a manuscript already published in *Estuarine, Coastal and Shelf Science*.

√ Felisa Rey, Gina M. Silva Neto, Rui Rosa, Henrique Queiroga, Ricardo Calado (2015). *Laboratory trials reveal that exposure to extreme raining events prior to metamorphosis affects the post-settlement performance of an estuarine crab. Estuarine Coastal and Shelf Science* 154:179–183
doi: 10.1016/j.ecss.2015.01.005

Part IV Concluding Remarks and Future Studies

This chapter summarises the main findings of the individual chapters in Part II and III. It highlights the significance of these findings for a better understanding of the reproduction, embryonic development and early benthic performance of a marine invertebrate with a bi-

phasic life cycle. This Part also points out future directions on the study of maternal effects and settlement dynamics.

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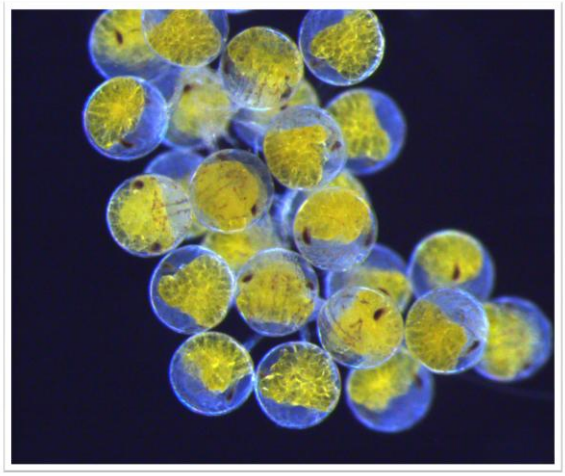

PART II

MATERNAL PROVISIONING: A BIOCHEMICAL PERSPECTIVE

2.1. FATTY ACIDS OF HIGHLY-PACKED EMBRYOS OF CARCINUS MAENAS *REVEAL HOMOGENOUS MATERNAL PROVISIONING AND NO WITHIN-BROOD VARIATION AT HATCHING*

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2.1.1 ABSTRACT

The embryonic development of decapod crustaceans relies on the use of yolk reserves supplied to offspring through maternal provisioning. An unequal partitioning of nutritional reserves during oogenesis, as well as the experience of contrasting environmental conditions during incubation, can be a source of within-brood variability. Ultimately, this potential variability may promote the occurrence of newly hatched larvae with contrasting yolk reserves and an unequal ability to endure starvation and/or suboptimal feeding during their early pelagic life. The present study evaluated maternal provisioning through the analysis of fatty acid (FA) profiles in newly extruded embryos of *Carcinus maenas*, as well as the dynamics of such provisioning during embryogenesis, taking into account the position occupied by developing embryos within the brooding chamber (left external, left internal, right external and right internal). The FA profiles surveyed during the present work revealed a uniform transfer of maternal reserves from the female to the whole mass of embryos and a homogeneous embryonic development within the brooding chamber. Although *C. maenas* presents a highly packed mass of embryos that is unequally distributed within its brooding chamber, this feature is not a source of within-brood variability during incubation. This finding contrasts with data already recorded for larger sized brachyuran crabs and allows us to infer that maternal behaviour of *C. maenas* promotes a homogeneous lipid catabolism along embryogenesis.

2.1.2 INTRODUCTION

Decapod crustaceans have become popular model organisms to address important topics in marine ecology, such as those related with parental care (Fernández et al. 2000). Females of the majority of these species (the main exception being the Dendrobranchiata) brood their embryos in their abdomen for variable periods of time, thus generating a favourable environment to their offspring (at least until hatching). This form of parental care is known to present significant energetic costs due to the active brooding behaviour displayed by ovigerous females (Fernández et al. 2000; Fernández and Brante 2003).

As embryonic development in decapod crustaceans is lecitothrophic, the quantitative and qualitative transfer of nutrients during oogenesis, from the female to what will later be the yolk reserves of developing embryos, plays a key role in embryogenesis (Rosa et al. 2007). Maternal provisioning to developing embryos has been extensively investigated in brachyuran crabs, namely through biochemical analysis of female gonads and newly extruded embryos (e.g., Rosa and Nunes 2003; Smith et al. 2004; Rosa et al. 2005; Wu et al. 2007; Li et al. 2012). Nonetheless, to date, no study has ever attempted to determine if maternal provisioning in brachyuran crabs may eventually be a source of offspring variability, namely in species brooding large numbers of embryos. In other words, do females provide all embryos within a brood with comparable yolk reserves?

Another potential source of offspring variability, already highlighted for brachyuran crabs, is associated with the supply of oxygen to developing embryos during their incubation in the brooding chamber of females. Indeed, embryos in the periphery of the brood are likely to be more easily oxygenated than those located at inner regions (Fernández et al. 2003; Fernández and Brante 2003). Brachyuran crabs commonly display highly packed embryos, but unlike other benthic marine invertebrates (e.g., opisthobranchs) do not display any gel covering them. This gel enhances the passive provision of oxygen to embryos located in the inner regions of the brood (Strathmann & Strathmann 1995, Lee & Strathmann 1998). In this way, while suitable levels of oxygenation may be difficult to achieve in inner embryos of highly packed broods, female brachyuran crabs have evolved active brooding behaviours (e.g., abdominal flapping) that assure a suitable supply of oxygen to the whole brood (Baeza & Fernández 2002).

Oxygen limitation may delay embryonic development (Hartnoll and Paul 1982; Cohen and Strathmann 1996; Brante et al. 2003; Fernández et al. 2003) with hypoxia-induced metabolic suppression decreasing the potential for synthesis (Zhou et al. 2001; Hochachka and Somero 2002; Alter et al. 2015). In this way, if embryos located in the inner regions are not supplied with similar levels of oxygen to the ones in the outer regions, their energetic reserves will be catabolized at a slower rate (Chaffee and Strathmann 1984; Fernández et al. 2003). Under this scenario, significant shifts are

expected to occur in the biochemical profile of inner and outer embryos of brachyuran crabs along the incubation period.

In the present study, we used the fatty acid (FA) profile of *Carcinus maenas* embryos as a biochemical proxy to evaluate if maternal provisioning during oogenesis was homogenous and the whole mass of newly extruded embryos displays a similar FA profile. We also used the same biochemical proxy to evaluate if at the end of the incubation period the location of developing embryos within the brooding chamber may act as a source of within-brood variability (e.g., condition the catabolism of FAs during embryogenesis). The following null hypotheses were tested: 1) maternal provisioning during oogenesis in *C. maenas* is homogenous and the whole mass of newly extruded embryos displays a similar FA profile; and 2) the location of developing embryos within the brooding chamber of females does not condition their FA profile by the end of embryogenesis (prior to hatching). Numerous studies have already addressed the biochemical dynamics of FA recorded during embryogenesis in brachyuran crabs (e.g., Rosa et al. 2005; Rosa et al. 2007; Li et al. 2012). In this way, the shifts in the pool of FAs present in early and late stage embryos are not discussed in the present study.

2.1.3 MATERIALS AND METHODS

2.1.3.1 SAMPLING

Ovigerous females of *C. maenas* were collected between March and April 2012 in Canal de Mira, Ria de Aveiro (40° 37’ 17’’ N, 8° 44’ 56’’ W), a coastal lagoon in the Northwestern coast of Portugal. Females were transported to the laboratory and sorted according to their carapace width (CW) (measured between the first pair of lateral spines) and the development stage of their embryos. Developing embryos were classified according to the following criteria (Rosa et al. 2007): stage 1 (newly extruded embryos) - uniform yolk and absence of cleavage and eyes; stage 3 (embryos ready to hatch in < 48 h) nearly no yolk present and embryo fully developed. Ten females with an average CW of 44.1 ± 0.7 mm were haphazardly selected, five brooding embryos in stage 1 and five brooding embryos in stage 3. The brooding chamber of each female was divided in two sides, left (L) and right (R), and each side in two regions, external (E) (where embryos are in the

periphery of the egg mass - outer embryos) and internal (I) (where embryos are located in the inner region of the egg mass - inner embryos), resulting in a total of four areas: left external (LE), left internal (LI), right external (RE), and right internal (RI). The embryos from each area were carefully removed with a pair of forceps. Overall, a total of 4 areas within the brooding chamber x 2 embryonic stages x 5 females = 40 samples of embryos collected for analysis. Immediately after collection 30 embryos from each sample were measured using a stereomicroscope and their volume determined using the formula $V=4/3(r^3)$ for spheroid embryos. All embryos samples were freeze-dried and stored at -32 °C for later biochemical analysis.

2.1.3.2 FATTY ACID ANALYSIS

Total lipids extracts were obtained using the Bligh and Dyer (1959) method. Briefly, freeze-dried embryos were resuspended in 1 mL of ultrapure water and 3.75 mL chloroform/methanol 1:2 (v/v) was added to the suspension that was vortexed and incubated on ice for 30 min. An additional volume of 1.25 mL chloroform was added along with 1.25 mL of ultrapure water. Following vigorous vortexing, samples were centrifuged at 1000 rpm for 5 min at room temperature to obtain a two-phase solution: an aqueous top phase and an organic bottom phase, from which lipids were retrieved. Lipid extracts were dried with a nitrogen flow and stored at -32 °C for posterior analysis.

In order to quantify the total amount of phospholipids (PL), a phosphorus assay was performed according to Bartlett and Lewis (1970). To quantify the total PL extract, 5% of the sample volume was used, and dried with a nitrogen flow. Perchloric acid (70%) was added to the samples, which were then incubated for 1 h at 180 °C. After incubation, 3.3 mL of water, 0.5 mL of ammonium molybdate (2.5%, m/V) and 0.5 mL of ascorbic acid (10%, m/v) were added to each sample, followed by incubation during 10 min at 100 °C in a water bath. Standards from 0.1 to 3.0 µg of phosphate underwent the same treatment as the samples. Absorbance of the mixtures was measured at 800 nm, at room temperature, in a microplate UV-vis spectrophotometer.

FAs were analysed by gas chromatography-mass spectrometry (GC-MS) after transesterification of embryos' total lipid extracts (30 µg of total PL). FA methyl esters (FAMEs) were prepared using a methanolic solution of potassium hydroxide (2 M) according to the previously described method by Aued-Pimentel et al. (2004). The FAMEs were resuspended in 40 µL of hexane, with 2 µL of this hexane solution being used for GC-MS analysis on an Agilent Technologies 6890N Network (Santa Clara, CA) equipped with a DB-1 column with 30 m of length, 0.25 mm of internal diameter, and 0.1 µm of film thickness (J&W Scientific, Folsom, CA). The GC-MS was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range *m/z* 40-500 in a 1 s cycle in a full scan acquisition mode. The oven temperature was programmed from an initial temperature of 40 °C, standing at this temperature for 0.5 min and following a linear increase to 220 °C at 20 °C/min, a linear increase at 2 °C/min to 240 °C, and 5 °C/min until reaching 250 °C. The injector was set at 220 °C and the detector at 230 °C. Helium was used as the carrier gas at a flow rate of 1.7 mL/min. The identification of FAMEs was performed by comparing the retention time and mass spectrum of each FAMEs relative to 34 mixed FAMEs standards (C6 – C24, Supelco 37 Component FAMEs Mix) and confirmed by comparison with the chemical database Wiley and the spectral library "The AOCS Lipid Library" (Christle 2012).

2.1.3.3 DATA ANALYSIS

After checking for assumptions (using Shapiro-Wilks and Levene's test to check for normality and homogeneity of variance, respectively), we tested for significant differences in the volume of stage 1 and stage 3 embryos using a two-way ANOVA with the side of the brooding chamber where the embryo was located (with two levels: right and left) and the location of the embryo within the brood (with two levels: inner and outer region of the brood) being used as categorical factors. The two-way ANOVAs were performed using STATISTICA v8 (StatSoft Inc., USA).

The relative content of each FA was expressed as the percentage of the total pool of FAs. For statistical analysis, only FAs representing more than 1% of the total pool of FAs were considered. For a better understanding of our results, only the classes of FAs containing

the most abundant FAs were considered, namely: Saturated FA (SFA), Monounsaturated FA (MUFA), Polyunsaturated FA (PUFA), Highly-unsaturated FA (HUFA), Epoxy FA (EpFA) and Branched FA (BrFA)). While Polyunsaturated FA are commonly defined as all FAs with ≥ 2 double bonds, in the present study we discriminate between PUFA (FAs with 2 or 3 double bonds) and HUFA (FAs with ≥ 4 double bonds). Multivariate statistical analyses were performed to detect the existence of significant differences in the: 1) FA profile of stage 1 embryos; 2) FA class profile of stage 1 embryos; 3) FA profile of stage 3 embryos; and 4) FA class profile of stage 3 embryos from the four different areas of the brooding chamber (LE, LI, RE and RI). Prior to the statistical analysis, in order to down-weight the contributions of quantitatively dominant FA, the raw data matrix was log (x +1) transformed. Following this transformation, a new matrix was assembled using Bray-Curtis similarity coefficient. Two-way crossed analyses of similarities (ANOSIM) were used to statistically test for significant differences between the percentage of individual FAs and FA classes in stage 1 and 3 embryos located in the four areas of the brooding chamber (LE, LI, RE and RI). The factors used in the ANOSIM analysis were side of the brooding chamber (with two levels: left and right) and region of the brooding chamber (with two levels: external and internal). ANOSIM calculates a global R statistic that assesses the differences in variability between groups, as compared to within groups, and checks for the significance of R using permutation tests. All multivariate statistical tests were performed with Primer 6.1 with PERMANOVA add-on (Primer-E Ltd. Plymouth, UK).

2.1.4 RESULTS

While the volume of embryos of *C. maenas* doubled during incubation, no significant differences were detected in the average volume of stage 1 or stage 3 embryos brooded on each region of the brooding chamber (LE, LI, RE and RI) (P = 0.652 and P = 0.845; V = 0.013 ± 0.001 mm^3 and V = 0.026 ± 0.002 mm^3, for stage 1 and stage 3 embryos, respectively).

The most relevant FAs recorded from each area of the brooding chamber, as well as for the whole mass of embryos, are summarized in Tables 2.1.1 and 2.1.2 (embryos in stage 1 and 3, respectively). The five most abundant FAs, representing > 50% of the total pool of

FAs recorded, were the same in embryos in stage 1 and 3 (Fig. 2.1.1), namely 16:0 (palmitic acid, PA), 16:1*n*-7 (palmitoleic acid), 18:1*n*-9 cis (oleic acid), 20:5*n*-3 (EPA) and 22:6*n*-3 (DHA).

In stage 1 embryos, the most abundant FA class was MUFA (representing around 40% of the total pool of FAs) (22.82 ± 1.92% for SFA, 40.09 ± 3.97% for MUFA and 22.77 ± 3.34% for HUFA) (Table 2.1.1). On the other hand, in stage 3 embryos the contribution of MUFA decreased, reaching values more similar to those of SFA and HUFA (27.60 ± 2.87% for SFA, 32.44 ± 2.50% for MUFA and 29.14 ± 3.92% for HUFA) (Table 2.1.2).

The ANOSIM analysis of embryos in stage 1 showed no significant effect of the factor side or region in FA profiles (Side: R = - 0.170, P = 0.969; Region: R = - 0.182, P = 0.969) and FA classes (Side: R = - 0.096, P = 0.837; Region: R = - 0.170, P = 0.969). Furthermore, the same analysis in embryos in stage 3 did not reveal the existence of any significant effect of factor side or region in FA profiles (Side: R = - 0.156, P = 0.955; Region: R = - 0.128, P = 0.948) and FA classes (Side: R = - 0.176, P = 0.988; Region: R = - 0.142, P = 0.942).

Table 2.1.1 Fatty acid (FA) composition (expressed as % of total pool of FAs) of stage 1 embryos of the green crab *Carcinus maenas* from four different areas within the brooding chamber (left external (LE), left internal (LI), right external (RE), right internal (RI)) and average (± SD) of total embryonic mass. Values are averages (± SD) of embryos from five different females (*n*=5).

Fatty Acid	Left External LE	Left Internal LI	Right External RE	Right Internal RI	Embryonic mass
14:0	0.96 ± 0.22	0.84 ± 0.08	1.05 ± 0.11	1.04 ± 0.22	0.97 ± 0.18
16:0	15.94 ± 0.53	15.05 ± 0.78	15.66 ± 1.72	16.53 ± 1.71	15.75 ± 1.31
18:0	4.16 ± 0.63	3.96 ± 0.33	4.07 ± 0.71	4.03 ± 0.49	4.04 ± 0.51
Σ SFA[a]	**23.09 ± 0.99**	**21.82 ± 0.56**	**22.84 ± 2.50**	**23.76 ± 2.77**	**22.82 ± 1.92**
16:1*n*-7	15.87 ± 2.65	15.01 ± 2.39	15.79 ± 2.36	16.46 ± 2.51	15.77 ± 2.34
18:1*n*-9*c*	11.21 ± 1.29	10.95 ± 1.60	11.06 ± 1.69	10.89 ± 1.82	11.00 ± 1.48
18:1*n*-7	4.75 ± 0.54	4.65 ± 0.77	4.50 ± 0.60	4.95 ± 0.58	4.69 ± 0.60
20:1*n*-9	2.14 ± 1.04	2.19 ± 1.15	2.21 ± 1.21	2.08 ± 1.06	2.15 ± 1.03
20:1*n*-7	3.26 ± 0.97	3.33 ± 1.04	3.25 ± 1.09	3.13 ± 1.00	3.24 ± 0.94
Σ MUFA[b]	**40.17 ± 4.77**	**39.71 ± 4.30**	**40.12 ± 3.94**	**40.69 ± 4.19**	**40.09 ± 3.97**
18:2*n*-6	0.84 ± 0.31	0.81 ± 0.27	0.67 ± 0.32	0.85 ± 0.38	0.78 ± 0.30
18:3*n*-3	1.13 ± 0.14	1.12 ± 0.18	1.19 ± 0.16	1.17 ± 0.17	1.15 ± 0.16
Σ PUFA[c]	**6.08 ± 1.92**	**6.63 ± 1.67**	**6.41 ± 1.24**	**6.01 ± 1.47**	**6.35 ± 1.48**
20:4*n*-6	2.05 ± 0.29	2.13 ± 0.30	2.80 ± 1.47	2.22 ± 0.48	2.30 ± 0.79
20:5*n*-3	8.46 ± 1.88	8.64 ± 1.72	8.02 ± 2.10	8.22 ± 1.43	8.38 ± 1.67
22:5*n*-3	2.10 ± 0.18	2.32 ± 0.49	2.07 ± 0.22	1.86 ± 0.24	2.09 ± 0.33
22:6*n*-3	8.34 ± 3.38	8.79 ± 3.18	8.15 ± 3.30	7.96 ± 3.81	8.30 ± 3.16
Σ HUFA[d]	**22.66 ± 3.60**	**23.80 ± 3.05**	**22.72 ± 3.33**	**21.79 ± 4.14**	**22.77 ± 3.34**
BrFA 1	1.95 ± 0.32	1.86 ± 0.20	1.89 ± 0.22	1.97 ± 0.23	1.92 ± 0.23
BrFA 2	1.57 ± 0.17	1.50 ± 0.16	1.54 ± 0.16	1.62 ± 0.24	1.56 ± 0.18
Σ BrFA[e]	**5.68 ± 0.52**	**5.29 ± 0.35**	**5.47 ± 0.84**	**5.58 ± 1.32**	**5.53 ± 0.80**
EpFa 1	1.52 ± 0.41	1.81 ± 0.39	1.58 ± 0.4	1.43 ± 0.50	1.60 ± 0.42
Σ EpFA[f]	**1.74 ± 0.37**	**2.04 ± 0.35**	**1.82 ± 0.42**	**1.66 ± 0.46**	**1.83 ± 0.40**

Abbrevations: Saturated FA (SFA), Monounsaturated FA (MUFA), Polyunsaturated FA (PUFA), Highly-unsaturated FA (HUFA), Branched FA (BrFA) and Epoxy FA (EpFA) represent the sum of all FAs identified as can see below:

[a] SFA: 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0, 22:0

[b] MUFA: 14:1*n*-5, 15:1*n*-1, 16:1*n*-7, 16:1*n*-5, 7-methyl-hexadec-6-enoate, 17:1*n*-9, 17:1*n*-8, 18:1*n*-9*c*, 18:1*n*-9*t*, 18:1*n*-7, 18:1*n*-5, 19:1*n*-9, 19:1*n*-8, 20:1*n*-9, 20:1*n*-7, 22:1*n*-11, 22:1*n*-9

[c] PUFA: 18:2*n*-6, 18:3*n*-3, 18:2*n*-3, 19:2*n*-7, 20:2*n*-9, 20:2*n*-7, 20:2*n*-6, 20:3*n*-4, 22:2*n*-9, 22:3*n*-6

[d] HUFA: 20:4*n*-6, 20:5*n*-3, 21:5*n*-3, 21:6, 22:4*n*-6, 22:5*n*-6, 22:5*n*-3, 22:6*n*-3

[e] BrFA: 4,8,12-trimethyl-tridecanoate, 9-methyl-tetradecanoate, 12-methyl-tetradecanoate (anteiso), 13-methyl-tetradecanoate (iso), 14-methyl-pentadecanoate (iso), 10-Methyl hexadecanoate, 14-methyl-hexadecanoate (anteiso), 15-methyl-hexadecanoate (iso), 15-methyl-heptadecanoate (anteiso), 16-methyl-heptadecanoate (iso), 16-methyl-octadecanoate (anteiso), 17-methyl-octadecanoate (iso)

[f] EpFA: 10,13-epoxy-11,12-dimethyl-octadecadienoate, 12,15-epoxy-13,14-dimethyl-eicosadienoate

BrFA 1: 14-methyl-hexadeanoate (anteiso); BrFA 2: 15-methyl-hexadeanoate (iso); EpFA 1: 12,15-epoxy-13,14-dimethyl-eiosadienoate

Table 2.1.2 Fatty acid (FA) composition (expressed as % of total pool of FAs) of stage 3 embryos of the green crab *Carcinus maenas* from four different areas within the brooding chamber (left external (LE), left internal (LI), right external (RE), right internal (RI)) and average (± SD) of total embryonic mass. Values are averages (± SD) of embryos from five different females (*n*=5).

Fatty Acid	Left External LE	Left Internal LI	Right External RE	Right Internal RI	Embryonic mass
14:0	0.48 ± 0.26	0.75 ± 0.42	0.55 ± 0.26	0.37 ± 0.18	0.53 ± 0.31
16:0	17.82 ± 3.26	19.09 ± 2.80	18.96 ± 3.05	17.93 ± 1.94	18.74 ± 2.62
18:0	6.04 ± 0.43	6.34 ± 0.41	6.16 ± 0.48	6.34 ± 0.52	6.23 ± 0.44
Σ SFA[a]	**26.44 ± 3.39**	**28.55 ± 2.87**	**27.74 ± 3.59**	**26.66 ± 2.08**	**27.60 ± 2.87**
16:1*n*-7	9.20 ± 1.34	10.06 ± 3.47	9.81 ± 1.04	8.75 ± 1.78	9.53 ± 2.01
18:1*n*-9*c*	12.07 ± 1.49	11.28 ± 1.20	11.86 ± 0.60	12.37 ± 1.61	11.97 ± 1.27
18:1*n*-7	5.71 ± 0.91	5.35 ± 0.73	5.91 ± 0.69	5.94 ± 0.83	5.75 ± 0.77
20:1*n*-9	1.23 ± 0.53	1.20 ± 0.45	1.28 ± 0.56	1.21 ± 0.55	1.22 ± 0.48
20:1*n*-7	2.32 ± 0.89	2.06 ± 0.52	2.25 ± 0.86	2.27 ± 0.81	2.21 ± 0.72
Σ MUFA[b]	**32.19 ± 1.45**	**32.10 ± 4.29**	**32.84 ± 1.35**	**32.13 ± 2.60**	**32.44 ± 2.50**
18:2*n*-6	0.96 ± 0.43	0.77 ± 0.35	1.00 ± 0.87	1.00 ± 0.80	0.93 ± 0.61
18:3*n*-3	0.72 ± 0.24	0.75 ± 0.13	0.69 ± 0.18	0.60 ± 0.12	0.68 ± 0.17
Σ PUFA[c]	**4.54 ± 1.28**	**4.19 ± 0.90**	**4.15 ± 1.29**	**4.28 ± 1.02**	**4.25 ± 1.05**
20:4*n*-6	2.52 ± 0.92	2.85 ± 1.28	2.47 ± 0.42	2.28 ± 1.25	2.52 ± 0.97
20:5*n*-3	15.45 ± 1.96	14.48 ± 2.83	15.24 ± 1.54	16.20 ± 2.21	15.29 ± 2.10
22:5*n*-3	1.32 ± 0.68	1.23 ± 0.31	1.14 ± 0.51	1.24 ± 0.38	1.19 ± 0.45
22:6*n*-3	9.81 ± 2.36	8.78 ± 2.60	8.82 ± 3.23	9.63 ± 1.46	9.15 ± 2.32
Σ HUFA[d]	**30.04 ± 2.08**	**28.44 ± 6.43**	**28.73 ± 3.59**	**30.29 ± 3.43**	**29.14 ± 3.92**
BrFA 1	1.72 ± 0.31	1.63 ± 0.20	1.63 ± 0.39	1.61 ± 0.34	1.66 ± 0.30
BrFA 2	1.58 ± 0.26	1.54 ± 0.17	1.49 ± 0.49	1.64 ± 0.35	1.54 ± 0.32
Σ BrFA[e]	**5.44 ± 0.51**	**5.50 ± 0.82**	**5.24 ± 1.21**	**5.48 ± 0.98**	**5.34 ± 0.86**
EpFa 1	0.99 ± 0.42	0.75 ± 0.16	0.85 ± 0.34	0.90 ± 0.19	0.85 ± 0.28
Σ EpFA[f]	**1.13 ± 0.39**	**0.95 ± 0.25**	**1.10 ± 0.37**	**0.96 ± 0.17**	**1.01 ± 0.29**

Abbrevations: See Table 2.1.1

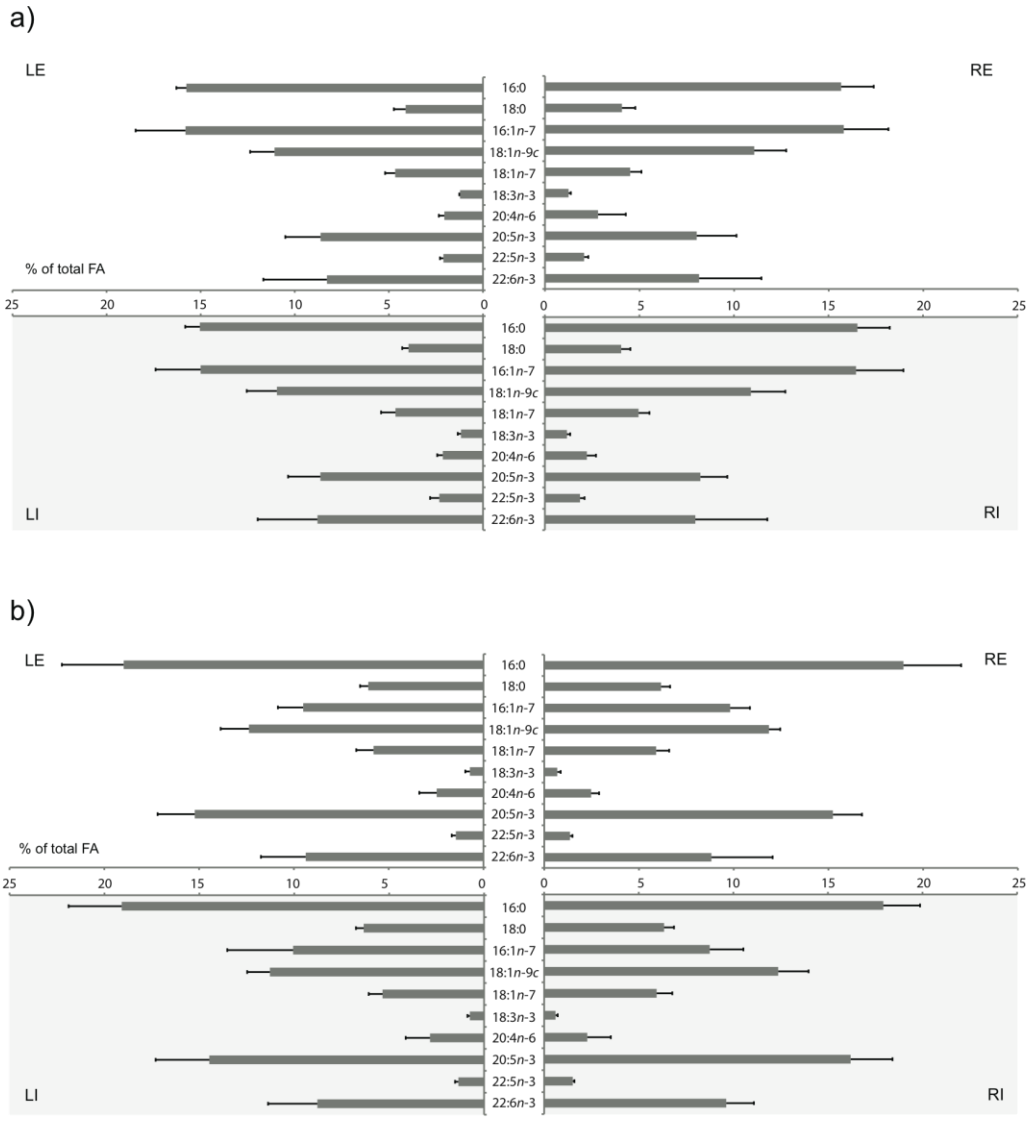

Figure 2.1.1 Fatty acid (FA) profile (expressed as % of the total pool of FAs) of the most abundant FAs (> 1% of the total pool of FAs) in stage 1 (a) and stage 3 (b) embryos of the green crab *Carcinus maenas* on different areas of the brooding chamber: left external (LE), left internal (LI), right external (RE) and right internal (RI). Values are averages (± SD) of embryos from five different females (n=5).

2.1.5 DISCUSSION

The results reported in the present study allow us to accept our first null hypothesis: maternal provisioning during oogenesis in *C. maenas* was homogenous and the whole mass of newly extruded embryos displays a similar FA profile. This finding does not agree with the study by Leal et al. (2013), where significant differences were recorded in the FA profile of newly extruded embryos (stage 1) across the brooding chamber of the European clawed lobster *Homarus gammarus*. As our work is solely the third study addressing this topic in decapods (the work by Pochelon et al. (2011) focused a more advanced embryonic stage and therefore cannot be compared with ours) it may be too speculative to advance any solid argument on why within-brood variation was not recorded in newly extruded embryos of *C. maenas*. Nevertheless, it must be highlighted that *C. maenas* and *H. gammarus* belong to different infraorders, Brachyura and Astacidae, respectively (De Grave et al. 2009). In this way, it is possible that the metabolic pathways involved in lipid accumulation during oogenesis in these two species may somehow differ. Maternal provisioning is influenced by maternal nutritional status (Tuck et al. 1997; Racotta et al. 2003), environmental conditions and feeding regimes (Calado et al. 2010; Brillon et al. 2005) and factors related with the biology of the species (e.g., time between moults or subsequent broods) (Verísimo et al. 2011). During ovarian maturation, as much as 60% of the lipid content of female decapods may be transferred to their oocytes (Herring 1973). The source of these lipids is still unclear. They may be mobilized from reserves stored in the hepatopancreas (Harrison 1990) or directly derived from the ingestion of food (Clarke 1982). Different lipidomic pathways during ovarian maturation might justify a dissimilar response in within-brood provisioning between decapod species. Lipids originating in the hepatopancreas are not fully depleted during ovarian maturation (Tuck et al. 1997) and are more homogeneous than those which are derived from dietary resources. In this way, species displaying a prevalence of resource allocation pathways originating in lipid reserves present in the hepatopancreas are likely more prone to display an homogeneous embryonic provisioning. In the present study, and despite the remarkable diversity of dietary items known to be consumed by an opportunistic feeder such as *C. maenas* (Baeta et al. 2006, Chaves et al. 2010), it is clearly shown that maternal provisioning is not a source of offspring variability and that the incorporation of lipid

reserves in developing oocytes must occur in a synchronous and uniform way during gonadal maturation.

Our data also allow us to accept our second null hypothesis affirming that the location of developing embryos within the brooding chamber did not condition their FA profile by the end of embryogenesis (prior to hatching). Previous studies by Baeza and Fernández (2002) and Fernández et al. (2003) reported dramatic differences in oxygen availability between the centre and the periphery of the brooding chamber of two large sized brachyuran crabs (*Cancer setosus* and *Homalaspis plana*). Indeed, these differences are even more accentuated during early embryonic development (Fernadez and Brante 2003). Such differences in oxygen levels within the brooding chamber invariably lead to contrasting metabolic rates (Alter et al. 2015), thus increasing energy demand and SFA oxidation (Leal et al. 2013). Overall, as oxygen levels influence the embryonic development (Hartnoll & Paul 1982), inner embryos should present a delay when compared with those from the periphery (Fernández et al. 2003). In our study, the FA analysis performed showed the existence of a synchronous embryonic development in the four brooding areas surveyed. In *C. maenas* the oxygen condition in the embryo mass is known to change drastically between early and late embryonic development (Fernández & Brante 2003). The absence of an asynchronous trend in developing embryos suggests that *C. maenas* exerts an efficient oxygen supply to the embryonic mass being incubated through well-known maternal behavioural patterns: abdominal flapping and pleopod movements (Baeza and Fernández 2002; Brante et al. 2003; Fernández and Brante 2003; Silva et al. 2007), which promote an intense water renovation (Fernández et al. 2006).

Although female ventilation must be the main responsible for a homogeneous embryonic development, other factors may have also contributed, although likely in a lesser extent, to such a synchronous embryonic development. The increase in embryo volume during embryogenesis enlarges the embryonic surface area for gas exchange (Chaffee & Strathmann 1984) and facilitates the passive supply of oxygen into the mass of developing embryos (Fernández et al. 2006). On the other hand, the presence of branched, cyclopropyl and odd-numbered FAs evidences the presence of a stable population of

bacteria on developing embryos (Dalsgaard et al. 2003). The role of the bacterial communities present in developing embryos is still largely unknown. The presence of bacterial films covering developing embryos of marine invertebrates has been commonly associated with negative effects: decrease in gaseous exchange, reduction of embryo development and even death (Fisher 1976; Biermann et al. 1992; Przeslawski and Benkendorff 2005; Silva et al. 2007). Nevertheless, symbiotic bacteria may also prevent the infection by pathogenic fungi on the surface of crustacean embryos (Gil-Turnes et al. 1989; Gil-Turnes and Fenical 1992). In this regard, the presence of a structurally stable bacterial population during the embryogenesis of *C. maenas* (as revealed by the steady levels of branched, cyclopropyl and odd-numbered FAs) could impair the proliferation of deleterious microrganisms and fungi, thus exerting a protective function and indirectly favouring oxygen diffusion (Cohen and Strathmann 1996; Cronin and Seymour 2000; Peters et al. 2012).

Fernández *et al.* (2003) also suggested that asynchronous development could imply an asynchronous hatching. Although ovigerous females of *C. maenas* release their larvae during two or more main events (Zeng and Naylor 1997), according to our results, these extended hatching events are probably not consequence of asynchronous embryonic development. The rhythms of larval release are likely associated with tidal cycles, in order to facilitate the export of these larvae from estuarine waters into the ocean (Queiroga et al. 1997, Anger 2001).

In conclusion, the present study demonstrated that maternal investment in *C. maenas* was uniformly distributed among newly extruded embryos and that despite displaying a highly packed mass of embryos, those incubated in inner and outer regions of the brood displayed similar biochemical profiles by the end of embryogenesis. Future studies should try to confirm if this apparent efficiency displayed by ovigerous females of *C. maenas* in providing suitable levels of oxygen to developing embryos are an exception or a rule within the Brachyura. By using similar sized species to *C. maenas*, as well as larger and smaller sympatric crab species, researchers may be able to investigate if adult body size is the key variable ruling the efficiency of these brooding behaviours. Moreover, it may also

be possible to investigate if this efficiency was shaped along the natural history of the Brachyura and is somehow reflected in their phylogeny.

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2.2 BIOCHEMICAL PROFILE OF CARCINUS MAENAS *EMBRYOS IS UNAFFECTED BY FEMALE SIZE BUT VARIES SIGNIFICANTLY OVER CONSECUTIVE YEARS IN A COASTAL LAGOON*

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2.2.1 ABSTRACT

Maternal effects play a crucial role on the phenotype of marine invertebrates, as they may act crossways along their whole life history and ultimately shape population dynamics. Understanding the processes through which maternal effects condition offspring phenotype is paramount to understand the dynamics of marine invertebrates with bi-phasic life cycles. In the present study, we use the keystone species *Carcinus maenas* as a model to analyse the influence of maternal effects in offspring phenotype. We sampled over two consecutive years (2012 and 2013), wild ovigerous females from two well-differentiated size classes (small and large) brooding newly extruded (stage 1) and ready to hatch embryos (stage 3). Embryos in stage 1 were used as proxy for maternal investment in offspring, while those at stage 3 were used to infer the quality of newly hatched larvae. The lipidomic profiles of both embryonic stages were surveyed through the quantification of phospholipids and fatty acid profiles. Results showed that maternal size was not a reliable indicator of maternal provision or larval quality at hatching. On the other side, interannual variability played a significant role in the phenotypic plasticity of the offspring. The biochemical profile of stage 3 embryos was shaped by environmental conditions prevailing during the incubation period and highlight the need to interpret with caution studies that overlook temporal variability.

2.2.2 INTRODUCTION

Maternal effects are one of the most important sources of variability in offspring phenotypes in marine invertebrates (Bernardo 1996), acting at several ecological levels (e.g., hatchability, larval survival, success at settlement) (Marshall et al. 2008). The complex life cycle of marine invertebrates and the constraints to monitor their full life history in natural systems as long limited the study of maternal effects in these taxa. However, maternal effects can be easily carried from one life stage to the next (e.g., from embryos to larvae and even juveniles; carry-over effects) which play an important role in marine population dynamics (Pechenik 2006, Marshall & Morgan 2011).

Although the relationship between female size and fecundity is widely accepted, with large females commonly being more fecund (Hines 1986, Tallack 2007), the relationship between female size and offspring fitness is still an open discussion (e.g., Moland et al. 2010; Wieland and Siegstad 2012; Koopman and Siders 2013; Swiney et al. 2013). In decapod crustaceans, some authors suggest a positive relationship between female size and offspring quality. This theory has been justified by the fact that larger females tend to moult less frequently than small females (Ouellet & Plante 2004), hence they can invest more energy into reproduction and consequently generate offspring with better quality (Sibert et al 2004; Moland et al 2010; Sato and Suzuki 2010; Wieland and Siegstad 2012). However, other studies found no relationship whatsoever between female size and offspring quality (Koopman & Siders 2013, Swiney et al. 2013).

During oogenesis females transfer their energetic reserves to developing oocytes depleting their own internal reserves (Harrison 1990) or directly allocating them through the digestion of dietary prey (Smith et al. 2004). Biochemical composition of embryos reflects the nutritional status of the female (Racotta et al. 2003, Smith et al. 2004, Calado et al. 2010). Although energy available in the yolk will be used by embryos according to their genetic program (Rosa et al. 2007), yolk catabolism is not isolate of environmental factors. In this sense, environmental and trophic conditions (e.g., available and quality food (Guisande & Harris 1995), temperature (Wear 1974) or salinity (Bas & Spivak 2000)) affect oogenesis, embryogenesis and larval quality (Giménez & Anger 2001) and thus may condition early larval performance and survival. In particular, estuarine areas are influenced by environmental conditions, as air temperature or precipitation, promoting shifts in water conditions (Rodrigues 2012, Álvarez et al 2013) than can affect metabolism during embryogenesis.

Carcinus maenas (Linnaeus, 1758) is the most characteristic decapod on north-western Atlantic estuaries and rocky shores. Females carry the embryos in broods until hatching, when larvae migrate to the ocean to develop (Queiroga et al. 1997), being the last larval stage (megalopa) the one which returns to adult habitats to settle (Domingues et al. 2011). Estuarine crabs, as euryhaline species, are physiologically well adapted to strong shifts in salinity through their efficient osmoregulation processes. Nevertheless, during incubation,

developing embryos remain attached to female pleopods and are directly exposed to external medium. Only a permeable membrane separates the embryo from the medium surround (Goudeau 1983). While adult crabs can live over extended periods in water at low salinity (Broekhuysen 1936), reproduction, embryogenesis, and larval development require a higher salt concentrations (Nagaraj 1993, Morgan 1995), but little is known about osmoregulation in embryos, their tolerance to salinity fluctuations and how these may interfere with the catabolism of embryonic reserves during incubation.

In the present study we compared maternal provisioning and embryonic catabolism of *C. maenas* during the early reproductive season of two consecutive years (2012 and 2013) in Ria de Aveiro (Portugal), using biochemical tools. The quantification of phospholipids (PLs) and analysis of fatty acid (FA) profiles of newly extruded (stage 1) and close to hatch embryos (stage 3) were surveyed from two well-differentiated size classes (small and large females) in 2012 and 2013. FA profiles of early embryonic stages can be used as a proxy of maternal provisioning, while those of late stage embryos reflect lipid dynamics during embryonic development and provide an insight on the lipid profile of newly hatched larvae. The following null hypotheses were tested: 1) maternal provisioning is similar in small and large sized females; 2) maternal provisioning does not differ among consecutive years; 3) FA catabolism is similar in embryos incubated by small and large females; and 4) FA catabolism of embryos during the incubation does not differ among different years.

2.2.3 MATERIALS AND METHODS

2.2.3.1 SAMPLING

Ovigerous females of *C. maenas* were collected using trawl nets in Ria de Aveiro, a shallow coastal lagoon on north-western Portugal (40° 37’ 17’’ N, 8° 44’ 56’’ W). Sampling was performed during early reproductive season (late March and early April) of 2012 and 2013. In each year, two criteria were used to classify collected females: their carapace width (CW, measured between the first pair of lateral spines of the carapace) and the development stage of embryos being brooded. Females were classified as small when displaying a CW < 40 mm or large when CW ≥ 40 mm. Developing embryos were classified according to the following criteria (as defined by Rosa et al., 2007): stage 1 (newly extruded embryos) -

uniform yolk and absence of cleavage and eyes; stage 3 (embryos ready to hatch in < 48 h) nearly no yolk present and embryo fully developed. In each year, twenty females were haphazardly selected, ten small females (2012 – CW: 36.94 ± 2 .07 mm; 2013 – CW: 34.11 ± 4.54 mm) and ten large females (2012: CW 44.12 ± 2.73 mm; 2013: CW 44.66 ± 2.96 mm), which five were brooding embryos in stage 1 and five in stage 3. Overall, 2 consecutive years (2012 and 2013) x 2 female sizes (small and large) x 2 embryonic stages (stage 1 and stage 3) x 5 replicates = 40 samples of embryos collected for analysis. The embryos of each female were carefully removed with fine forceps. After collection, all samples were freeze-dried and stored at - 32 °C for later biochemical analysis.

2.2.3.2 FATTY ACID ANALYSIS

Total lipids extracts were obtained using the Bligh and Dyer (1959) method. Briefly, freeze-dried embryos were resuspended in 1 mL of ultrapure water and 3.75 mL chloroform/methanol 1:2 (v/v) was added to the suspension that was vortexed and incubated on ice for 30 min. An additional volume of 1.25 mL chloroform was added along with 1.25 mL of ultrapure water. Following vigorous vortexing, samples were centrifuged at 1000 rpm for 5 min at room temperature to obtain a two-phase solution: an aqueous top phase and an organic bottom phase, from where lipids were retrieved. Lipid extracts were dried with a nitrogen flow and stored at -32 °C for posterior analysis.

In order to quantify the total amount of phospholipids, a phosphorus assay was performed according to Bartlett and Lewis (1970). The lipid extracts were resuspended in 300 µL of chloroform and 20 µL of the samples were used. After being dried with a nitrogen flow, the samples were resuspended in 0.650 mL of perchloric acid (70%), being incubated for 1 h at 180 °C. A total of 3.3 mL ultrapure water, 0.5 mL ammonium molybdate (2.5%) and 0.5 mL ascorbic acid (10%) were added to each sample, followed by incubation during 5 min at 100 °C in a water bath. Standards from 0.1 to 3.0 µg of phosphate (standard solution of $NaH_2PO_4.2H_2O$, 439 mg L^{-1} of water, i.e. 100 µg of phosphorus mL^{-1}) underwent the same treatment as the samples. Absorbance of standards and samples was measured at 800 nm, at room temperature, in a microplate UV-vis spectrophotometer.

FAs were analysed by gas chromatography-mass spectrometry (GC-MS) after transesterification of total lipid extracts (30 µg of total PL). FA methyl esters (FAMEs) were prepared using a methanolic solution of potassium hydroxide (2 M) according to the previously described method by Aued-Pimentel et al. (2004). The FAMEs were resuspended in 40 µL of hexane, with 2 µL of this hexane solution being used for GC-MS analysis on an Agilent Technologies 6890N Network (Santa Clara, CA) equipped with a DB-1 column with 30 m of length, 0.25 mm of internal diameter, and 0.1 µm of film thickness (J&W Scientific, Folsom, CA). The GC-MS was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range *m/z* 40-500 in a 1 s cycle in a full scan mode acquisition. The oven temperature was programmed from an initial temperature of 40 °C, standing at this temperature for 0.5 min and following a linear increase to 220 °C at 20 °C/min, a linear increase at 2 °C/min to 240 °C, and 5 °C/min until reaching 250 °C. The injector was set at 220 °C and the detector at 230 °C. Helium was used as the carrier gas at a flow rate of 1.7 mL/min. The identification of FAMEs was performed by comparing the retention time and mass spectrum of each FAME relative to 34 mixed FAME standards (Supelco 37 Component FAME Mix) and confirmed by comparison with the chemical database Wiley and the spectral library "The AOCS Lipid Library" (Christle 2012).

2.2.3.3 ENVIRONMENTAL DATA

Environmental data (precipitation and air temperature) were acquired through an automatic weather station (AWS) located in Campus de Santiago, University of Aveiro (40° 38´ 36´ N; 8° 39´36´´ W; altitude: 6 m above sea level). This AWS is integrated in the national network of AWS of Instituto Nacional do Mar e da Atmosfera (Portugal). Precipitation and air temperature data were recorded during the period of January to April 2012 and 2013, every 10 min by two sensors 1.5 m apart. Precipitation values were measured using a pluviometer (Pronamic® Pró) and displayed as monthly-accumulated data (mm). Air temperature values were measured with the sensor Vaisala HUMICAP® HMP155 with protector shield. These data were displayed by monthly averages (°C).

We used air temperature as a proxy for sea surface temperature (SST), once both measures are positively correlated in this estuarine system (Álvarez et al 2013). Moreover, near-bed

temperature at Ria de Aveiro show nearly the same value as SST, indicating that solar heating affects whole water column (Álvarez et al 2013).

2.2.3.4 DATA ANALYSIS

The content of each FA was transformed in µg per unit of dry weight (DW) of embryo (mg), (µg mg^{-1} DW), using as reference the quantification of PLs on each sample, as PLs comprise the major lipid classes in brachyuran crab embryos. The sum of the total pool of FAs and the head group represents the total PL quantified for each sample.

For statistical analysis, in the FA class analysis only the FA classes containing the most abundant FAs were consider, namely: Saturated FA (SFA), Monounsaturated FA (MUFA), Polyunsaturated FA (PUFA), Highly-unsaturated FA (HUFA), Branched FA (BrFA) and Epoxy FA (EpFA). While Polyunsaturated FA are commonly defined as all FA with ≥ 2 double bonds, in the present study we discriminate between PUFA (FAs with 2 or 3 double bonds) and HUFA (FAs with ≥ 4 double bonds).

We analysed FA classes and total amount of PLs in stage 1 and stage 3 embryos using a two-way ANOVA analysis with female size (with two levels: small and large) and reproductive year (with two levels: 2012 and 2013) as factors. Post-hoc Tukey HSD test was used when ANOVA results revealed significant differences ($P < 0.05$). Prior to analysis, we tested for deviations from normality in the response variable using the Shapiro test and equality of variance using the Levene´s test. These statistical analysis were performed with the software R (R Development Core Team 2011).

Multivariate statistical analyses were performed to detect the existence of significant differences in FA profiles (only the twenty more abundant FAs were used) and FA class profiles of stage 1 and stage 3 embryos from females with different sizes and from different reproductive years. Prior to statistical analysis, in order to down-weight the contributions of quantitatively dominant FAs, the raw data matrix was log (x +1) transformed. Following this transformation, a new matrix was assembled using Bray-Curtis similarity coefficient. Two-way analyses of similarities (ANOSIM) were used to test for significant differences

between the individual FAs and FA classes of stage 1 and stage 3 embryos. The factors used in the ANOSIM analysis were female size (with two levels: small and large) and reproductive year (with two levels: 2012 and 2013). ANOSIM calculates a global R statistic that assesses the differences in variability between groups, as compared to within groups, and checks for the significance of R using permutation tests (Clarke & Gorley 2006). In order to visualize inter-individual differences in FA profiles of stage 3 embryos, a Principal Coordinate Analysis (PCO) was performed, representing differences between reproductive years along the first two axes. Differences in FA profiles recorded between reproductive years were explored using the similarity percentages routine (SIMPER), the FAs which contributed in more than 50% of difference between groups were presented in the PCO graph. All multivariate statistical tests were performed with Primer 6.1 with PERMANOVA add-on (Primer-E Ltd. Plymouth, UK).

Statistical analysis of environmental data (precipitation and air temperature) was carried out with pairwise comparisons. Mann-Whitney test was used to compare the monthly differences between consecutive years (2012 and 2013). The level of statistical significance was $P < 0.05$.

2.2.4 RESULTS

The two-way ANOVA performed to compare the FA classes of stage 1 embryos (Fig. 2.2.1a) showed no significant differences between small and large females. In factor year significant differences were found for EpFA ($P = 0.023$). The interaction between both factors (size and year) was significant in the FA classes MUFA, BrFA and EpFA ($P= 0.016$; $P = 0.025$; $P = 0.032$, respectively). The post-hoc analysis identified significant differences between pairs of groups in BrFA (small females from 2012 and 2013, $P = 0.041$) and EpFA (large females from 2012 and 2013, $P= 0.016$). Concerning stage 3 embryos (Fig. 2.2.1b), no significant differences were recorded between small and large females, except for EpFA ($P = 0.018$). Additionally, the FA classes SFA, MUFA and HUFA presented significantly lower values in 2013 than in 2012 ($P < 0.0001$; $P = 0.018$; $P < 0.001$, respectively).

The two-way ANOVA performed to compare the total amount of PLs in stage 1 embryos (Table 2.2.1) showed no significant differences between female sizes or reproductive years.

For stage 3 embryos (Table 2.2.2), no significant differences were recorded for factor female size although embryos from 2013 displayed a significantly lower total amount of PLs than those from 2012 (P = 0.002).

The ANOSIM analysis of FA profiles for stage 1 embryos showed a significant effect on factor female size (R = 0.208; P = 0.014) and no significant effect on factor reproductive year (R = 0.126; P = 0.108). The ANOSIM analysis of FA profiles in stage 3 embryos, showed no significant effect of the factor female size (R = 0.040; P = 0.346) and a significant effect of factor reproductive year (R = 0.494; P = 0.001). The ANOSIM analysis of the FA class profiles in stage 1 embryos showed no significant effect of the factor female size (R = 0.206; P = 0.056) and significant effect of the factor reproductive year (R = 0.210; P = 0.030). In stage 3 embryos, the ANOSIM analysis of the FA class profiles revealed no significant effect of the factor female size (R = - 0.008; P = 0.502) and significant effect of the factor reproductive year (R = 0.426; P = 0.002) (Table 2.2.3).

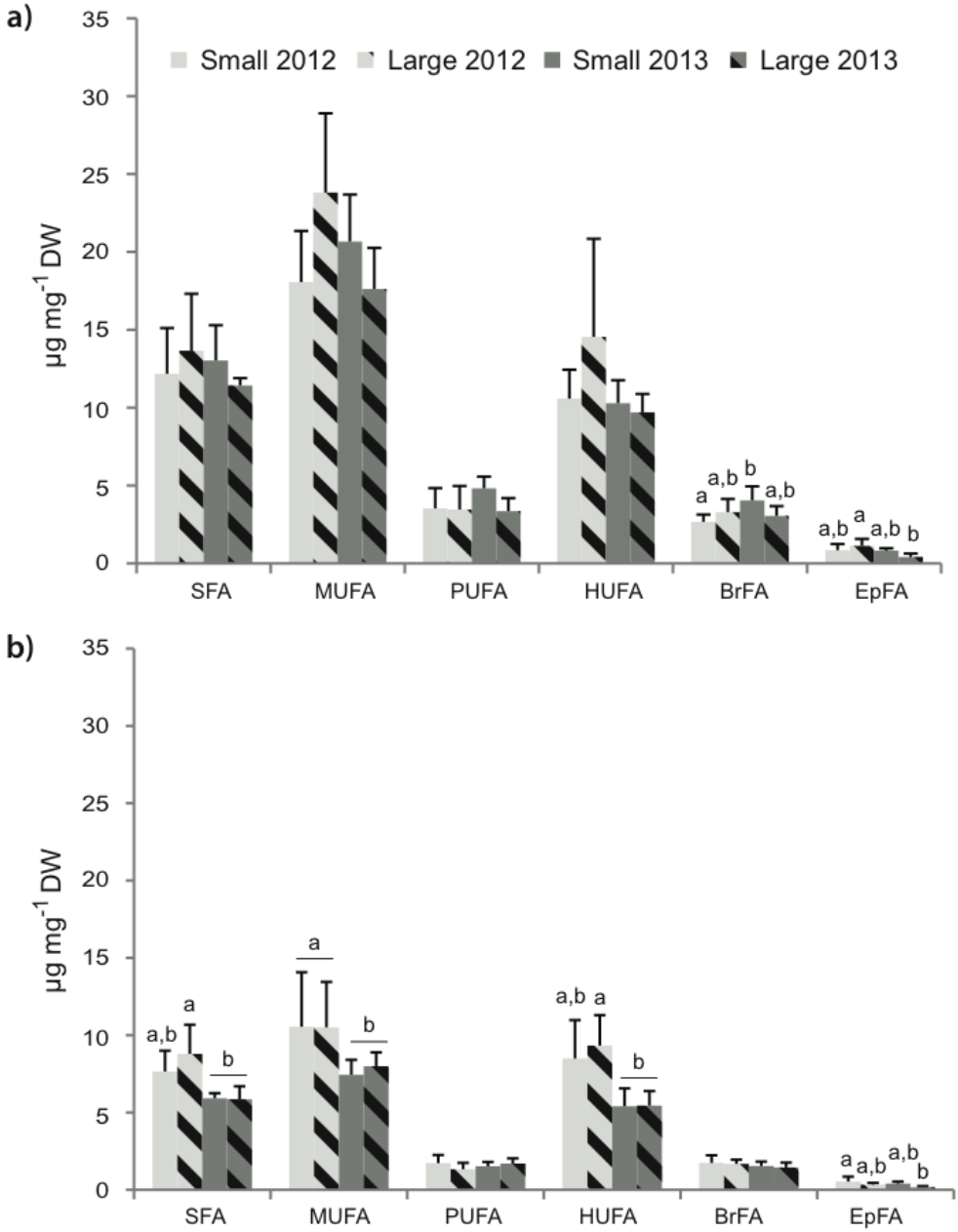

Figure 2.2.1 (a) Fatty acid (FA) class profiles of *Carcinus maenas* embryos in stage 1 and (b) in stage 3, from small and large females sampled during two consecutive years (2012 and 2013) (µg mg^{-1} DW). Error bars represent standard deviation of five independent samples. Small letters above bars indicate means that are significantly different from each other, in the same FA class (Tukey-HSD, $P < 0.05$).

Abbreviations:

SFA: Saturated FA; MUFA: Monounsaturated FA; PUFA: Polyunsaturated FA; HUFA: Highly-unsaturated FA; BrFA: Branhed FA; EpFA: Epoxy FA

The PCO analysis of stage 3 embryos revealed high variability between embryos incubated during different reproductive years (Fig. 2.2.2). The first two axes of the PCO analysis explained > 77 % of FA variation (PCO axis 1: 65.1 %; PCO axis 2: 12.4 %). The individual FAs that contributed to distinguish both reproductive years in more than 50 % were: eicosapentaenoic acid (20:5*n*-3, EPA), docosahexaenoic acid (22:6*n*-3, DHA), oleic acid (18:1*n*-9*c*), palmitic acid (16:0) and palmitoleic acid (16:1*n*-7) (complete list in Table 2.2.4).

The precipitation graph (Fig. 2.2.3a) shows that during the incubation period, precipitation was significantly higher in 2012 than 2013, except for April when the precipitation in 2012 was higher than 2013 (January $P < 0.001$; February $P < 0.001$; March $P < 0.001$; April $P < 0.05$). Monthly averages of air temperature (Fig. 2.2.3b) show significantly lower temperatures in 2012 than 2013, except for March (January $P < 0.001$; February $P < 0.05$; April $P < 0.01$).

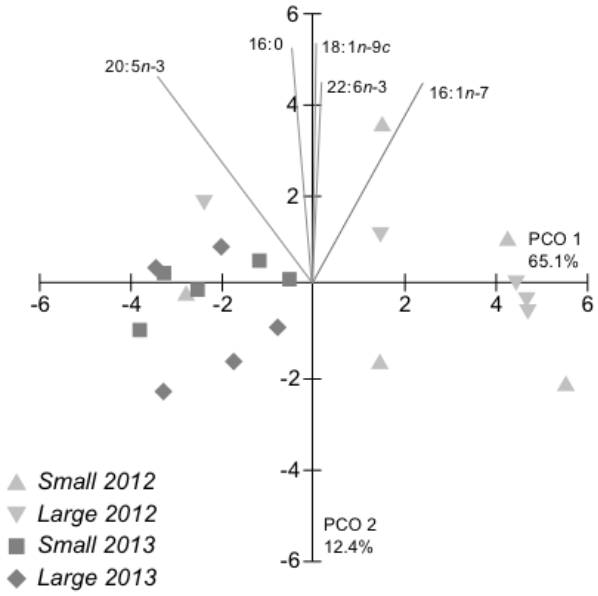

Figure 2.2.2 Principal coordinates analysis (PCO) comparing fatty acid (FA) profile of *Carcinus maenas* embryos in stage 3, from small and large females sampled during two consecutive years (2012 and 2013). Vectors displayed represent individual FAs that contributed in more than 50% to distinguish between from 2012 and 2013.

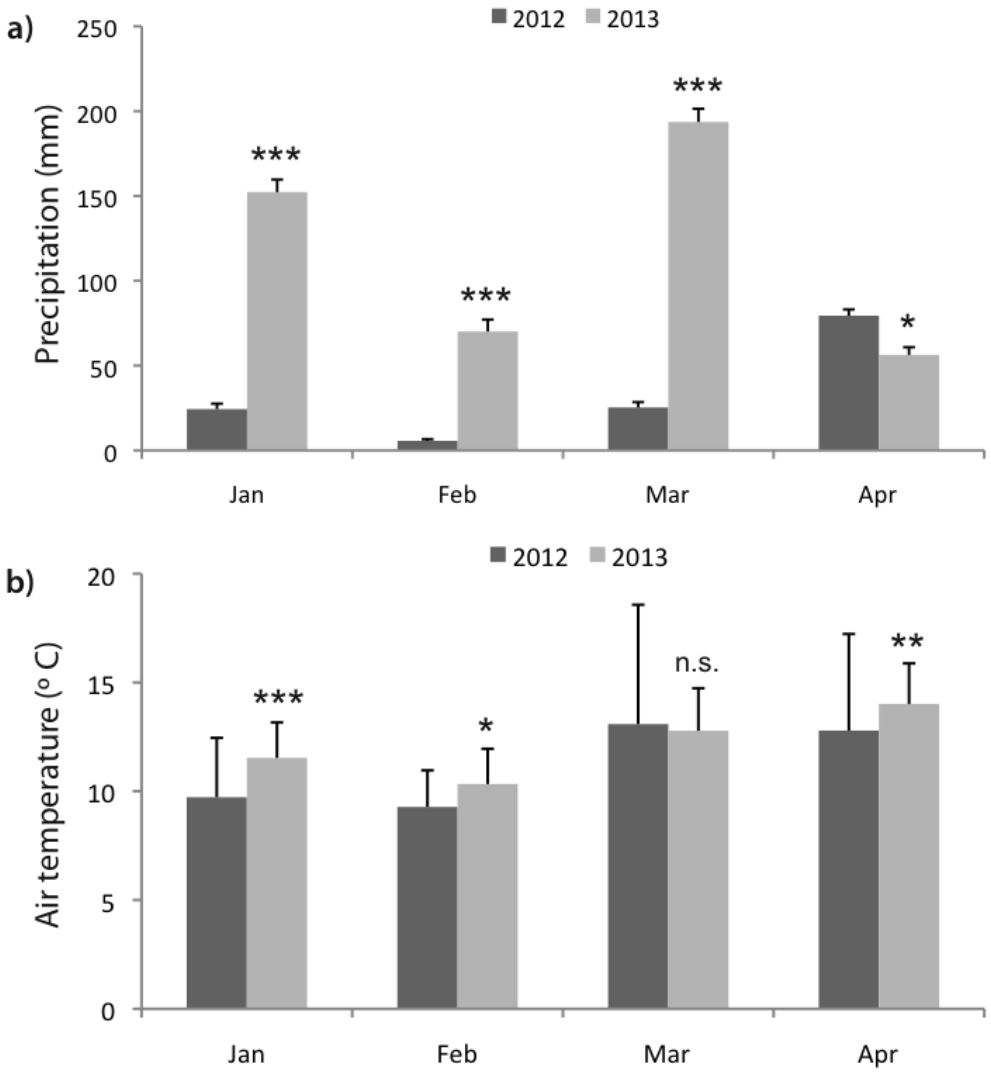

Figure 2.2.3 (a) Monthly accumulated precipitation data (mm) and (b) monthly average air temperature (°C) of two consecutive years (2012 and 2013) from January to April in Ria Aveiro (Portugal). Error bars represent standard deviation of two sensors. Significant differences are marked with asterisks (Mann-Whitney test, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$); n.s.: statistically not significant.

Table 2.2.1 Fatty acid composition and total phospholipid (PL) quantification (µg mg^{-1} DW) of stage 1 embryos of the green crab *Carcinus maenas* recorded from two different female sizes (small and large) and during two consecutive years (2012 and 2013). Values show average (± SD) of embryos from five different females (*n*=5).

	2012		2013	
	Small	Large	Small	Large
14:0	0.52 ± 0.14	0.57 ± 0.12	0.61 ± 0.14	0.53 ± 0.07
15:0	0.52 ± 0.12	0.52 ± 0.10	0.71 ± 0.17	0.60 ± 0.19
16:0	8.28 ± 2.05	9.47 ± 2.72	8.70 ± 1.72	7.66 ± 0.73
18:0	2.25 ± 0.60	2.42 ± 0.70	2.22 ± 0.34	1.98 ± 0.14
Σ SFA[1]	**12.18 ± 2.93**	**13.66 ± 3.66**	**13.03 ± 2.27**	**11.42 ± 0.48**
16:1n-7	6.42 ± 1.78	9.34 ± 2.05	8.17 ± 1.58	6.59 ± 2.03
18:1n-9c	4.74 ± 0.89	6.65 ± 2.05	5.29 ± 0.81	4.79 ± 0.54
18:1n-7	3.14 ± 0.90	2.85 ± 1.00	2.83 ± 0.82	2.36 ± 0.36
20:1n-9	1.03 ± 0.13	1.22 ± 0.54	1.20 ± 0.48	1.01 ± 0.56
20:1n-7	1.49 ± 0.23	1.90 ± 0.58	1.37 ± 0.46	1.52 ± 0.44
Σ MUFA[2]	**18.07 ± 3.28**	**23.81 ± 5.09**	**20.66 ± 3.01**	**17.63 ± 2.63**
18:2n-6	0.49 ± 0.11	0.50 ± 0.36	0.48 ± 0.18	0.49 ± 0.17
18:3n-3[a]	0.89 ± 0.66	0.00 ± 0.00	0.70 ± 0.30	0.40 ± 0.18
18:3n-3[b]	0.37 ± 0.11	0.69 ± 0.18	0.77 ± 0.39	0.48 ± 0.18
22:2n-9	0.38 ± 0.17	0.55 ± 0.33	0.82 ± 0.30	0.44 ± 0.21
Σ PUFA[3]	**3.53 ± 1.30**	**3.46 ± 1.51**	**4.82 ± 0.76**	**3.35 ± 0.84**
20:4n-6	0.61 ± 0.24	1.35 ± 0.32	0.76 ± 0.21	1.09 ± 0.64
20:5n-3	5.22 ± 1.15	5.15 ± 2.40	4.75 ± 1.07	4.29 ± 0.79
22:5n-3	1.17 ± 0.36	1.24 ± 0.25	1.03 ± 0.20	0.93 ± 0.25
22:6n-3	2.34 ± 0.48	5.33 ± 3.36	2.42 ± 0.75	2.52 ± 0.68
Σ HUFA[4]	**10.58 ± 1.86**	**14.55 ± 6.29**	**10.29 ± 1.47**	**9.69 ± 1.19**
BrFA 1	1.12 ± 0.18	1.15 ± 0.34	1.55 ± 0.37	1.14 ± 0.04
BrFA 2	0.71 ± 0.14	0.95 ± 0.33	1.19 ± 0.47	0.88 ± 0.17
Σ BrFA[5]	**2.67 ± 0.47**	**3.29 ± 0.84**	**4.04 ± 0.91**	**3.05 ± 0.62**
EpFA 1	0.73 ± 0.39	0.99 ± 0.41	0.76 ± 0.11	0.39 ± 0.18
Σ EpFA[6]	**0.85 ± 0.40**	**1.12 ± 0.45**	**0.82 ± 0.15**	**0.41 ± 0.22**
Σ (n-3)	10.71 ± 2.28	13.06 ± 6.15	10.40 ± 1.70	9.04 ± 0.97
Σ (n-6)	2.20 ± 0.68	3.21 ± 0.99	2.89 ± 0.35	2.61 ± 0.72
PL	74.82 ± 13.46	93.69 ± 26.33	83.97 ± 10.45	71.20 ± 2.64

Abbreviations:
Saturated FA (SFA), Monounsaturated FA (MUFA), Polyunsaturated FA (PUFA), Highly-unsaturated FA (HUFA), Branhed FA (BrFA) and Epoxy FA (EpFA) represent the sum of all FAs identified as can see below:

[1] SFA: 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0, 22:0

[2] MUFA: 14:1*n*-5, 15:1*n*-1, 16:1*n*-13*t*, 16:1*n*-7, 16:1*n*-5, 7-methyl-hexadec-6-enoate, 17:1*n*-9, 17:1*n*-8, 18:1*n*-9*c*, 18:1*n*-9*t*, 18:1*n*-7, 18:1*n*-5, 19:1*n*-9*c*, 19:1*n*-8, 20:1*n*-9, 20:1*n*-7, 22:1*n*-11, 22:1*n*-9

[3] PUFA: 16:3*n*-6, 18:2*n*-6, 18:3*n*-3[a] (9*c*, 12*c*, 15*c* - octadecatrienoate), 18:3*n*-3[b] (9*c*, 11*t*, 15*c* - octadecatrienoate), 18:3*n*-1, 19:2*n*-7, 20:2*n*-9, 20:2*n*-7, 20:2*n*-6, 20:3*n*-4, 22:2*n*-9, 22:3*n*-6

[4] HUFA: 18:4*n*-3, 20:4*n*-6, 20:5*n*-3, 21:5*n*-3, 21:6, 22:4*n*-6, 22:5*n*-6, 22:5*n*-3, 22:6*n*-3

[5] BrFA: 4,8,12-trimethyl-tridecanoate, 12-methyl-tetradecanoate (anteiso), 13-methyl-tetradecanoate (iso), 14-methyl-pentadecanoate (iso), 10-methyl hexadecanoate, 14-methyl-

hexadecanoate (anteiso), 15-methyl-hexadecanoate (iso), 16-methyl-heptadecanoate (iso), 16-methyl-octadecanoate (anteiso), 17-methyl-octadecanoate (iso)
[6]EpFA: 10,13-epoxy-11,12-dimethyl-octadecadienoate, 12,15-epoxy-13,14-dimethyl-eicosadienoate
BrFA 1: 14-methyl-hexadeanoate (anteiso)
BrFA 2: 15-methyl-hexadeanoate (iso)
EpFA 1: 12,15-epoxy-13,14-dimethyl-eiosadienoate

Table 2.2.2 Fatty acid composition and total phospholipid (PL) quantification (µg mg^{-1} DW) of stage 3 embryos of the green crab *Carcinus maenas* recorded from two different female sizes (small and large) and two consecutive years (2012 and 2013). Values show average (± SD) of embryos from five different females (*n*=5).

	2012		2013	
	Small	Large	Small	Large
14:0	0.19 ± 0.12	0.18 ± 0.09	0.22 ± 0.06	0.26 ± 0.08
15:0	0.25 ± 0.06	0.23 ± 0.06	0.25 ± 0.07	0.21 ± 0.13
16:0	4.94 ± 0.88	5.96 ± 1.32	3.80 ± 0.19	3.81 ± 0.55
17:0	0.35 ± 0.12	0.34 ± 0.06	0.27 ± 0.05	0.24 ± 0.06
18:0	1.82 ± 0.46	1.99 ± 0.45	1.24 ± 0.18	1.24 ± 0.20
Σ SFA[1]	**7.65 ± 1.35**	**8.79 ± 1.89**	**5.92 ± 0.33**	**5.85 ± 0.85**
16:1n-7	3.09 ± 1.30	3.15 ± 1.18	2.29 ± 0.29	2.87 ± 0.75
18:1n-9c	3.34 ± 1.08	3.83 ± 0.96	2.28 ± 0.57	2.35 ± 0.23
18:1n-7	1.99 ± 0.68	1.84 ± 0.46	1.28 ± 0.09	1.21 ± 0.07
20:1n-9	0.55 ± 0.23	0.40 ± 0.19	0.36 ± 0.06	0.27 ± 0.03
20:1n-7	0.85 ± 0.35	0.74 ± 0.33	0.58 ± 0.16	0.63 ± 0.10
Σ MUFA[2]	**10.55 ± 3.52**	**10.50 ± 2.94**	**7.44 ± 0.97**	**7.99 ± 0.90**
18:2n-6	0.23 ± 0.19	0.29 ± 0.17	0.11 ± 0.07	0.25 ± 0.06
18:3n-3*	0.22 ± 0.09	0.23 ± 0.08	0.21 ± 0.06	0.29 ± 0.14
Σ PUFA[3]	**1.72 ± 0.53**	**1.33 ± 0.42**	**1.52 ± 0.28**	**1.70 ± 0.33**
20:4n-6	0.68 ± 0.27	0.78 ± 0.24	0.39 ± 0.06	0.43 ± 0.10
20:5n-3	4.56 ± 1.53	4.79 ± 0.69	2.71 ± 0.53	2.30 ± 0.60
22:4n-6	0.28 ± 0.10	0.22 ± 0.08	0.25 ± 0.03	0.19 ± 0.08
22:5n-3	0.58 ± 0.33	0.40 ± 0.16	0.47 ± 0.09	0.35 ± 0.09
22:6n-3	2.10 ± 0.64	2.98 ± 1.01	1.37 ± 0.51	1.98 ± 0.47
Σ HUFA[4]	**8.49 ± 2.48**	**9.33 ± 1.97**	**5.40 ± 1.16**	**5.44 ± 0.94**
BrFA 1	0.47 ± 0.09	0.48 ± 0.07	0.40 ± 0.08	0.37 ± 0.05
BrFA 2	0.69 ± 0.22	0.52 ± 0.10	0.55 ± 0.07	0.50 ± 0.09
Σ BrFA[5]	**1.73 ± 0.50**	**1.67 ± 0.28**	**1.54 ± 0.28**	**1.44 ± 0.31**
EpFA 1	0.45 ± 0.29	0.29 ± 0.13	0.39 ± 0.13	0.14 ± 0.06
Σ EpFA[6]	**0.53 ± 0.32**	**0.33 ± 0.12**	**0.40 ± 0.13**	**0.15 ± 0.08**
Σ (n-3)	7.97 ± 2.23	8.50 ± 1.91	5.14 ± 1.19	5.16 ± 0.90
Σ (n-6)	1.55 ± 0.50	1.43 ± 0.52	1.16 ± 0.12	1.32 ± 0.31
PL	47.90 ± 11.19	49.81 ± 11.28	34.74 ± 3.82	35.26 ± 3.75

Abbreviations: See Table 2.2.1

Table 2.2.3 Similarity values (ANOSIM) between fatty acid (FA) profiles and FA class profiles in embryos of *Carcinus maenas* in stage 1 and stage 3 from two female sizes (small and large females) and two reproductive years (2012 and 2013).

	FA profile				**FA class profile**			
	Stage 1		Stage 3		Stage 1		Stage 3	
	R	P	R	P	R	P	R	P
Female size	0.208	0.014	0.040	0.346	0.206	0.056	- 0.008	0.502
Reproductive year	0.126	0.108	0.494	0.001	0.210	0.030	0.426	0.002

Table 2.2.4 Similarity percentage analysis (SIMPER) identifying which fatty acids (FAs), contribute to the differences recorded in the FA profiles of stage 3 embryos of *Carcinus maenas* from two consecutive years (2012 and 2013).

	2012 *vs* 2013	
	Stage 3	
Fatty acids	Ind (%)	Cum (%)
20:5*n*-3	14.88	14.88
22:6*n*-3	9.81	24.69
18:1*n*-9*c*	9.67	34.36
16:0	9.20	43.57
16:1*n*-7	9.16	52.72
18:0	7.05	59.77
18:1*n*-7	6.71	66.49
20:4*n*-6	4.60	71.09
20:1*n*-7	4.42	75.51
20:1*n*-9	3.52	79.03
EpFA 1	3.36	82.39
22:5*n*-3	3.02	85.42
18:2*n*-6	2.50	87.92
BrFA 1	2.16	90.08

Abbreviations:
EpFA 1: 12,15-epoxy-13,14-dimethyl-eiosadienoate
BrFA 1: 14-methyl-hexadeanoate (anteiso)

2.2.5 DISCUSSION

The results of this study allow us to accept our two first null hypotheses: maternal provisioning is not significantly different between small and large females and it does not differ between consecutive years. Several studies focusing decapod crustacean have correlated positively female body size and offspring quality (Moland et al. 2010, Sato &

Suzuki 2010). Large females moult with less frequency than small females therefore they can invest more energy in their offspring (Ouellet & Plante 2004, Hartnoll 2006). Although ANOSIM analysis of stage 1 embryos showed significant differences in FA profiles between females with different sizes, the low value of *R* (*R* = 0.208) indicates a high within group variability, with the differences being recorded likely resulting from the natural variation (e.g., genetic, physiological or environmental) present in the biological material that was surveyed. Maternal provisioning is influenced by maternal diets (Racotta et al. 2003, Calado et al. 2010). Absence of differences among size classes suggests that during the storage of energetic reserves females of different sizes most likely had access to similar dietary items (in quality and quantity) (Baeta et al. 2006). *Carcinus maenas* is an opportunistic feeder with a high position in the food web of estuarine environments (Baeta et al. 2006, Chaves et al. 2010). Therefore, available food during winter months is likely to have enough quality to assure the energy resources required to undergo ovarian maturation.

On the other hand, the absence of differences in maternal provisioning among years can be explained by the fact of this study being performed during early reproductive season. Ria de Aveiro is a temperate estuary highly influenced by seasonal variations that promote contrasting differences in environmental conditions, food webs and hydrologic regimens (Lopes et al. 2007, Rodrigues et al. 2012). In winter months, a lower variability of potential food items is known to occur in the study area than during late reproductive season (over summer months) (Cunha et al. 1999, Lopes et al. 2007); overall, under this scenario, interannual qualitative and quantitative variability is less likely to be noticed on food items known to be part of *C. maenas* diet.

The absence of significant differences between small and large females in stage 3 embryos confirms our third null hypothesis: lipid catabolism is similar in embryos being brooded by females with different sizes. This finding demonstrates that the efficiency of incubation processes in *C. maenas* is independent from female body size. The development of highly-packed embryos, such as those displayed by the studied species, depends of a suitable provisioning of oxygen within the brood mass (Strathmann & Chaffee 1984, Cohen & Strathmann 1996, Fernández et al. 2003). Brachyuran crab females perform active brooding behaviours during incubation to ventilate the embryos and provide suitable levels

of oxygen to support catabolism (Fernández et al. 2000). This maternal care is associated with a substantial energy consumption (Fernández et al. 2000), which could have dramatic consequences for non-feeding brooding female crabs (Howard 1982, Baeta et al. 2006) if they were not highly effective. In line with other studies performed on decapod crustaceans (Koopman & Siders 2013, Swiney et al. 2013), our study confirms that maternal size is not a source of offspring variability, at least during embryonic development, in *C. maenas*.

Our results from the comparison of FA profiles of stage 3 embryos from 2012 and 2013 allow us to reject our last null hypothesis: lipid catabolism differs between consecutive years in embryos being brooded. As the FA profile of newly extruded embryos (stage 1) was similar among years, the differences recorded for embryos about to hatch (stage 3) should be the result of differences during embryogenesis promoted by contrasting environmental conditions. The embryogenesis of *C. maenas* lasts between 6 - 11 weeks at temperatures commonly recorded in the study area during the early reproductive season (10 – 13 °C) (Wear 1974, Hartnoll & Paul 1982). The precipitation graph (Fig. 2.2.3a) shows that during this period (January to April) precipitation was higher in 2013 than 2012, except during April. Rodrigues (2012) proposed that an increase of precipitation of about 20% leads to a decrease of the salinity about 2 in our study site. This suggests that in 2013 the embryos that were sampled in stage 3 were exposed to a lower salinity during most of their embryogenesis than those from 2012. Salinity plays an important role during embryogenesis and low pre-hatching salinity is known to increase the duration of embryonic development due to the energetic costs of osmoregulation (Giménez and Anger 2001). In this way, at low salinities, embryos need to allocate higher energetic resources to cope with osmotic stress (Giménez & Anger 2001), which can explain the lower levels of several FA classes (i.e., SFA, MUFA and HUFA) in stage 3 embryos from 2013. SFA and MUFA are the most abundant FA classes in triacylglycerols, which are the main source of energy to fuel the embryonic development (Sasaki et al. 1986). In stage 3 embryos from 2013, the low amount of SFA and MUFA suggests an additional use of these FA classes for energetic requirements during embryogenesis (Fischer et al. 2009), likely conditioned by osmoregulation process. HUFA play a role in cell differentiation and are incorporated in biological membranes throughout embryonic catabolism of brachyuran crabs (Cahu et al.

1995; Fischer et al. 2009). Previous studies performed on decapod crustaceans showed a selective retention of HUFA during embryogenesis (Rosa et al. 2007, Rey et al. 2015). The lower levels of essential FAs (e.g., ARA, EPA, DHA) in stage 3 embryos from 2013 suggests an use of these FAs as complementary energetic source, likely giving origin to larvae with a lower fitness in their early pelagic life (Nates and McKenney Jr 2000; Anger 2001; Rosa et al. 2005; Giménez 2006).

Furthermore, PLs comprise the major lipid class in brachyuran embryos, playing an essential role on embryonic development (Tocher 1995, Coutteau et al. 1997). The lower quantity of PLs displayed by stage 3 embryos from 2013, could also be a reflection of osmotic stress (Giménez and Anger 2001). In this sense, osmotic stress may produce compensatory modifications of the N metabolism due to shifts in energy utilization by embryos (Schoffeniels & Gilles 1970). Hence, the low levels of PLs in 2013 compared to 2012 could show a plastic response in the use of energy for osmoregulation. This mechanism can generate larvae with lower levels of C and N, due to higher respiration rate at low salinities (Giménez and Anger 2001), which will display lower chances of survival in the pelagic environment (Anger 2001).

In conclusion, while the biochemical profile of *C. maenas* embryos was not related with female size, interannual variability was recorded in the FA profile of embryos about to hatch. Natural conditions prevailing during embryonic development (e.g., salinity, temperature) shaped the catabolism of developing embryos and can significantly affect the biochemical composition of newly hatched larvae (as these will closely reflect the profile of stage 3 embryos). This finding suggests a plastic response in embryogenesis by *C. maenas* as a function of environmental conditions in the maternal habitat.

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2.3 NO MATTER IF YOU ARE BIG OR SMALL, FATTY ACID PROFILES REVEAL THAT LATE BREEDING FEMALES OF CARCINUS MAENAS *DISPLAY A HIGHER MATERNAL PROVISIONING*

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2.3.1 ABSTRACT

Maternal effects and larval experience are considered the most important sources of variability on offspring phenotypes in marine invertebrates with bi-phasic life cycle, influencing subsequent life history stages and population dynamics. The complex life cycle of marine invertebrates make difficult to find a direct connection between these effects and individual performance. Maternal provisioning during oogenesis is the starting point of a series of processes that will ultimately affect offspring phenotype. In decapod crustaceans females transfer most of their internal reserves to the egg yolk, with larval fitness at hatching varying with its biochemical composition and catabolism during embryogenesis. In this study, we analysed the effect of female size and reproductive season on the fatty acid (FA) profile of newly extruded (stage 1) and ready to hatch (stage 3) embryos. We surveyed embryonic FA profile of *Carcinus maenas* females during two consecutive reproductive seasons early reproductive season (ERS) and late reproductive season (LRS)). Results showed that female size played no significant effect on the FA profile of embryos. However, reproductive season had a significant effect on embryos FAs, with those from LRS displaying higher lipid content, namely a larger amount of phospholipids and highly-unsaturated FA (HUFA), when compared with embryos from ERS. The relevant role played by HUFA in early larval life suggests that reproductive season acts as source of variability in offspring phenotype and performance, with maternal effects in *C. maenas* being mostly linked with the nutritional status of females rather than their size.

2.3.2 INTRODUCTION

Do larger females produce a better offspring? It is certainly difficult to find only one answer to this question, namely when addressing marine invertebrates with complex life cycles. Organisms allocate their energy between survival, growth and reproduction. Although with considerable differences between groups, decapod crustaceans allocate a high proportion of their energy to reproductive investment (e.g., gonad maturation, parental care) (Hartnoll 2006). As the energy allocated to reproduction is not available for somatic growth, this partitioning may promote contrasting performances in females displaying different sizes, as small females need to invest more energy in somatic growth

(Green et al. 2014). Eventually, this constraint can be reflected in offspring, namely through a lower fecundity or reproductive investment in embryonic reserves (Ramirez Llodra 2002, Tallack 2007). Fecundity depends on environmental condition (e.g., food availability, temperature), as well as on species biology (e.g., body size, reproductive period) (Hines 1982, Ouellet & Plante 2004). In decapod crustaceans, small females display a lower fecundity, in part due to their hard exoskeleton which physically constraints available incubation space (Hines 1982, Ramirez Llodra 2002). However, knowledge on the relationship between female size and reproductive investment is still limited. While some studies concluded that larger females are able to produce offspring with better fitness (Sibert et al 2004; Moland et al 2010; Sato and Suzuki 2010; Wieland and Siegstad 2012), others found no relationship between female size and offspring fitness (Koopman & Siders 2013, Swiney et al. 2013). Moreover, Gardner (1997) even recorded a decline in the fecundity displayed by larger crabs due to an increase in embryos size.

The role that females phenotype plays in offspring fitness is complex and difficult to unravel (Bernardo 1996, Marshall et al. 2008). Maternal features interact with several factors (e.g., environmental conditions, food availability, natural selection, reproductive selection) which drives female features (e.g., size) to act as important sources of variability in offspring phenotypes and performance (Marshall et al. 2008, Renborg et al. 2014). In species displaying extended reproductive periods, shifts in biotic and abiotic factors may affect embryonic and larval quality (Wu et al. 2010, Andrés et al. 2010, Verísimo et al. 2011, Rotllant et al. 2014). Indeed, these shifts may reflect phenotypic differences in offspring that may influence settlement and juvenile performance (Giménez & Anger 2003, Giménez 2010) and, consequently, populations dynamics (Burgess & Marshall 2011).

The green crab *Carcinus maenas* (L.) display a bi-phasic life cycle, with a benthic juvenile/adult and planktonic larval stages. The tolerance displayed by these species to a range of temperatures, salinities and other abiotic factors, as well as its high fecundity and long larval development, makes *C. maenas* one of the most successful coastal marine invaders in the word (Yamada 2001, Klassen & Locke 2007, Darling et al. 2008). This species presents a long reproductive period (Queiroga 1995, Yamada 2001, Leignel et al.

2014), separated by two reproductive seasons: an early reproductive season (ERS) during winter and a late reproductive season (LRS) over the summer (Lyons et al. 2012). The contrasting oceanographic conditions prevailing during the reproductive season of *C. maenas*, force newly hatched larvae released during ERS or LRS to develop under different environmental and trophic scenarios (e.g., temperature, food availability) (Oliveira et al. 2009, Álvarez et al. 2013). These variations may contribute to seasonal fluctuations in larval quality, settlement performance and recruitment (Giménez 2010, Calado & Leal 2015).

In the present study, the fatty acid (FA) profiles of *C. maenas* embryos were used to evaluate female reproductive investment over the reproductive season; ovigerous females displaying different sizes (small *vs.* large) and brooding either early or late stage embryos were used to estimate reproductive investment and determine the FA profile of developing embryos over ERS and LSR. The FA profiles of early stage embryos (stage 1) where employed as a proxy of maternal provisioning, while the FA profile of late stage embryos (stage 3) was employed as a proxy for the quality of newly hatched larvae. The following null hypotheses were tested: 1) maternal provisioning is homogeneous between small and large females; 2) maternal provisioning does not differ between ERS and LRS; 3) FA profiles of ready to hatch embryos does not differ between embryos from small and large females; and 4) FA profiles of ready to hatch embryos does not differ between ERS and LRS.

2.3.3 MATERIALS AND METHODS

2.3.3.1 SAMPLING

Ovigerous females of *C. maenas* were collected using trawl nets in Ria de Aveiro (Portugal) (40° 37’ 17’’ N, 8° 44’ 56’’ W), a coastal lagoon in the Atlantic west coast of Portugal. Female crabs were collected during the early reproductive season (ERS, during March 2013) and late reproductive season (LRS, during July 2013). In each reproductive season two criteria were used to classify collected females: their carapace width (CW, measured between the first pair of lateral spines of the carapace) and the development stage of

embryos being brooded. Females were classified as small when displaying a CW < 40 mm or large when CW ≥ 40 mm. Developing embryos were classified according to the following criteria (as defined by Rosa et al., 2007): stage 1 (newly extruded embryos) - uniform yolk and absence of cleavage and eyes; stage 3 (embryos ready to hatch in < 48 h) nearly no yolk present and embryo fully developed. In each reproductive season, twenty females were haphazardly selected, ten small females (ERS – CW: 34.11 ± 4.54 mm; LRS – CW: 35.86 ± 2.89 mm) of which five were brooding embryos in stage 1 and five in stage 3, and ten large females (ERS – CW: 44.66 ± 2.96 mm; LRS – CW: 45.02 ± 2.89 mm) of which five were brooding embryos in stage 1 and five in stage 3. Overall, 2 reproductive seasons (ERS and LRS) x 2 female sizes (small and large) x 2 embryonic stages (stage 1 and stage 3) x 5 replicates = 40 samples of embryos were collected for analysis. The embryos of each female were carefully removed with fine forceps. Immediately after collection 30 embryos from each sample were measured using a stereomicroscope and their volume determine using the formula $V = 4/3 (r^3)$ for spheroid embryos. After collection, all samples were freeze-dried and stored at - 32 °C for later biochemical analysis.

2.3.3.2 FATTY ACID ANALYSIS

Total lipids extracts were obtained using the Bligh and Dyer (1959) method. Briefly, freeze-dried embryos were resuspended in 1 mL of ultrapure water and 3.75 mL chloroform/methanol 1:2 (v/v) were added to the suspension that was vortexed and incubated on ice for 30 min. An additional volume of 1.25 mL chloroform was added along with 1.25 mL of ultrapure water. Following vigorous vortexing, samples were centrifuged at 1000 rpm for 5 min at room temperature to obtain a two-phase solution: an aqueous top phase and an organic bottom phase, from which lipids were retrieved. Lipid extracts were preserved in a nitrogen atmosphere at - 32 °C for posterior analysis.

In order to quantify the total amount of phospholipids, a phosphorus assay was performed according to Bartlett and Lewis (1970). The lipid extracts were resuspended in 300 µL of chloroform and 20 µL of the samples were used. After being dried with a nitrogen flow, the samples were resuspended in 0.650 mL of perchloric acid (70%), being incubated for 1 h at

180 °C. A total of 3.3 mL ultrapure water, 0.5 mL ammonium molybdate (2.5%) and 0.5 mL ascorbic acid (10%) were added to each sample, followed by incubation during 5 min at 100 °C in a water bath. Standards from 0.1 to 3.0 µg of phosphate (standard solution of $NaH_2PO_4.2H_2O$, 439 mg L^{-1} of water, i.e. 100 µg of phosphorus mL^{-1}) underwent the same treatment as the samples. Absorbance of standards and samples was measured at 800 nm, at room temperature, in a microplate UV-vis spectrophotometer.

Total FAs were analysed by gas chromatography-mass spectrometry (GC-MS) after transesterification of total lipid extract (20 µg of total phospholipids). FA methyl esters (FAMEs) were prepared using a methanolic solution of potassium hydroxide (2 M) according to the previously described method by Aued-Pimentel et al. (2004). The FAMEs were resuspended in 40 µL of hexane, with 2 µL of this hexane solution being used for GC-MS analysis on an Agilent Technologies 6890N Network (Santa Clara, CA) equipped with a DB-1 column with 30 m of length, 0.25 mm of internal diameter, and 0.1 µm of film thickness (J&W Scientific, Folsom, CA). The GC was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range *m/z* 40-500 in a 1 s cycle in a full scan mode acquisition. The oven temperature was programmed from an initial temperature of 90 °C, standing at this temperature for 0.5 min and following a linear increase to 220 °C at 20 °C/min, a linear increase at 2 °C/min to 240 °C, and 5 °C/min until reaching 250 °C. The injector was set at 220 °C and the detector at 230 °C. Helium was used as the carrier gas at a flow rate of 1.7 mL/min. The identification of FAMEs was performed by comparing the retention time and mass spectrum of each FAME, that was analysed with MS spectra of commercial FAME standards (Supelco 37 Component FAME Mix) and confirmed by comparison with the chemical database Wiley and the spectral library "The AOCS Lipid Library" (Christle 2012).

2.3.3.3 DATA ANALYSIS

Differences in the volume of early stage and late stage embryos sampled from females with different sizes and during different reproductive seasons were determined by a two-way ANOVA (factor female size: small and large; factor reproductive season: ERS and LRS) using the software R (R Development Core Team 2011). Prior to analysis, deviations from

normality in the response variable were tested using the Shapiro test and homogeneity of variance using the Levene´s test. Post hoc Tukey HSD test was used when the two-way ANOVA revealed significant differences ($p < 0.05$). Comparisons between the volume of early and late stage embryos were not perform as the significant increase in egg volume recorded during the embryogenesis of decapod crustaceans is already well documented (Sibert et al. 2004, Calado et al. 2005, Rosa et al. 2007).

The relative content of each FA was transformed in µg per unit of dry weight (DW) of embryos´ sample (mg), (µg mg^{-1} DW), using as reference the phospholipid quantification of each sample, since phospholipids comprise the major lipid classes in embryos. The sum of the total pool of FAs and the head group represents the total phospholipids quantified by embryos´ sample.

For statistical analysis, only the twenty more abundant FAs of the total pool were considered when comparing these molecules individually; for statistical analysis of FA class profiles all FAs were used. For a better understanding of our results, only the classes of FAs containing the most abundant FAs were considered, namely: Saturated FA (SFA), Monounsaturated FA (MUFA), Polyunsaturated FA (PUFA), Highly-unsaturated FA (HUFA), Branched FA (BrFA) and Epoxy FA (EpFA). While Polyunsaturated FA are commonly defined as all FAs with ≥ 2 double bonds, in the present study we discriminate between PUFA (FAs with 2 or 3 double bonds) and HUFA (FAs with ≥ 4 double bonds). Multivariate statistical analyses were performed to detect the existence of significant differences in overall FA profile of: 1) stage 1 embryos from small and large females; 2) stage 1 embryos from ERS and LRS; 3) stage 3 embryos from small and large females; and 4) stage 3 embryos from the ERS and LRS. Prior to statistical analysis, in order to down-weight the contributions of quantitatively dominant FAs, the raw data matrix was log (x +1) transformed. Following this transformation, a new matrix was assembled using Bray-Curtis similarity coefficient. Two-way analyses of similarities (ANOSIM) were used to test for significant differences between the individual FAs of stage 1 and stage 3 embryos. The factors used in the ANOSIM analysis were female size (with two levels: small and large) and reproductive season (with two levels: early and late). ANOSIM calculates a global R statistic that assesses

the differences between groups, where values close to one indicate maximum differences between groups and values near zero suggest complete groups overlap (Clarke & Gorley 2006). In order to visualize inter-individual differences in FA profiles, a Principal Coordinate Analysis (PCO) was performed, representing differences between reproductive seasons along the first two axes. Differences in FA profiles recorded between reproductive seasons were explored using the similarity percentages routine (SIMPER). All multivariate statistical tests were performed with Primer 6.1 with PERMANOVA add-on (Primer-E Ltd. Plymouth, UK).

A two-way ANOVA was used to determine the influence of maternal size and reproductive season in FA class profiles, *n*-3 and *n*-6 FAs in stage 1 and stage 3 embryos. Assumptions and post hoc comparisons were performed as described above for the two-way ANOVA performed to compare the volume of early and late stage embryos.

2.3.4 RESULTS

Embryos displayed an average (± standard deviation (SD)) volume of 0.0146 ± 0.0013 mm^3 in stage 1 and 0.0263 ± 0.0020 mm^3 in stage 3 (Table 2.3.1), no significant differences were recorded in the volume of embryos in the same stage originating from females with different sizes (stage 1: $P = 0.53$; stage 3: $P = 0.43$) or from different reproductive seasons (stage 1: $P = 0.19$; stage 3: $P = 0.31$).

The FA profile of embryos recorded from different female sizes (small and large), as well as and during ERS and LRS, are summarized in Table 2.3.2 (stage 1 embryos) and Table 2.3.3 (stage 3 embryos). The two-way ANOSIM analysis of the FA profiles of stage 1 embryos showed a significant effect of factor female size ($R = 0.186$, $P = 0.018$), as well as a significant effect of factor reproductive season ($R = 0.472$, $P = 0.001$). Concerning embryos in stage 3, the ANOSIM analysis of FA profiles showed no significant effect of the factor female size and a significant effect of the factor reproductive season (Size: $R = 0.078$, $P = 0.206$; Season: $R = 0.590$, $P = 0.001$).

The two-way ANOVA performed to analyse FA class profiles (Fig. 2.3.1) revealed no significant differences in embryos from small and large females in both embryonic stages; the sole exception recorded was in MUFA from stage 1 embryos, which presented significant differences between small and large females during LRS. The analysis of variance performed to compare the pool of *n*-3 FAs revealed no significant effects promoted by female size, for both embryonic stages (stage 1: $P = 0.071$; stage 3: $P = 0.555$). However, reproductive season significantly affected the levels of *n*-3 FAs present in embryos (stage 1: $P < 0.001$; stage 3: $P = < 0.001$). Concerning *n*-6 FAs, no significant differences were recorded in stage 1 embryos promoted by any of the factors being surveyed, while significant differences were recorded in stage 3 embryos sampled during ERS and LRS (stage 1- Size: $P = 0.058$, Season: $P = 0.846$; stage 3- Size: $P = 0.546$, Season: $P = 0.011$) (Table 2.3.2 and 2.3.3, stage 1 and 3, respectively).

The PCO analysis revealed the existence of a high variability in the FA profiles of stage 1 and stage 3 embryos sampled during ERS and LRS. In stage 1 embryos the first two axes of the PCO analysis of the FA profile explained > 66% of the FAs variation (PCO axis 1: 50.8%, PCO axis 2: 15.7%, Fig. 2.3.2.a), while in stage 3 embryos the first two axis explained > 73% (PCO axis 1: 60.9%, PCO axis 2: 12.6%, Fig. 2.3.2.b). The SIMPER analysis showed that the individual FAs which contributed more than 50% to discriminated between ERS and LRS in stage 1 embryos were: eicosapentaenoic acid (20:5*n*-3, EPA), docosahexaenoic acid (22:6*n*-3, DHA), stearic acid (18:0), palmitoleic acid (16:1*n*-7), palmitic acid (16:0), docosadienoic acid (22:2*n*-9), 12,15-epoxy-13,14-dimethyl-eicosadienoate (EpFA 1) and vaccenoic acid (18:1*n*-7) (complete list in Table 2.3.4). In stage 3 embryos, the individual FAs which contributed more than 50% to discriminated between ERS and LRS were: EPA, DHA, 16:0, 18:0, oleic acid (18:1*n*-9*c*) and 16:1*n*-7 (complete list in Table 2.3.4).

Table 2.3.1 Embryonic volume (mm^3) of *Carcinus maenas* embryos in stage 1 and stage 3, from small and large females sampled during consecutive reproductive seasons (early reproductive season (ERS) and late reproductive season (LRS)). Values are averages (± SD) of 30 embryos from five different females (*n*=5).

	ERS		LRS	
	Small	Large	Small	Large
Stage 1	0.014 ± 0.002	0.014 ± 0.001	0.015 ± 0.001	0.015 ± 0.002
Stage 3	0.025 ± 0.002	0.027 ± 0.001	0.027 ± 0.003	0.027 ± 0.002

Table 2.3.2 Fatty acid (FA) composition (µg mg^{-1} DW) of stage 1 embryos of the green crab *Carcinus maenas* recorded from two different female sizes (small and large) and two reproductive seasons (early reproductive season (ERS) and late reproductive season (LRS)). Values are averages (± SD) of embryos from five different females (*n*=5).

	ERS		LRS	
	Small	Large	Small	Large
16:0	8.70 ± 1.72	7.66 ± 0.73	11.42 ± 1.80	9.84 ± 1.87
18:0	2.22 ± 0.34	1.98 ± 0.14	4.44 ± 0.94	3.67 ± 0.77
Σ SFA[1]	**13.03 ± 2.27**	**11.42 ± 0.48**	**19.06 ± 3.35**	**15.85 ± 2.82**
16:1*n*-7	8.17 ± 1.58	6.59 ± 2.03	8.07 ± 1.76	4.93 ± 2.83
18:1*n*-9*c*	5.29 ± 0.81	4.79 ± 0.54	5.65 ± 1.23	4.47 ± 0.83
18:1*n*-7	2.83 ± 0.82	2.36 ± 0.36	3.36 ± 0.58	2.69 ± 0.43
20:1*n*-9	1.20 ± 0.48	1.01 ± 0.56	1.72 ± 0.42	1.35 ± 0.22
20:1*n*-7	1.37 ± 0.46	1.52 ± 0.44	1.45 ± 0.36	1.14 ± 0.25
Σ MUFA[2]	**20.66 ± 3.01**	**17.63 ± 2.63**	**22.22 ± 3.78**	**15.73 ± 3.01**
18:2*n*-6	0.48 ± 0.18	0.49 ± 0.17	0.56 ± 0.06	0.30 ± 0.05
18:3*n*-3[a]	0.70 ± 0.30	0.40 ± 0.18	0.71 ± 0.07	0.35 ± 0.07
18:3*n*-3[b]	0.77 ± 0.39	0.48 ± 0.18	0.75 ± 0.27	0.59 ± 0.12
22:2*n*-9	0.82 ± 0.30	0.44 ± 0.21	1.17 ± 0.47	0.88 ± 0.32
Σ PUFA[3]	**4.82 ± 0.76**	**3.35 ± 0.84**	**5.70 ± 1.57**	**3.95 ± 0.83**
20:4*n*-6	0.76 ± 0.21	1.09 ± 0.64	0.68 ± 0.44	0.66 ± 0.29
20:5*n*-3	4.75 ± 1.07	4.29 ± 0.79	9.24 ± 1.99	7.30 ± 1.26
22:5*n*-3	1.03 ± 0.20	0.93 ± 0.25	1.37 ± 0.29	0.97 ± 0.24
22:6*n*-3	2.42 ± 0.75	2.52 ± 0.68	4.93 ± 1.59	4.58 ± 1.63
Σ HUFA[4]	**10.29 ± 1.47**	**9.69 ± 1.19**	**18.13 ± 3.91**	**14.85 ± 3.33**
BrFA 1	1.55 ±0.37	1.14 ± 0.04	1.37 ± 0.25	0.96 ± 0.17
BrFA 2	1.19 ± 0.47	0.88 ± 0.17	1.13 ± 0.26	0.87 ± 0.14
Σ BrFA[5]	**4.04 ± 0.91**	**3.05 ± 0.62**	**3.72 ± 0.80**	**2.68 ± 0.36**
EpFA 1	0.76 ± 0.11	0.39 ± 0.18	1.05 ± 0.47	0.91 ± 0.35
Σ EpFA[6]	**0.82 ± 0.15**	**0.41 ± 0.22**	**1.25 ± 0.49**	**0.98 ± 0.39**
Σ (*n*-3)	10.40 ± 1.70	9.04 ± 0.97	18.17 ± 4.28	14.59 ± 3.24
Σ (*n*-6)	2.89 ± 0.35	2.61 ± 0.72	3.22 ± 0.68	2.39 ± 0.59

Abbrevations: Saturated FA (SFA), Monounsaturated FA (MUFA), Polyunsaturated FA (PUFA), Highly-unsaturated FA (HUFA), Branched FA (BrFA) and Epoxy FA (EpFA) represent the sum of all FAs identified as can see below:

[1] SFA: 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0, 22:0

[2] MUFA: 14:1*n*-5, 15:1*n*-1, 16:1*n*-13*t*, 16:1*n*-7, 16:1*n*-5, 7-methyl-hexadec-6-enoate, 17:1*n*-8, 18:1*n*-9*c*, 18:1*n*-9*t*, 18:1*n*-7, 19:1*n*-9*c*, 19:1*n*-8, 20:1*n*-9, 20:1*n*-7, 22:1*n*-11, 22:1*n*-9

[3] PUFA: 16:3*n*-6, 18:2*n*-6, 18:3*n*-3[a] (9*c*, 12*c*, 15*c* - octadecatrienoate), 18:3*n*-3[b] (9*c*, 11*t*, 15*c* - octadecatrienoate), 20:2*n*-9, 20:2*n*-7, 20:2*n*-6, 20:3*n*-4, 22:2*n*-9, 22:3*n*-6

[4] HUFA: 18:4*n*-3, 20:4*n*-6, 20:5*n*-3, 21:5*n*-3, 22:4*n*-6, 22:5*n*-6, 22:5*n*-3, 22:6*n*-3

[5] BrFA: 4,8,12-trimethyl-tridecanoate, 12-methyl-tetradecanoate (anteiso), 13-methyl-tetradecanoate (iso), 14-methyl-pentadecanoate (iso), 10-Methyl hexadecanoate, 14-methyl-hexadecanoate (anteiso), 15-methyl-hexadecanoate (iso), 16-methyl-heptadecanoate (iso), 16-methyl-octadecanoate (anteiso), 17-methyl-octadecanoate (iso)

[6]EpFA: 10,13-epoxy-11,12-dimethyl-octadecadienoate, 12,15-epoxy-13,14-dimethyl-eicosadienoate

BrFA 1: 14-methyl-hexadeanoate (anteiso)

BrFA 2: 15-methyl-hexadeanoate (iso)

EpFA 1 – 12,15-epoxy-13,14-dimethyl-eiosadienoate

Table 2.3.3 Fatty acid (FA) composition (µg mg^{-1} DW) of stage 3 embryos of the green crab *Carcinus maenas* recorded from two different female sizes (small and large) and two reproductive seasons (early reproductive season (ERS) and late reproductive season (LRS)). Values are averages (± SD) of embryos from five different females (*n*=5).

	ERS		LRS	
	Small	Large	Small	Large
16:0	3.80 ± 0.19	3.81 ± 0.55	5.73 ± 1.16	5.86 ± 0.91
18:0	1.24 ± 0.18	1.24 ± 0.20	2.46 ± 0.67	2.25 ± 0.41
Σ SFA[1]	**5.92 ± 0.33**	**5.85 ± 0.85**	**9.50 ± 1.98**	**9.35 ± 1.47**
16:1*n*-7	2.29 ± 0.29	2.87 ± 0.75	2.34 ± 0.64	2.60 ± 0.66
18:1*n*-9*c*	2.28 ± 0.57	2.35 ± 0.23	2.78 ± 0.58	3.03 ± 0.32
18:1*n*-7	1.28 ± 0.09	1.21 ± 0.07	1.60 ± 0.45	1.58 ± 0.26
20:1*n*-9	0.36 ± 0.06	0.27 ± 0.03	0.67 ± 0.43	0.56 ± 0.25
20:1*n*-7	0.58 ± 0.16	0.63 ± 0.10	0.75 ± 0.41	0.71 ± 0.20
Σ MUFA[2]	**7.44 ± 0.97**	**7.99 ± 0.90**	**8.94 ± 2.30**	**9.15 ± 1.23**
18:2*n*-6	0.11 ± 0.07	0.25 ± 0.06	0.27 ± 0.08	0.27 ± 0.17
18:3*n*-3[a]	0.22 ± 0.08	0.11 ± 0.03	0.34 ± 0.17	0.21 ± 0.22
18:3*n*-3[b]	0.21 ± 0.06	0.29 ± 0.14	0.35 ± 0.09	0.35 ± 0.13
22:2*n*-9	0.15 ± 0.15	0.21 ± 0.07	0.33 ± 0.16	0.21 ± 0.14
Σ PUFA[3]	**1.52 ± 0.28**	**1.70 ± 0.33**	**2.46 ± 0.70**	**1.91 ± 0.95**
20:4*n*-6	0.39 ± 0.06	0.43 ± 0.10	0.60 ± 0.18	0.56 ± 0.21
20:5*n*-3	2.71 ± 0.53	2.30 ± 0.60	5.09 ± 1.49	4.63 ± 1.08
22:5*n*-3	0.47 ± 0.09	0.35 ± 0.09	0.56 ± 0.16	0.49 ± 0.08
22:6*n*-3	1.37 ± 0.51	1.98 ± 0.47	2.95 ± 0.77	2.74 ± 0.72
Σ HUFA[4]	**5.40 ± 1.16**	**5.44 ± 0.94**	**9.97 ± 2.46**	**9.03 ± 1.54**
BrFA 1	0.55 ± 0.07	0.50 ± 0.09	0.48 ± 0.26	0.60 ± 0.14
BrFA 2	0.40 ± 0.08	0.37 ± 0.05	0.56 ± 0.14	0.53 ± 0.11
Σ BrFA[5]	**1.54 ± 0.28**	**1.44 ± 0.31**	**1.58 ± 0.28**	**1.70 ± 0.35**
EpFA 1	0.39 ± 0.13	0.14 ± 0.06	0.42 ± 0.21	0.25 ± 0.14
Σ EpFA[6]	**0.40 ± 0.13**	**0.15 ± 0.08**	**0.45 ± 0.18**	**0.25 ± 0.14**
Σ (*n*-3)	5.14 ± 1.19	5.16 ± 0.90	9.66 ± 2.56	8.70 ± 1.75
Σ (*n*-6)	1.16 ± 0.12	1.32 ± 0.31	1.79 ± 0.25	1.47 ± 0.45

Abbreviations: See Table 2.3.2

Table 2.3.4 Similarity percentage analysis (SIMPER) identifying the fatty acids (FAs) that contribute to the differences recorded in FA profiles of embryos in stage 1 and stage 3 of *Carcinus maenas* sampling during two consecutive reproductive seasons (early reproductive season (ERS) and late reproductive season (LRS)).

	ERS *vs* LRS Stage 1			ERS *vs* LRS Stage 3	
Fatty acids	Ind (%)	Cum (%)	Fatty acids	Ind (%)	Cum (%)
20:5*n*-3	9.91	9.91	20:5*n*-3	12.97	12.97
22:6*n*-3	9.00	18.91	22:6*n*-3	9.37	22.34
18:0	8.42	27.32	16:0	9.11	31.45
16:1*n*-7	7.69	35.01	18:0	9.11	40.56
16:0	5.25	40.27	18:1*n*-9*c*	4.88	45.44
22:2*n*-9	3.85	44.12	16:1*n*-7	4.72	50.16
EpFA 1	3.71	47.83	20:1*n*-9	4.04	54.21
18:1*n*-7	3.53	51.36	18:1*n*-7	3.42	57.63
20:4*n*-6	3.39	54.75	20:1*n*-7	2.81	60.44
18:1*n*-9*c*	3.34	58.09	EpFA 1	2.67	63.11
21:5*n*-3	3.15	61.24	20:4*n*-6	2.45	65.56
20:1*n*-7	2.76	64.00	17:0	2.45	68.00
22:3*n*-6	2.46	66.46	18:3*n*-3[a]	2.42	70.43
14:0	2.41	68.87	22:2*n*-9	2.23	72.66
BrFA 1	2.33	71.20	18:2*n*-6	2.08	74.74
22:5*n*-3	2.31	73.51	BrFA 2	2.07	76.81
18:3*n*-3[a]	2.28	75.79	18:3*n*-3[b]	2.03	78.84
18:3*n*-3[b]	2.28	78.06	BrFA 1	2.02	80.85
BrFA 2	2.15	80.21	22:5*n*-3	1.90	82.76
20:2*n*-9	2.04	82.25	20:2*n*-6	1.82	84.57
15:0	1.69	83.94	15:0	1.80	86.38
20:2*n*-6	1.68	85.63	22:3*n*-6	1.80	88.17
18:4*n*-3	1.64	87.27	18:4*n*-3	1.60	89.77
18:2*n*-6	1.63	88.89	7-Me-16:1*n*-10	1.51	91.29
17:0	1.60	90.49			

Abbreviations:
BrFA 1: 14-methyl-hexadeanoate (anteiso)
BRFA 2: 15-methyl-hexadeanoate (iso)
EpFA 1: 12,15-epoxy-13,14-dimethyl-eiosadienoate
7-Me-16:1*n*-10: 7-methyl-hexadec-6-enoate.
18:3*n*-3[a] (9*c*, 12*c*, 15*c* - octadecatrienoate)
18:3*n*-3[b] (9*c*, 11*t*, 15*c* - octadecatrienoate)

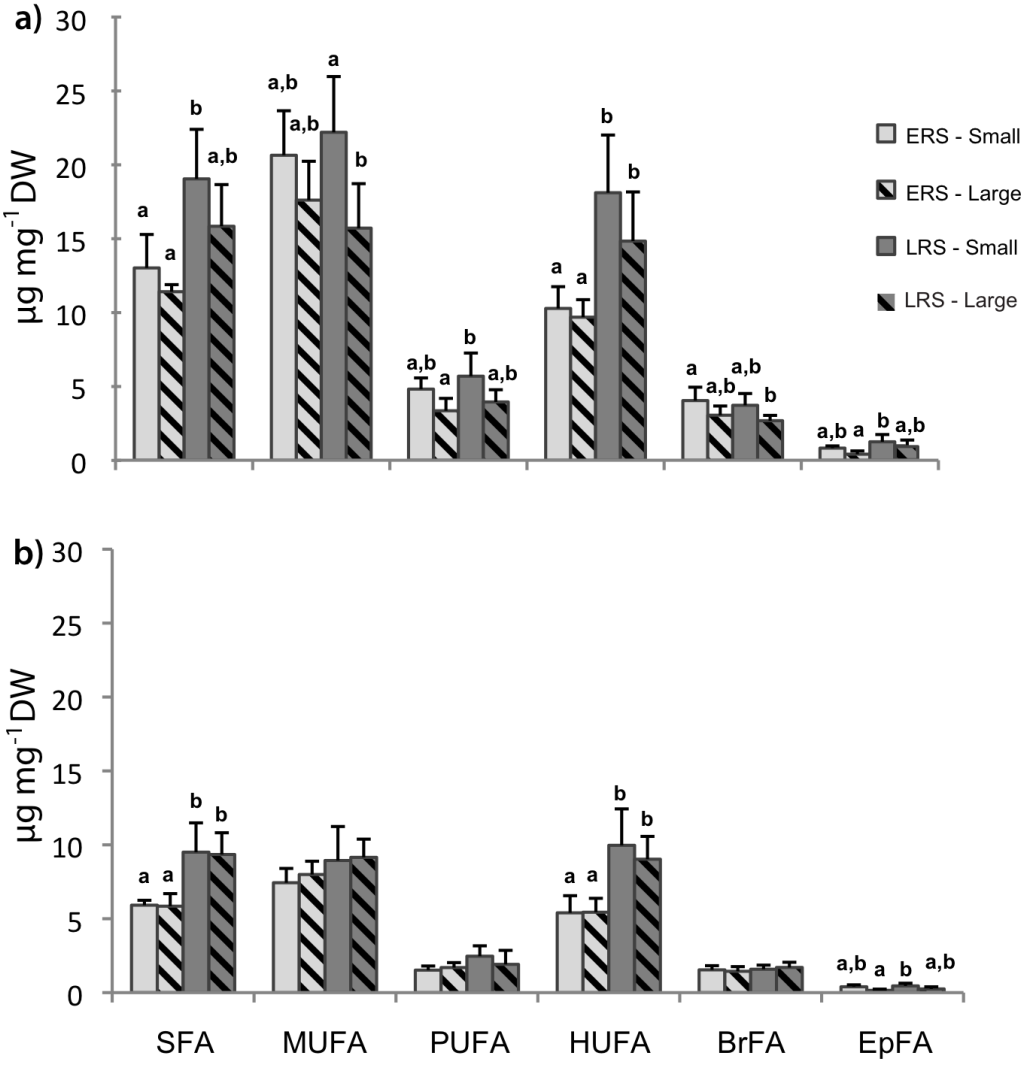

Figure 2.3.1 (a) Fatty acid (FA) class profiles of *Carcinus maenas* embryos in stage 1 and (b) stage 3, from small and large females sampled during consecutive reproductive seasons, early reproductive season (ERS) and late reproductive season (LRS) (µg mg^{-1} DW). Error bars represent standard deviation of five independent samples. Small letters on the top of the bars indicate means that are significantly different from each other, in the same FA class (Tukey-HSD, $P < 0.05$).

Abbreviations: SFA – saturated FA; MUFA – monounsaturated FA; PUFA – polyunsaturated FA; HUFA – Highly-unsaturated FA; BrFA – Branched FA; EpFA – Epoxy FA.

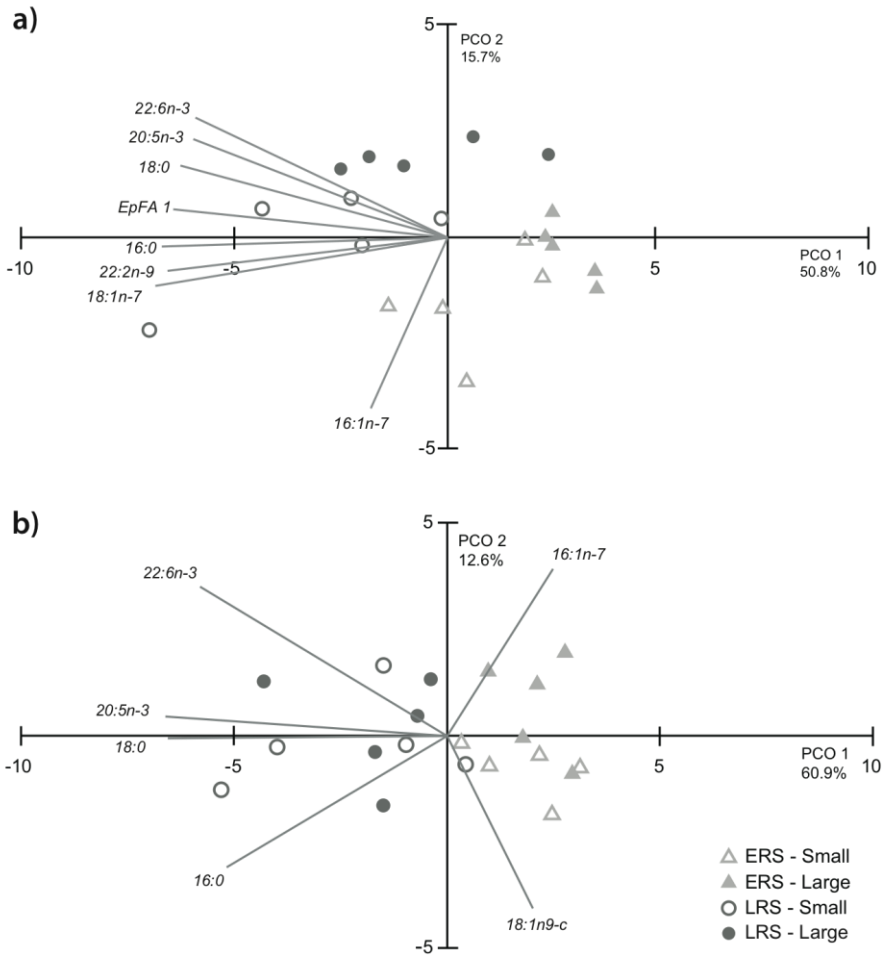

Figure 2.3.2 (a) Principal coordinates analysis (PCO) comparing the fatty acid (FA) composition of *Carcinus maenas* embryos in stage 1 and (b) stage 3, from small and large females sampled during consecutive reproductive seasons, early reproductive season (ERS) and late reproductive season (LRS). Vectors displayed represent individual FAs that contributed in more than 50% to distinguish between ERS and LRS samples.

Abbreviations:

EpFA 1: 12,15-epoxy-13,14-dimethyl-eiosadienoate

2.3.5 DISCUSSION

Embryo and offspring size have long been used as proxies to measure parental investment in both theoretical and empirical studies (Bernardo 1996, Rius et al. 2010). Furthermore, in marine invertebrates, it has been suggested that embryonic features reflected larval fitness and settlement performance (Sasaki et al. 1986, Giménez & Anger 2001, 2003, Sibert et al. 2004). In the present study, the analysis of the volume displayed by *C. maenas* embryos showed an increase of ~ 45% during the embryogenesis. This expected finding is likely related with embryonic development and increase in water content as a consequence of osmotic water uptake and/or metabolic water retention (Charmantier & Charmantier-Daures 2001, Calado et al. 2005, Rosa et al. 2007). However, egg size was not influenced by female size, with the positive relation between female size and embryo size reported for decapod species (Sibert et al. 2004, Moland et al. 2010) not being recorded. On the other hand, egg size was not influenced by reproductive season. In species that reproduce throughout the year, it is common to record females producing larger embryos during cooler winter months (winter eggs) and smaller embryos during warmer summer months (winter eggs) (Sampedro et al. 1997, Oh & Hartnoll 2004); these previous authors refer to this strategy as a way to maximise fecundity, but this feature was not recorded for *C. maenas* in the present study. These results indicated that egg size is not always related with egg energy content (Calado et al. 2005, Fischer et al. 2009) and may not be a suitable proxy to infer offspring quality.

The results reported in the present work allow us to accept our first null hypothesis and reject the second one: maternal provisioning is homogeneous between small and large females but differs between ERS and LRS. These results suggest that maternal size is not a key factor in maternal provisioning of *C. maenas* embryos. Although, in stage 1 embryos the analysis of FA profiles showed significant differences for factor female size ($P = 0.018$), the low value displayed by R ($R = 0.186$) indicates that this difference may rather be the result of random grouping and natural variability of our samples (Clarke & Gorley 2006). Moreover, the ANOVA analysis only revealed significant differences among female sizes for MUFA present in stage 1 embryos from LRS.

Most lipidomic metabolism of brachyuran crab females is destined to store lipid reserves, which will be used over embryogenesis to fuel embryonic development (Herring 1974, Guisande & Harris 1995). *Carcinus maenas* is an opportunistic feeder with at least 104 families of marine organisms being known to be part of its natural diet (Baeta et al. 2006, Leignel et al. 2014). Once yolk composition is influence by maternal status and feeding regimens (Tuck et al. 1997, Racotta et al. 2003, Calado et al. 2010), the absence of differences in maternal provisioning between females with different sizes suggests that small and large females have access to diets of similar quality (Baeta et al. 2006). An indicator of quality in reproductive female diet is the amount of HUFA available for maternal provisioning, as a diet with low levels of these FAs can decrease the reproductive performance and negatively affect embryonic development (Cahu et al. 1995). The lack of differences in HUFA content recorded between embryos originating from small and large females confirms the existence of a similar and size-independent maternal provisioning in *C. maenas* (Koopman & Siders 2013, Swiney et al. 2013).

Furthermore, the FA profiles of stage 1 embryos showed significant shifts between ERS and LRS, suggesting an enhancement of maternal provisioning along the reproductive season. Females that reproduce during ERS have not yet built up enough reserves in their bodies to allocate to reproduction, mostly due to less favourable environmental conditions (e.g., lower temperature, lower food available) than those experienced by females during LRS (Verísimo et al. 2011). Estuaries, as Ria de Aveiro, are highly productive ecosystems, whose dynamics are influenced by environmental gradients (Rodrigues et al. 2012, Basset et al. 2013). The succession of seasons generates a progression of environmental conditions (e.g., temperature, hydrological regimens, nutrients availability) (Lopes et al. 2007, Álvarez et al. 2013), determining the dynamics of biological communities (Moreira et al. 1993, Cunha et al. 1999, Leandro et al. 2006). These seasonal variations promote changes in estuarine food webs, which can condition the quality and availability of certain prey types for *C. maenas* females (Baeta et al. 2006). As explained above, shifts in food quantity and/or quality promote differential female investments in the build-up of yolk reserves (Guisande & Harris 1995). Therefore, it is legitimate to affirm that the biochemical profile

of the yolk present in stage 1 embryos reflects seasonal shifts in the nutritional value and availability of food for *C. maenas* females.

The analysis of individual FA and FA class profiles in stage 3 embryos allow us to accept the third null hypothesis and reject the fourth: FA profiles of ready to hatch embryos did not differ between small and large females, but changed over the reproductive season. The majority of decapod crustaceans brood highly-packed embryo masses which increase in volume during embryogenesis (Rosa et al. 2007). Since oxygen is a limiting factor in egg masses of marine invertebrates, ovigerous females of brachyuran crabs perform specific and energetically demanding behaviours to provide suitable levels of oxygen to the whole embryonic mass (Fernández et al. 2000). According with our data, while small females have less experience in reproduction (it is possible that some of the small females sampled in the present study were primiparous, once the size of female maturity is 15 - 31 mm (Crothers 1967)), this is not a disadvantage for their offspring. The mode of growth of decapod crustaceans is through successive moults. Reproductive females face a trade-off between amount of energy for reproduction and growth (Green et al. 2014). Large females tend to moult less often, consequently have more energetic resource to invest in reproduction (Ouellet & Plante 2004). This has commonly been the rationale used to justify a lower reproductive investment in smaller females (Calder 1984). In this way, as already proposed by Marshall et al. (2010), the idea that small females produce a constrained offspring goes against an evolutionary perspective. Indeed, if offspring from small females have a lower fitness because they are smaller or have lower energy levels, females should profit more by allocating resources to their own somatic growth to overcome the low quality of their offspring. In this sense, if larvae from smaller females presented a lower fitness, the resources that young females allocate to reproduction will never be positively reflected in adult populations.

Seasonal analysis of FA profiles showed an increase of energetic reserves devoted to reproduction in embryos from LRS (Andrés et al. 2010, Verísimo et al. 2011). This difference may be explained by shifts in environmental conditions (e.g., temperature), which shape food quality and availability. A higher amount of phospholipids in embryos

from LRS indicate an important investment of breeding females on their offspring (Clarke 1982). Differences in maternal provisioning may directly affect later life history stages (Giménez 2006), as during early larval life lipid reserves may determine the chances of survival under suboptimal trophic and environmental conditions (Coutteau et al. 1997, Palacios et al. 1999). The presence of higher levels of important FAs (e.g., HUFA, DHA, EPA, *n*3 FAs) in stage 3 embryos from LRS suggests that offspring spawned during this period will likely display a higher fitness and be more resilient to suboptimal conditions (e.g., starvation periods) (Anger 2001, Torres et al. 2002).

Maternal effects play a key role on offspring phenotype, influencing important stages of their life cycle and acting as source of variation in marine populations (Pechenik 2006, Marshall et al. 2008, 2010, Fischer et al. 2011). In this sense, offspring success can be determined before hatching. As already referred, the European populations of *C. maenas* show a reproductive period with two breeding seasons (winter and summer) (Queiroga 1995, Neves et al. 2006, Lyons et al. 2012). Newly hatched larvae will therefore have to endure contrasting oceanographic conditions according to the timing of their release over extended reproductive seasons (Villegas-Ríos et al. 2011, Álvarez et al. 2013). Larvae hatching during ERS will likely face lower water temperatures and a reduced food availability (Oliveira et al. 2009, Álvarez et al. 2013), which will consequently result in an extended period of pelagic life in the plankton (Dawirs 1985). A longer pelagic development may increase dispersal distances (Shanks et al. 2003), but may also reduce offspring fitness (Burgess et al. 2012). Nevertheless, *C. maenas* is likely to achieve a fine tuned balance between larval dispersal and quality at settlement that allows this species to be a successful marine invader (Yamada 2001, Leignel et al. 2014).

In conclusion, this study suggests that the timing of offspring production along the reproductive season can play a more important role in the shaping of adult populations of *C. maenas* than female size. Future studies should focus in the dissociation of maternal effects and larval history in the post-settlement success of benthic marine invertebrates with complex life cycles.

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*2.4 UNRAVELLING POLAR LIPIDS DYNAMICS DURING EMBRYONIC DEVELOPMENT OF TWO SYMPATRIC BRACHYURAN CRABS (*CARCINUS MAENAS *AND* NECORA PUBER*) USING LIPIDOMICS*

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Omega 3

Mass spectrometry

2.4.1 ABSTRACT

Embryogenesis is an important stage of marine invertebrates with bi-phasic life cycles, as it conditions their larval and adult life. Throughout embryogenesis, phospholipids (PL) play a key role as an energy source, as well as constituents of biological membranes. However, the dynamics of PL during embryogenesis in marine invertebrates is still poorly studied. The present work used a lipidomic approach to determine how polar lipid profiles shift during embryogenesis in two sympatric estuarine crabs, *Carcinus maenas* and *Necora puber*. The combination of thin layer chromatography, liquid chromatography – mass spectrometry and gas chromatography – mass spectrometry allowed us to achieve an unprecedented resolution on PL classes and molecular species present on newly extruded embryos (stage 1) and those near hatching (stage 3). Embryogenesis proved to be a dynamic process, with four PL classes being recorded in stage 1 embryos (68 molecular species in total) and seven PL classes at stage 3 embryos (98 molecular species in total). The low interspecific difference recorded in the lipidomic profiles of stage 1 embryos appears to indicate the existence of similar maternal investment. The same pattern was recorded for stage 3 embryos revealing a similar catabolism of embryonic resources during incubation for both crab species.

2.4.2 INTRODUCTION

The European green crab *Carcinus maenas* and the velvet swimming crab *Necora puber* are two sympatric brachyuran crabs that commonly occur in estuarine habitats of Western Europe (D'Udekem d'Acoz 1999). *Carcinus maenas* is a keystone species commonly used as a model in ecological studies, whereas *N. puber* is a commercially important species for coastal fisheries. Brachyuran crabs commonly display a bi-phasic life cycle, with a pelagic larval phase developing in open ocean waters and a benthic post-larval phase that occurs in coastal and estuarine habitats (Giménez 2010). Decapod embryos are incubated in the abdomen of females (with the exception of penaeiod shrimps); being lecithotrophic throughout their embryonic development, they solely rely on the catabolism of yolk reserves originating from maternal investment for energy and organogenesis (Anger 2001).

Lipid reserves catabolism is the main energetic pathway that fuels embryonic development in decapod crustaceans (Sasaki et al. 1986). However, most studies performed so far on crustacean embryos solely focus their fatty acid (FA) profile (Rosa et al. 2007) and commonly overlook polar lipids. Phospholipids (PL) are important polar lipids being the major constituent of biological membranes and involved in a range of cellular functions (e.g., stabilization of proteins within the membrane, protein folding and cofactors in enzymatic reactions) (Lykidis 2007). Moreover, they are also precursors of biologically active mediators which play important functions at metabolic and physiologic levels (e.g., eicosanoids, diacylglycerols, inositol phosphates) (Tocher et al. 2008). PL are also essential for the absorption, transport and storage of lipids, acting as a rich source of essential FAs (EFAs) more than neutral lipids such as triacylglycerols (TAG) (Tocher 1995).

In the present study we used a lipidomic approach, combining the use of thin layer chromatography (TLC), liquid chromatography – mass spectrometry (LC-MS/MS) and gas chromatography – mass spectrometry (GC-MS), to analyse the PL profile of *C. maenas* and *N. puber* during embryogenesis. We compared resource partitioning and maternal investment between these two sympatric and phylogenetically close species (both are members of family Portunidae (Board 2015)) by analysing newly extruded embryos. At this stage, lipid profiles closely reflect maternal diets and can be considered a reliable proxy of quantitative and qualitative maternal investment (Harrison 1990, Calado et al. 2010). Additionally, we also analysed embryos close to hatching in order to unravel any interspecific differences in lipid dynamics during embryogenesis, with emphasis to PL classes and their molecular species.

2.4.3 MATERIALS AND METHODS

2.4.3.1 SAMPLING

Ovigerous females of *C. maenas* and *N. puber* (carapace width, average ± SD, 50.2 ± 2.5 mm) were collected in the mussel beds of Mira Channel, Ria de Aveiro (Portugal) (40°38'26.30"N, 8°43'58.90"W) during March (early spring) 2012. Embryos were classified according to Rosa *et al*. (2007): stage 1 (newly extruded embryos) - uniform yolk, no

cleavage or eyes; stage 3 (embryos ready to hatch in < 48 h) nearly no yolk present and embryo fully developed. Three females carrying embryos at stage 1 and three carrying embryos at stage 3 were selected for each species and their egg mass removed with fine forceps. All collected samples were freeze-dried and stored at -32 °C for biochemical analysis.

2.4.3.2 LIPID EXTRACTION

The Bligh and Dyer method (1959) was used to isolate total lipids from embryos. Samples were resuspended in glass centrifuge tubes using 1 mL of ultrapure water and 3.75 mL of chloroform/methanol (1:2, V/V) and incubated on ice for 30 min. The samples were centrifuged at 2000 rpm for 10 min at room temperature to resolve a two–phase system: an aqueous upper phase which contained the non-lipid components and an organic lower phase from where the lipids were recovered. The extraction was repeated twice. The organic phases were dried under a nitrogen stream. Lipid extracts were preserved at -20 °C for further analysis.

2.4.3.3 QUANTIFICATION OF PHOSPHOLIPIDS BY PHOSPHORUS ASSAY

Quantification of PL in the total lipid extract and in the spots separated by TLC was performed according to Bartlett and Lewis (1970). Samples were put on acid-washed glass tubes and resuspended in 0.650 mL of perchloric acid (70%, m/V). Glass tubes were incubated for 60 min at 180 °C in a heating block. After incubation, 3.3 mL of water, 0.5 mL of ammonium molybdate (2.5%, m/V) and 0.5 mL of ascorbic acid (10%, m/v) were added. After each addition, the mixture was well homogenized in a vortex mixer and incubated during 5 min at 100 °C in a water bath. Standards from 0.1 to 3.0 µg of phosphate (standard solution of $NaH_2PO_4 \cdot 2H_2O$, 439 mg L^{-1} of water, i.e. 100 µg of phosphorus mL^{-1}) underwent the same treatment as the samples. Absorbance of standards and samples was measured at 800 nm, at room temperature, in a microplate UV-vis spectrophotometer. The relative abundance of each PL class was calculated by the relation of the amount of phosphorus in each spot to the phosphorus amount of the total lipid extract in the sample applied in the TLC spot.

2.4.3.4 SEPARATION OF PHOSPHOLIPIDS CLASSES BY THIN LAYER CHROMATOGRAPHY (TLC)

The TLC method was used to separate the PL from the total lipid extract using TLC silica gel plates with concentration zone. Initially, the plates were washed with chloroform/methanol (1:1, V/V) and activated (sprayed) with 2.3% of boric acid in ethanol and dried for 30 min at 100 °C in an oven. The samples (20 µL of chloroform solution containing 30 µg of PL) were applied on the TLC plate and eluted with chloroform/ethanol/water/triethylamine (30:35:7:35, V/V/V/V). After the elution, the PL spots were revealed by spraying with a primuline solution (50 µg in 10 mL of acetone/water, 80:20, V/V) and visualized with a UV lamp (246 and 366 nm) (Fuchs et al. 2011, Alves et al. 2013) The identification of PL spots was accomplished by using PL standards (phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), lysophosphatidylcholine (LysoPC), phosphatidylinositol (PI), cardiolipin (CL)) from Avanti® Polar Lipids, Inc. (Alabaster, AL, USA), applied on the TLC plate. Spots coincident with the migration of standards were scraped into glass tubes and quantified as described above.

2.4.3.5 HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY ELECTROSPRAY IONIZATION – MASS SPECTROMETRY (HILIC-ESI-MS) OF THE TOTAL LIPID EXTRACTS

HILIC analysis of total lipid extracts was performed on a Waters Alliance 2690 HPLC system (Waters Corp., Milford, MA, USA) coupled to a Finnigan LXQ electrospray linear ion trap mass spectrometer (Thermo Fisher, San Jose, CA, USA). Mobile phase A consisted of 50% acetonitrile, 25% methanol, and 25% water with 10 mM ammonium acetate, and mobile phase B consisted of 60% acetonitrile and 40% methanol with 10 mM ammonium acetate. The lipid extracts (25 µg) were diluted in mobile phase B (90 µL) and 10 µL of the reaction mixture was introduced into an Ascentis Si HPLC Pore column (150 mm x 1.0 mm, 3 µm; Sigma-Aldrich). The solvent gradient was programmed as follows: gradient started with 0% of A and 100% of B, linearly increased to 100% of A in 20 min, and isocratically held for 35 min, returning to the initial conditions in 5 min. The flow rate through the column was 7.5 µL min^{-1} obtained using a pre-column split (Accurate, LC Packings, San Francisco, CA, USA). PL internal standards were purchased from Avanti®

Polar Lipids, Inc. (Alabaster, AL, USA) and used without further purification: 1',3'-bis[1,2-dimyristoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (CL); 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (dMPC); 1-nonadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LysoPC); 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (dMPE); 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-myo-inositol) (dPPI); and N-(heptadecanoyl)-sphing-4-enine-1-phosphocholine (SM). Polar lipid analysis was carried out by negative-ion electrospray ionization mass spectrometry (ESI-MS) on the Finnigan LXQ linear ion trap mass spectrometer. The electrospray voltage was 4.7 kV, the capillary temperature was 275 °C, and the sheath gas (He) flow rate was 25 units. A precursor ion isolation width of 0.5 *m/z* units was used, with a 30 ms activation time for MS/MS experiments. Full scan MS spectra and MS/MS spectra were acquired with a maximum ionization time of 50 ms and 200 ms, respectively. The normalized collision energy (CE) varied between 17 and 20 (arbitrary units) for MS/MS. The data were acquired and the results were treated with the Xcalibur® Data System 2.0 (Thermo Scientific, San Jose, CA, USA)(Santinha et al. 2013).

2.4.3.6 FATTY ACID ANALYSIS BY GAS CHROMATOGRAPHY – MASS SPECTROMETRY (GC-MS)

Total FAs were analysed by GC-MS after transesterification of embryos' total lipid extracts (20 µg of total PL). FA methyl esters (FAMEs) were prepared using a methanolic solution of potassium hydroxide (2.0 M) according to the previously described method (Aued-Pimentel et al. 2004). FAMEs were resuspended in 40 µL of hexane, with 2 µL of this hexane solution being used for GC-MS analysis on an Agilent Technologies 6890N Network (Santa Clara, CA) equipped with a DB-1 column with 30 m of length, 0.25 mm of internal diameter, and 0.1 µm of film thickness (J&W Scientific, Folsom, CA). The GC-MS was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range *m/z* 40-500 in a 1 s cycle in a full scan acquisition mode. The oven temperature was programmed from an initial temperature of 90 °C, standing at this temperature for 0.5 min and following a linear increase to 220 °C at 20 °C/min, a linear increase at 2 °C/min to 240 °C, and 5 °C/min until reaching 250 °C. The injector and detector temperatures were 220 and 230 °C,

respectively. Helium was used as the carrier gas at a flow rate of 1.7 mL/min. FAME identification was performed by comparing their retention time and mass spectrum, which was analysed with MS spectra of commercial FAME standards (Supelco 37 Component FAME Mix) and confirmed by comparison with the chemical database Wiley and the spectral library "The AOCS Lipid Library" (Christle 2012). FA profile was classified in seven FA classes: Saturated FA (SFA), Monounsaturated FA (MUFA), Polyunsaturated FA (PUFA), Highly-unsaturated FA (HUFA), Branched FA (BrFA), Cyclic FA (CyFA) and Epoxy FA (EpFA). While polyunsaturated FAs are commonly defined as all FAs with ≥ 2 double bonds, in the present study we distinguish between PUFA (FAs with 2 or 3 double bonds) and HUFA (FAs with ≥ 4 double bonds).

2.4.3.7 STATISTICAL ANALYSIS

For PL quantification by LC-MS, the area of each molecular species in the spectra was transformed in their relative abundance, using the area of the PL internal standards as reference. The relative content of each PL class and FA was calculated as percentage of total PL and FA profiles, respectively. Differences in the relative abundance of PL classes, FA classes and molecular species of PL, were determined by 2-way ANOVA with interaction (factor crab species: *C. maenas* and *N. puber*; factor stage: stage1 and stage 3). Post hoc Tukey HSD test was used when ANOVA results revealed significant differences ($P < 0.05$). Statistical analyses of PL classes and their molecular species present in only one embryonic stage were performed using pairwise comparisons (Student´s *t*-test) between crab species. The sample size was identical in all treatments: n = 3. Prior to analysis, we tested for deviations from normality in the response variable with the Shapiro test and homogeneity of variance with the Levene´s test. The level of statistical significance was $P < 0.05$. The statistical analyses were performed using the statistical package R version 2.13.2 (R Development Core Team 2011).

2.4.4 RESULTS

The limited number of samples processed per crab species and embryonic stage (n=3) appeared to have no influence on our results given the little variation recorded among replicates. All significant differences recorded on the relative content of each PL class,

molecular species and FA are highlighted in the figures presented, along with accurate *P* values for each statistical test performed.

2.4.4.1 IDENTIFICATION OF PHOSPHOLIPIDS CLASSES AND QUANTIFICATION BY TLC

The fractioning of total lipid extracts in TLC plates showed two different profiles for each stage of embryogenesis (Fig. 2.4.1a and 2.4.1b). The separation by TLC enabled to identify phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM) as the PL classes present in the initial stage of embryogenesis (Fig. 2.4.1a). LysoPC was observed at stage 1 embryos of *C. maenas*, but not on *N. puber*. At the end of embryonic development (stage 3) the separation by TLC revealed two new classes: phosphatidylinositol (PI) and cardiolipin (CL) (Fig. 2.4.1b). In both crab species and stages, the most abundant PL classes were PCs (*C. maenas*: 60.76 ± 4.28% and 40.15 ± 1.83%; *N. puber*: 74.13 ± 2.74% and 51.40 ± 3.58%, at stage 1 and 3, respectively) and PEs (*C. maenas*: 21.61 ± 1.33% and 20.89 ± 2.02%; *N. puber*: 19.63 ± 2.62% and 19.90 ± 2.94%, at stage 1 and 3, respectively).

2.4.4.2 IDENTIFICATION OF MOLECULAR PROFILE IN PHOSPHOLIPIDS CLASSES BY HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (HILIC-ESI-MS)

In general, the HILIC-ESI-MS analysis confirmed the lipidomic profile revealed by TLC. However, this technique allowed to identify a new PL class in the embryos of *N. puber* at stage 1 which had not been previously detected by TLC: lysophosphatidylethanolamine (LysoPE) (see supplementary Fig. S1). A total of 98 molecular species were identified in the seven PL classes detected. In the next sections, the composition of the most abundant molecular species, namely the assignment of polar head groups and fatty acyl chain composition (determined by MS/MS (Pulfer & Murphy 2003)), is explained in detail. MS/MS spectra of each PL class can be seen in supplementary Fig. S2 for *C. maenas* and Fig. S3 for *N. puber*. The number of carbon atoms (C) and double bonds (N) (C:N) are described for all molecular species recorded, as well as the FA side chains of the diacyl,

plasmanyl or plasmenyl species (whenever possible) (total molecular species identified are listed in supplementary Table S4).

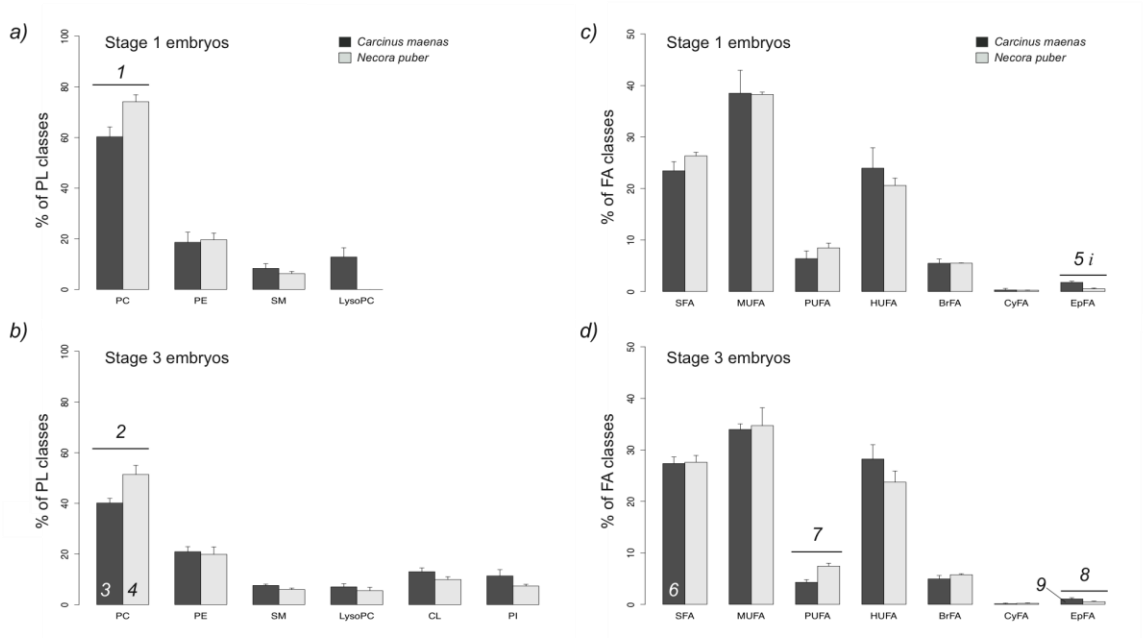

Figure 2.4.1 (a) Relative abundance of phospholipids classes separated by thin layer chromatography in embryos at stage 1 and (b) at stage 3, and (c) fatty acid (FA) class profiles in embryos at stage 1 and (d) stage 3 of *Carcinus maenas* and *Necora puber*. Error bars represent standard deviation of three independent samples. *P* values for each significant statistical test performed are represented in the figure with a number, with significant differences between crab species being represented on the top of the graph bars and significant differences between stages of the same crab species being represented within the bar of stage 3 embryos. Significant interaction between species and stage is represented with an *i* on the top of stage 1 bars. (Post hoc Tukey HSD, *1*: $P = 0.0027$; *2*: $P = 0.0095$; *3*: $P = 0.0002$; *4*: $P = 0.0001$; *5*: $P = 0.0001$; *6*: $P = 0.0266$; *7*: $P = 0.0175$; *8*: $P = 0.0361$; *9*: $P = 0.0050$). Interaction species *vs* stage (*i*): Epoxy FA ($P = 0.0081$).

Abbreviations: PC - phosphatidylcholine; PE - phosphatidylethanolamine; SM - sphingomyelin; LysoPC - Lysophosphatidylcholine; CL - cardiolipin; PI – phosphatidylinositol; SFA – Saturated FA; MUFA – Monounsaturated FA; PUFA – Polyunsaturated FA; HUFA – Highly-unsaturated FA; BrFA – Branched FA; CyFA – Cyclic FA; EpFA – Epoxy FA.

2.4.4.2.1 PHOSPHATIDYLCHOLINE AND LYSOPHOSPHATIDYLCHOLINE

PCs (Fig. 2.4.2a) were the most abundant PL class in both species and stages. Analysis of HILIC-ESI-MS spectra allowed the identification of 22 molecular species on this class and the determination of their molecular composition (C:N) (Table 2.4.1). In *C. maenas*, the most abundant species at stage 1 were observed at *m/z* 790.3 and *m/z* 776.3 (Fig. 2.4.2b), and identified as PC(32:1) or PC(O-33:1) and PC(31:1) or PC(O-32:1), respectively. Similarly, the most abundant molecular species in *N. puber* were seen at *m/z* 790.3 and *m/z* 864.3, with a possible composition of PC(32:1) or PC(O-33:1) and PC(38:6), respectively (Fig. 2.4.2b). At stage 3, the most abundant species were *m/z* 818.3, PC(34:1), and *m/z* 790.3 for *C. maenas* and *m/z* 790.3 and *m/z* 864.3 for *N. puber* (Fig. 2.4.2c).

The higher sensitivity of HILIC-ESI-MS, in comparison to TLC, allowed the identification of LysoPCs (Fig. 2.4.3a) also in stage 1 embryos of *N. puber*, (Fig. 2.4.3b). A total of 8 molecular species were identified by MS/MS (Table 2.4.2). At stage 1, the most abundant molecular species in *C. maenas* were at *m/z* 582.1, LysoPC(18:0), and at *m/z* 552.1, LysoPC(16:1), while in *N. puber* were at *m/z* 582.1 and *m/z* 574.1, LysoPC(18:4), (Fig. 2.4.3b). At stage 3 embryos the most abundant molecular species for both crab species were observed at *m/z* 582.1 and *m/z* 580.1, LysoPC(18:1), (Fig. 2.4.3c). At both embryonic stages the molecular composition of LysoPCs exhibited FAs from 15:0 to 19:1, including two possible LysoPC plasmanyl/plasmenyl species: LysoPC(O-16:0) and LysoPC(O-18:1) (Table 2.4.2).

2.4.4.2.2 PHOSPHATIDYLETHANOLAMINE AND LYSOPHOSPHATIDYLETHANOLAMINE

PEs (Fig. 2.4.4a) were the most specious PL class recorded. A total of 29 molecular species were identified (Table 2.4.3). In stage 1 embryos of *C. maenas* the most abundant species were at *m/z* 716.4, PE(34:1), and *m/z* 764.4, PE(38:5), (Fig. 2.4.4b), while in *N. puber* were at *m/z* 774.4, PE(38:0), PE(39:7), PE(O-39:0) or PE(O-40:7), and *m/z* 748.4, PE(37:6) or PE(O-38:6) (Fig. 2.4.4b). Stage 3 embryos of *C. maenas* displayed the ions at *m/z* 762.4, PE(38:6), and *m/z* 764.4 assigned as the most abundant molecular species, while for *N.*

puber were at *m/z* 748.4 and *m/z* 764.4 (Fig. 2.4.4c). According to MS/MS analysis of the ions attributed to the PE molecular species their compositions contained SFA, MUFA, PUFA and HUFA, from 14:0 to 22:6 (Table 2.4.3).

LysoPE (Fig. 2.4.5a) was one of the PL classes solely recorded in the last embryonic stage, with 9 molecular species (Table 2.4.4) being identified in stage 3 embryos of *C. maenas* and *N. puber* (Fig. 2.4.5b). The most abundant LysoPEs recorded were at *m/z* 498.2, LysoPE(20:5), and *m/z* 524.2, LysoPE(22:6), (Fig. 2.4.5b).

2.4.4.2.3 SPHINGOMYELIN

The LC-MS/MS spectra of SMs (Fig. 2.4.6a) revealed the presence of 9 molecular species (Table 2.4.5). For both *C. maenas* and *N. puber*, the most abundant molecular species at stage 1 and 3 embryos were at *m/z* 761.3, SM(d18:1/16:0, with the FAs in the positions *sn*-1 and *sn*-2, respectively) and *m/z* 719.3, SM(d18:1/13:0), (Fig. 2.4.6b and 2.4.6c, respectively). The SM species presented a fatty acyl combination of SFA and MUFA, with one PUFA. The FAs present in this PL class ranged from 13:0 to 19:1 (Table 2.4.5).

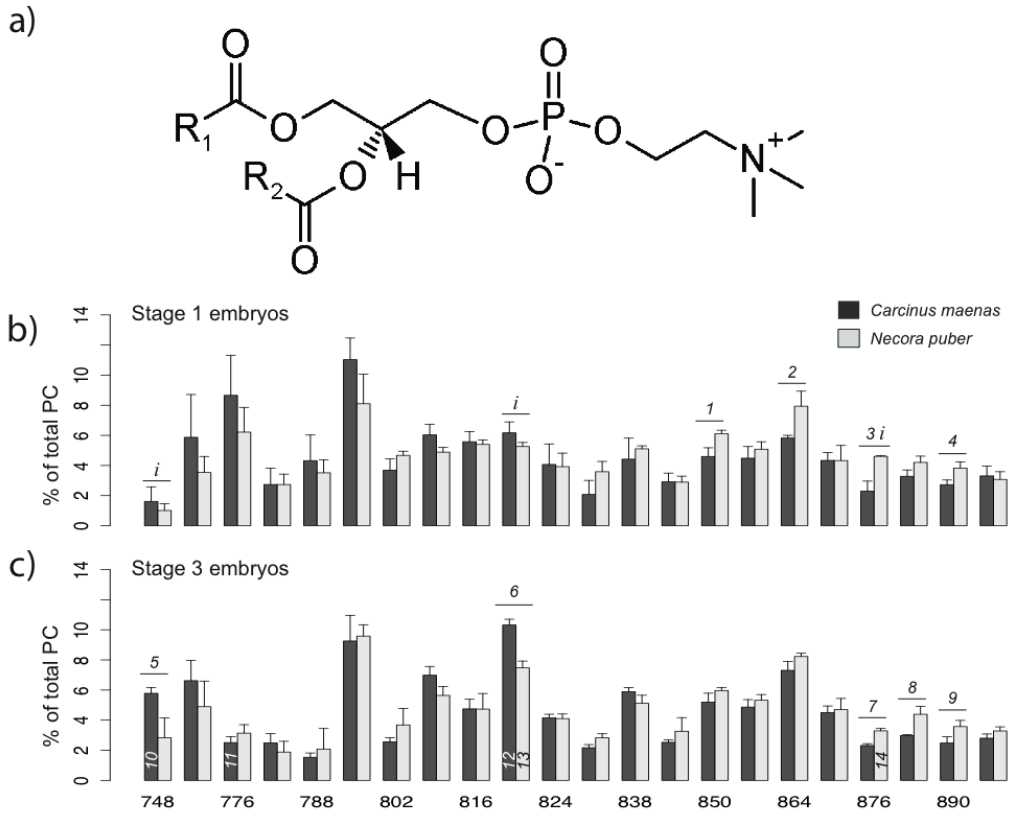

Figure 2.4.2 (a) General structure of phosphatidylcholine (PC). Error bars represent standard deviation of three independent samples. (b) Relative abundance of the $[M+CH_3COO]^-$ ions of the different molecular species of PC present in the total lipid extract from embryos of *Carcinus maenas* and *Necora puber* at stage 1 and (c) stage 3. *P* values for each significant statistical test performed are represented in the figure with a number, with significant differences between crab species being represented on the top of the graph bars and significant differences between stages of the same crab species being represented within the bar of stage 3 embryos. Significant interaction between species and stage is represented with an *i* on the top of stage 1 bars. (Post hoc Tukey HSD, *1*: $P = 0.0130$; *2*: $P = 0.0106$; *3*: $P = 0.0002$; *4*: $P = 0.0352$; *5*: $P = 0.0133$; *6*: $P = 0.0005$; *7*: $P = 0.0357$; *8*: $P = 0.0103$; *9*: $P = 0.0359$; *10*: $P = 0.0016$; *11*: $P = 0.0066$; *12*: $P = 2.98 \times 10^{-5}$; *13*: $P = 0.0025$; *14*: $P = 0.0071$). Interaction species *vs* stage (*i*): *m/z* 748.2 ($P = 0.0470$); *m/z* 818.3 ($P = 0.0089$); *m/z* 876.3 ($P = 0.0104$).

Table 2.4.1 Major molecular species of phosphatidylcholine identified by liquid chromatography – mass spectrometry in negative-ion mode in the embryos of *Carcinus maenas* and *Necora puber*.

m/z $[M+CH_3COO]^-$	Molecular species (C:N)
748.2	29:1 or O-30:1
762.3	30:1 or O-31:1
776.3	31:1 or O-32:1
778.3	31:0 or O-32:0
788.3	32:2 or O-33:2
790.3	32:1 or O-33:1
802.3	33:2 or O-34:2
804.3	33:1 or O-34:1
816.3	34:2 or O-35:2
818.3	34:1
824.3	35:5 or O-36:5
836.3	36:6
838.3	36:5
844.4	36:2
850.3	37:6 or O-38:6
852.3	37:5 or O-38:5
864.3	38:6
866.3	38:5
876.3	39:7
878.3	39:6 or O-40:6
890.3	30:9 or O-40:0 and 40:7
892.3	40:6

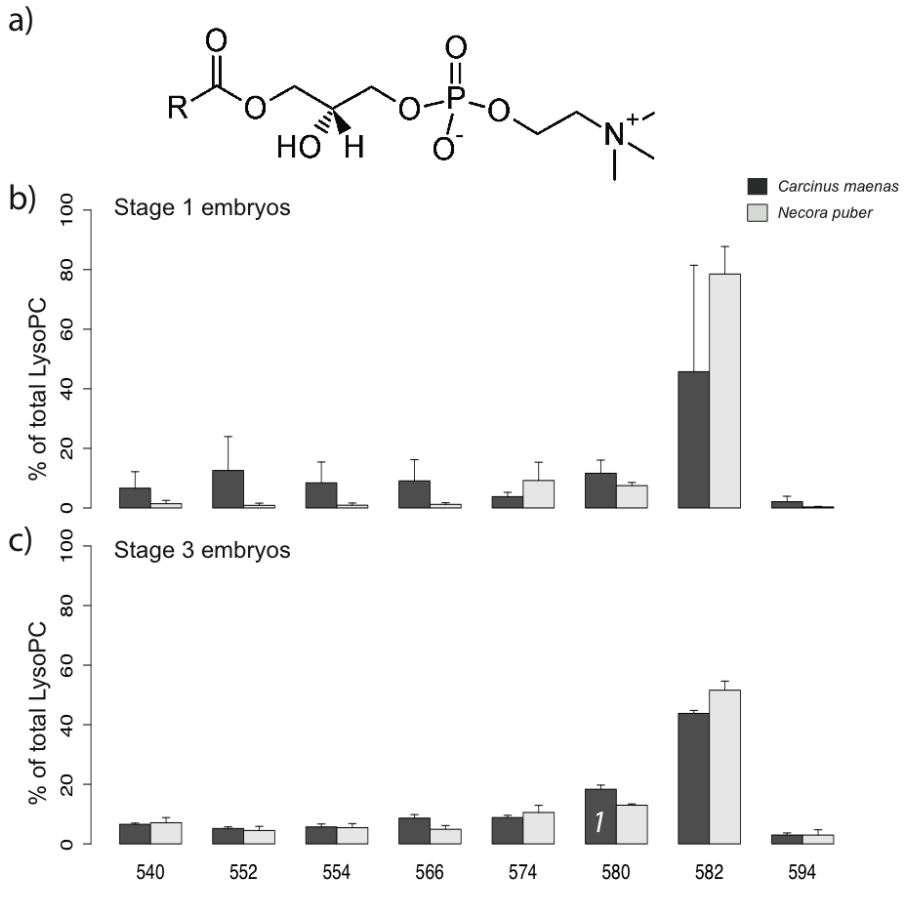

Figure 2.4.3 (a) General structure of lysophosphatidylcholine (LysoPC). Error bars represent standard deviation of three independent samples. (b) Relative abundance of the $[M+CH_3COO]^-$ ions of the different molecular species of LysoPC present in embryos of *Carcinus maenas* and *Necora puber* at stage 1 and (c) at stage 3. *P* values for each significant statistical test performed are represented in the figure with a number, significant differences between stages of the same crab species being represented within the bar of stage 3 embryos. (Post hoc Tukey HSD, *1*: *P* = 0.0354).

Table 2.4.2 Major molecular species of lysophosphatidylcholine identified by liquid chromatography – mass spectrometry in negative-ion mode in the embryos of *Carcinus maenas* and *Necora puber*.

m/z $[M+CH_3COO]^-$	Molecular species (C:N)
540.1	15:0 or O-16:0
552.1	16:1
554.1	16:0
566.1	17:1 or O-18:1
574.1	18:4
580.1	18:1
582.1	18:0
594.1	19:1

2.4.4.2.4 PHOSPHATIDYLINOSITOL

PIs (Fig. 2.4.7a) were one of the PL classes that were synthesized during embryogenesis, thus solely being recorded in stage 3 embryos. The LC-MS analysis identified a total of 15 molecular species (Table 2.4.6), with the ions at *m/z* 883.4, PI(38:5), and *m/z* 881.4, PI(38:6), being the most abundant in both crab species (Fig. 2.4.7b). The fatty acyl composition of PIs included SFA, MUFA, PUFA and HUFA ranging from 16:0 to 22:6 (Table 2.4.6).

2.4.4.2.5 CARDIOLIPIN

CLs (Fig. 2.4.8a) only appeared in the last embryonic stage. A total of 6 molecular species were identified (Table 2.4.7), the most abundant being at *m/z* 1355.2, CL(65:1), and *m/z* 1337.6, CL(64:0), in *C. maenas* and at *m/z* 1355.2 and *m/z* 1547.2, CL(80:14) in *N. puber* (Fig. 2.4.8b).

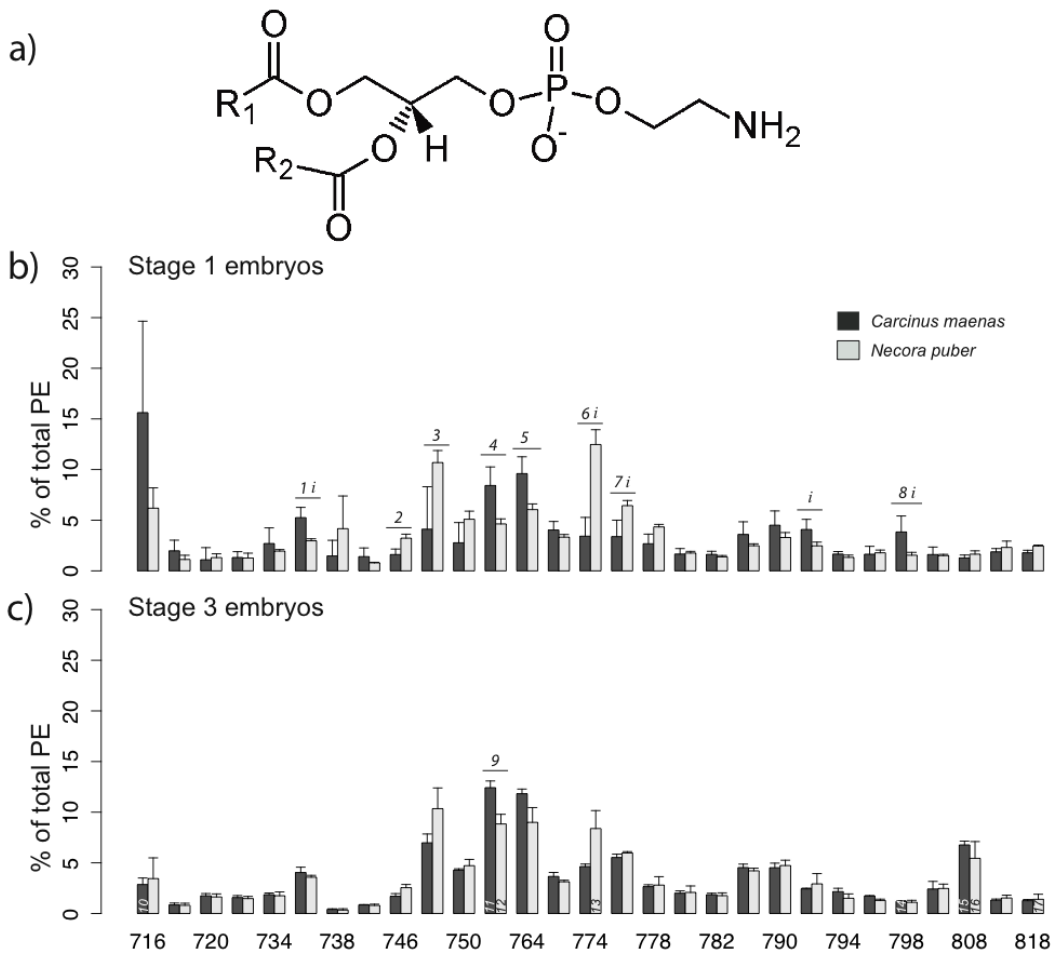

Figure 2.4.4 (a) General structure of phosphatidylethanolamine (PE). Error bars represent standard deviation of three independent samples. (b) Relative abundance of the $[M-H]^-$ ions of the different molecular species of PE present in embryos of *Carcinus maenas* and *Necora puber* at stage 1 and (c) at stage 3. *P* values for each significant statistical test performed are represented in the figure with a number, with significant differences between crab species being represented on the top of the graph bars and significant differences between stages of the same crab species being represented within the bar of stage 3 embryos. Significant interaction between species and stage is represented with an *i* on the top of stage 1 bars. (Post hoc Tukey HSD, *1*: $P = 0.0061$; *2*: $P = 0.0045$; *3*: $P = 0.0441$; *4*: $P = 0.0133$; *5*: $P = 0.0237$; *6*: $P = 0.0003$; *7*: $P = 0.0109$; *8*: $P = 0.0346$; *9*: $P = 0.0181$; *10*: $P = 0.0440$; *11*: $P = 0.0099$; *12*: $P = 0.0074$; *13*: $P = 0.0389$; 14: $P = 0.0167$; *15*: $P = 0.0003$; *16*: $P = 0.0032$; *17*: $P = 0.0089$). Interaction species *vs* stage (*i*): *m/z* 736.4 ($P = 0.0274$); *m/z* 774.4 ($P = 0.0146$); *m/z* 776.4 ($P = 0.0310$); *m/z* 792.4 ($P = 0.0389$); *m/z* 798.2 ($P = 0.0466$).

Table 2.4.3 Major molecular species of phosphatidylethanolamine identified by liquid chromatography – mass spectrometry in negative-ion mode in the embryos of *Carcinus maenas* and *Necora puber*.

m/z [M-H]$^-$	Molecular species (C:N)	Fatty acyl composition							
716.4	34:1	14:0/20:1;	16:0/18:1;	16:1/18:0					
718.4	34:0	16:0/18:0;	17:0/17:0						
720.4	35:6 and O-36:6	15:1/20:5;	O-16:1/20:5						
722.4	35:5 and O-36:5	15:0/20:5;	17:1/18:4						
734.4	36:6	14:1/22:5;	16:1/20:5;	O-16:1/20:4;	O-16:0/20:5;	O-18:1/18:4			
736.4	36:5	14:0/22:5;	14:1/22:4;	16:0/20:5;	16:1/20:4;	18:1/18:4			
738.4	36:4	16:0/20:4;	16:1/20:3;	18:3/18:1					
742.4	36:2	16:0/20:2;	16:1/20:1;	17:2/19:0;	18:0/18:2;	18:1/18:1			
746.4	36:0 and 37:7 and O-38:7	16:0/20:0;	17:0/19:0;	18:0/18:0;	15:1/22:6;	17:2/20:5;	O-16:1/22:6		
748.4	37:6 and O-38:6	15:0/22:6;	17:1/20:5;	17:2/20:4;	22:5/15:1;	O-16:0/22:6;	O-16:1/22:5	O-18:1/20:5	
750.4	37:5 and O-38:5	17:0/20:5;	17:1/20:4;	15:0/22:5;	15:1/22:4;	O-16:0/22:5;	O-16:1/22:4	O-18:0/20:5	O-18:1/20:4
762.4	38:6	16:0/22:6;	16:1/22:5;	18:1/20:5;	18:2/20:4				
764.4	38:5	16:0/22:5;	16:1/22:4;	18:0/20:5;	18:1/20:4;	18:2/20:3			
766.4	38:4	16:0/22:4;	18:0/20:4;	18:1/20:3;	18:2/20:2;	18:3/20:1;	18:4/20:0		
774.4	38:0 and 39:7 and O-39:0 and O-40:7	17:0/21:0;	17:1/22:6;	17:2/22:5;	18:0/20:0;	O-18:0/21:0;	O-18:1/22:6		
776.4	39:6 and O-40:6	17:0/22:6;	17:1/22:5;	O-18:0/22:6;	O-18:1/22:5				
778.4	39:5 and O-40:5	17:0/22:5;	17:1/22:4;	19:0/20:5;	O-18:0/22:5;	O-18:1/22:4			
780.4	39:4 and 40:4	17:0/22:4;	17:2/22:2;	19:0/20:4;	O-18:0/22:4				
782.4	39:3 and 40:10 and O-40:3	22:2/17:1;	22:3/17:0;	20:5/20:5;	O-18:1/22:2				
788.4	40:7 and 39:0	19:0/20:0;	20:5/20:2;	22:6/18:1					
790.4	40:6	18:0/22:6;	18:1/22:5;	20:1/20:5;	20:2/20:4				
792.4	40:5	18:0/22:5;	18:1/22:4;	20:4/20:1;	20:5/20:0				
794.4	40:4	18:0/22:4;	20:2/20:2;	20:4/20:0					
796.4	40:3	18:1/22:2;	20:1/20:2						
798.2	40:2	18:0/22:2;	18:1/22:1;	20:1/20:1;	20:2/20:0				
806.4	41:5	19:0/22:5;	21:0/20:5						
808.4	42:11	20:5/22:6							
816.4	42:7	20:1/22:6;	20:2/22:5						
818.4	42:6	20:0/22:6;	20:1/22:5;	20:2/22:4;	20:4/22:2				

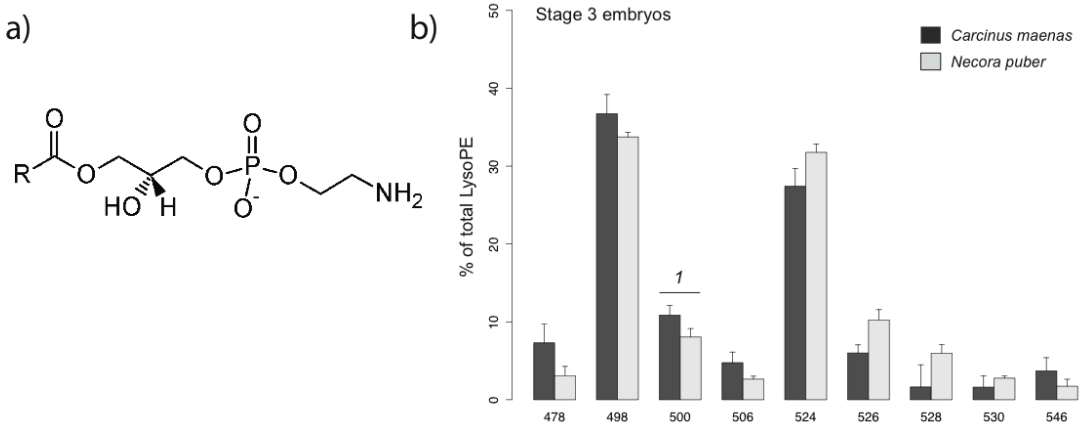

Figure 2.4.5 (a) General structure of lysophosphatidylethanolamine (LysoPE). Error bars represent standard deviation of three independent samples. (b) Relative abundance of the [M-H]$^-$ ions of the different molecular species of LysoPE present in embryos of *Carcinus maenas* and *Necora puber* at stage 3. *P* values for each significant statistical test performed are represented in the figure with a number, with significant differences between crab species being represented on the top of the graph bars. (*t*-test, *1*: *P* = 0.0435).

Table 2.4.4 Major molecular species of lysophosphatidylethanolamine identified by liquid chromatography – mass spectrometry in negative-ion mode in the embryos of *Carcinus maenas* and *Necora puber*.

m/z [M-H]$^-$	Fatty acid composition
478.2	18:1
498.2	20:5
500.2	20:4
506.2	20:1
524.2	22:6
526.2	22:5
528.2	22:4
530.2	22:3
546.2	22:2+O*

* 12,15-epoxy-13,14-dimethyl-eicosadienoate

2.2.4.3 ANALYSIS OF THE FATTY ACID PROFILE

The identification of the FA profile of the total lipid extract was performed by GC-MS analysis of FAMEs. This analysis corroborated the information on the FAs recorded in the PL classes described above. The relative quantification of FAs revealed that palmitic acid (16:0) was the most abundant FA in both embryonic stages of the two sympatric crab species (see supplementary Table S5 for a complete list of all FAs recorded). In *C. maenas*, the major FAs were 16:0 (16.31 ± 0.39%), 16:1*n*7 (15.37 ± 3.24%) at stage 1 and 16:0 (18.60 ± 1.28%), eicosapentaenoic acid (EPA, 20:5*n*-3) (14.32 ± 0.75%) at stage 3. However, in *N. puber* the most abundant FAs were 16:0 (18.86 ± 0.33%, 19.57 ± 0.34%) and 16:1*n*7 (17.21 ± 1.14%, 12.82 ± 2.91%) at stage 1 and 3 embryos, respectively. The analysis of FA classes (Fig. 2.4.1c and 2.4.1d) showed that MUFA were the most well represented in the pool of FAs (*C. maenas*: 38.51 ± 4.45%, 33.98 ± 1.05%; *N. puber* 38.26 ± 0.46%, 34.71 ± 3.47%, at stage 1 and 3, respectively), followed by HUFA (*C. maenas*: 23.95 ± 3.96%, 28.23 ± 2.76%; *N. puber* 20.61 ± 1.40%, 23.73 ± 2.16%, at stage 1 and 3, respectively) and SFA (*C. maenas*: 23.45 ± 1.75% 27.34 ± 1.27%; *N. puber* 26.33 ± 0.68%, 21.61 ± 1.29%, at stage 1 and 3, respectively). Within the HUFA, EPA (*C. maenas*: 9.02 ± 2.06%, 14.32 ± 0.75%; *N. puber* 8.17 ± 0.59%, 10.11 ± 1.10%, at stage 1 and 3, respectively) and docosahexaenoic acid (DHA, 22:6*n*-3) (*C. maenas*: 9.36 ± 4.02%, 9.63 ±

2.02%; *N. puber* 7.58 ± 0.83%, 8.01 ± 1.48%, stage 1 and 3, respectively) were the most abundant FAs.

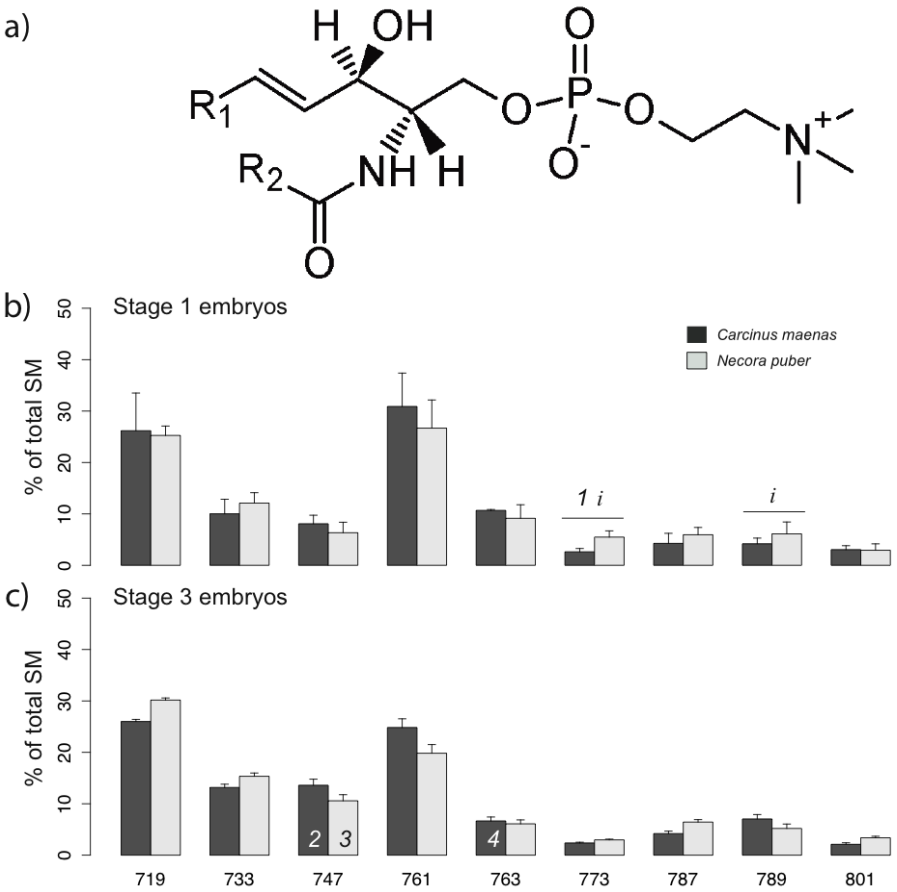

Figure 2.4.6 (a) General structure of sphingomyelin (SM). Error bars represent standard deviation of three independent samples. (b) Relative abundance of the $[M+CH_3COO]^-$ ions of the different molecular species of SM present in embryos of *Carcinus maenas* and *Necora puber* at stage 1 and (c) at stage 3. *P* values for each significant statistical test performed are represented in the figure with a number, with significant differences between crab species being represented on the top of the graph bars and significant differences between stages of the same crab species being represented within the bar of stage 3 embryos. Significant interaction between species and stage is represented with an *i* on the top of stage 1 bars. (Post hoc Tukey HSD, *1*: $P = 0.0043$; *2*: $P = 0.0115$; *3*: $P = 0.0440$; *4*: $P = 0.03889$). Interaction species *vs* stage (*i*): *m/z* 773.3 ($P = 0.0223$); *m/z* 789.3 ($P = 0.0449$).

Table 2.4.5 Major molecular species of sphingomyelin identified by liquid chromatography – mass spectrometry in negative-ion mode in the embryos of *Carcinus maenas* and *Necora puber*.

***m/z* $[M+CH_3COO]^-$**	**Molecular species (C:N)**	**Fatty acyl composition**
719.3	31:1	d18:1/13:0
733.3	32:1	d18:1/14:0
747.3	33:1	d18:1/15:0
761.3	34:1	d18:1/16:0
763.3	34:0	d18:0/16:0
773.3	35:2	d18:1/17:1
787.3	36:2	d18:0/18:2
789.3	36:1	d18:1/18:0
801.3	37:2	d18:1/19:1

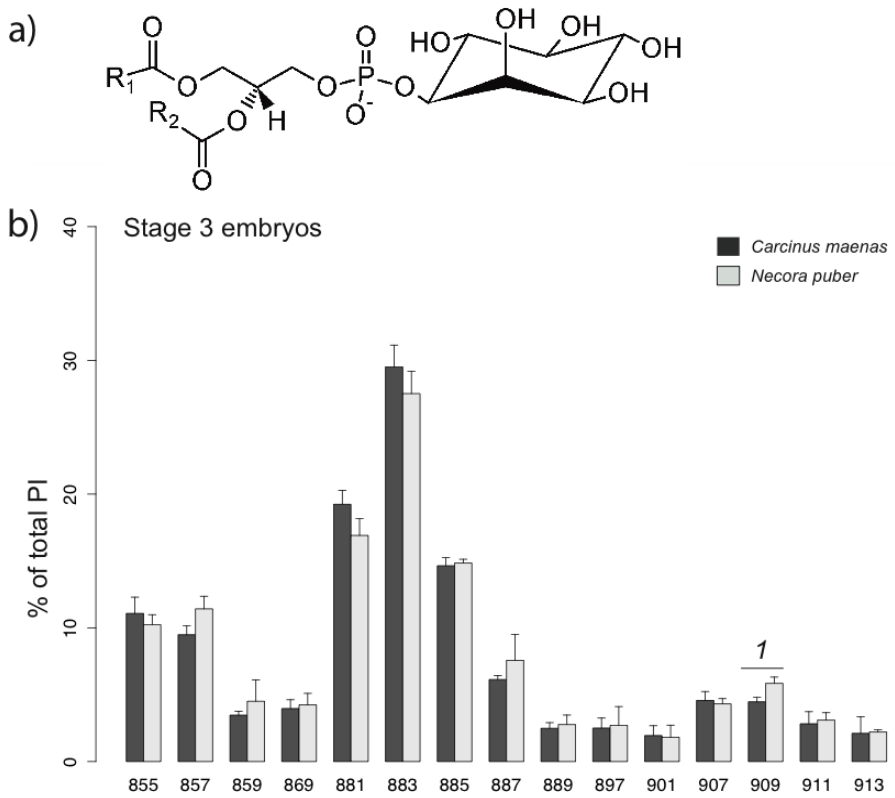

Figure 2.4.7 (a) General structure of phosphatidylinositol (PI). (b) Relative abundance of the $[M-H]^-$ ions of the different molecular species of PI present in embryos of *Carcinus maenas* and *Necora puber* at stage 3. Error bars represent standard deviation of three independent samples. *P* values for each significant statistical test performed are represented in the figure with a number, with significant differences between crab species being represented on the top of the graph bars. (*t*-test, *1*: *P* = 0.0175).

Table 2.4.6 Major molecular species of phosphatidylinositol identified by liquid chromatography – mass spectrometry in negative-ion mode in the embryos of *Carcinus maenas* and *Necora puber*.

m/z [M-H]$^-$	Molecular species (C:N)	Fatty acyl composition				
855.4	36:5	16:0/20:5;	16:1/20:4			
857.4	36:4	16:0/20:4;	16:1/20:3			
859.4	36:3	16:0/20:3;	16:1/20:2;	18:0/18:3;	18:1/18:2	
869.4	37:5	17:0/20:5;	17:1/20:4			
881.4	38:6	18:1/20:5;	18:2/20:4			
883.4	38:5	16:0/22:5;	18:0/20:5;	18:1/20:4		
885.4	38:4	16:0/22:4;	18:1/20:3;	18:2/20:2		
887.4	38:3	16:1/22:2;	18:0/20:3;	18:1/20:2		
889.4	38:2	16:1/22:1;	18:0/20:2;	18:1/20:1;	18:2/20:0	
897.5	39:5 or O-40:5	17:0/22:5;	17:1/22:4;	19:0/20:5;	O-18:0/22:5;	O-18:1/22:4
901.4	39:3 or O-40:3 and 40:10	17:1/22:2;	19:0/20:3;	20:5/20:5;	O-18:1/22:2	
907.5	40:7	18:1/22:6;	18:2/22:5;	20:2/20:5;	20:4/20:3	
909.5	40:6	18:0/22:6;	18:1/22:5			
911.4	40:5	18:0/22:5;	18:1/22:4;	20:0/20:5;	20:1/20:4;	20:2/20:3
913.4	40:4	18:0/22:4;	18:2/22:2;	20:0/20:4;	20:1/20:3;	20:2/20:2

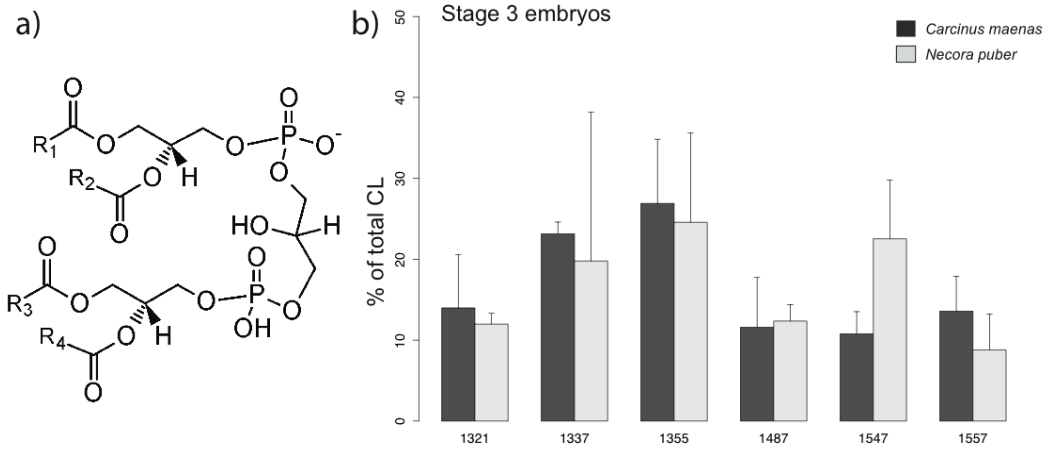

Figure 2.4.8 (a) General structure of cardiolipin (CL). (b) Relative abundance of the [M-H]$^-$ ions of the different molecular species of CL present in embryos of *Carcinus maenas* and *Necora puber* at stage 3. Error bars represent standard deviation of three independent samples.

Table 2.4.7 Major molecular species of cardiolipin identified by liquid chromatography – mass spectrometry in negative-ion mode in the embryos of *Carcinus maenas* and *Necora puber*.

m/z $[M-H]^-$	Molecular species (C:N)
1321.6	62:1
1337.6	64:0
1355.2	65:1
1487.6	74:3
1547.2	80:14
1557.1	80:9

2.4.5 DISCUSSION

The lipidomic approach employed in the present study allowed an unprecedented insight on the lipid dynamics during embryonic development in decapod crustaceans. TLC analysis revealed the biochemical evolution of lipids during embryogenesis at cellular level. PL classes present at stage 1 (PC, LysoPC, PE, SM) commonly play a key role on energetic and structural functions, while those that solely were recorded at stage 3 (LysoPE, PI and CL) may be involved in the neurologic and sensorial development of embryos about to hatch (Vance & Vance 2008).

The important role that PC plays during embryogenesis was reflected in our results, with their decrease in relative abundance from stage 1 to stage 3 embryos agreeing with data from other marine species (Fraser et al. 1988, Kattner et al. 1994). PC is recognized as the major component of biological membranes (Coutteau et al. 1997), therefore of paramount importance on cells biochemistry and physiology (D'Abramo et al. 1982, 1985). Additionally, PC also has a similar role in the transport of yolk components in developing embryos (Fraser et al. 1988). Although the catabolism of PC may provide a secondary energy source when TAG are depleted (Sasaki et al. 1986), it is most likely related with the provision of phosphate and choline. PCs likely play an important function as a source of EFAs for developing embryos (Fraser et al. 1988). PCs are rich in EPA and DHA, which are both required for cell differentiation and membrane formation during embryogenesis (Fischer et al. 2009). In line with the above, a possible consequence of

excessive or even complete catabolism of PLs for energy would be the loss of important HUFA. However, studies carried out on fish embryos and larvae showed a selective retention of the DHA liberated by the catabolism of PCs in neutral lipids and/or PEs (Fraser et al. 1988, Rønnestad et al. 1995). Such EFAs are also known to play a major role during late embryonic development and early larval life in brachyuran crabs (Andrés et al. 2010). The low variability in the relative abundance of PEs between embryonic stages, in both crab species, suggests a structural function of this PL class. PC and PE were the major components of polar lipids. As already referred above for PC, PE is also a key component of membrane bilayers. Moreover, the FA composition of the molecular species of PEs showed a high level of unsaturation, which is known to contribute to membrane fluidity, as this feature is largely determined by the degree of FA unsaturation and *n*-3 HUFA (Dalsgaard et al. 2003). Specifically, the role played by DHA and EPA on membrane flexibility has already been documented (Dalsgaard et al. 2003). In both *C. maenas* and *N. puber*, the molecular species of this class that display significant differences between stage 1 and 3 embryos (*m/z* 762.4 and *m/z* 808.4) possess EPA, DHA and/or arachidonic acid (ARA, 20:4*n*-6) in their composition. The selective retention of these FAs in the last stages of embryonic development may reflect a preparation for planktonic life. In decapod crustaceans, those EFAs are known to be decisive for larval fitness during early planktonic life stages (Anger 2001). These molecules influence early neural development and functions (Beltz et al. 2007), hatchability at the end of embryonic development (Xu et al. 1994), larval growth (Sulkin & McKeen 1999) and larval resistance to stress (Wickins et al. 1995). Other molecular species of LysoPEs include C_{20} and C_{22} HUFA, important FAs, such as ARA, EPA, DHA, docopentaenoic acid (DPA, 22:5) and 12,15-epoxy-13,14-dimethyl-eicosadienoate (Table 2.4.4), which are known to be precursors of key biomolecules. ARA and EPA are the precursors of highly bioactive derivatives, the eicosanoids (Tocher et al. 2008), whereas ARA is the precursor of prostaglandins, which are known to be involved in a number of vital pathways (e.g., reproduction, digestion, respiration, membrane permeability and fat dissolution) (Lilly & Bottino 1981).

PIs and CLs were not recorded in early stage embryos and thus must originate from the catabolism of yolk reserves provided through maternal investment. PIs are considered as key signalling molecules and are involved in secretory events, as well as intercellular signalling events (Barlow et al. 2010). The metabolism of inositol lipids is involved in the signal transduction of many hormones, neurotransmitters and growth factors (Berridge 1993, Strum et al. 1997). Qualitative requirements suggest that PC may be more important for growth, while PI may be relevant for organogenesis and tissue differentiation (Tocher et al. 2008). These features may explain their occurrences in different periods of embryogenesis (e.g., PCs are already present in newly extruded embryos).

In general, the analysis of the lipidomic profiles of developing embryos of *C. maenas* and *N. puber* did not reveal the existence of major interspecific differences. In the sampling site, these two species share the same estuarine habitats, which allow us to infer that they may have access to the same dietary resources. The scarce differences in the lipidomic profiles of embryos in the beginning of their development (stage 1) demonstrate a similar maternal investment in both crab species. Embryos of *C. maenas* and *N. puber* also appear to be programmed to catabolize embryonic resources in a similar pattern during the incubation period, as the incubation environment (in terms of female brooding chamber) is similar for both species (Wear 1974, Choy 1991). A follow up study surveying ovigerous females from these two species where they no longer occur in sympatry (*C. maenas* present in the inner regions of the coastal lagoon, which are more strongly influenced by fresh water runoffs, and *N. puber* located in a typically marine environment, the outer regions of the pier protecting the inlet of the coastal lagoon) will allow us to determine if the trends recorded in maternal investment and lipid catabolism during embryogenesis remain similar under contrasting environmental conditions.

Furthermore, the analysis of FA classes revealed scarce differences between both crab species. Decapod larvae generally exhibit a limited ability to introduce double bonds into the *n*-6 and *n*-3 position of C_{18}, C_{20}, and C_{22} FAs (Teshima et al. 1992). The presence of EPA and DHA in newly hatched larvae is known to originate from maternal lipids transferred at

oogenesis, as decapod embryos are lecitothrophic and cannot synthesize these FAs *de novo* (Kanazawa et al. 1979a, Kanazawa et al. 1979b). The selective retention of essential HUFA through embryogenesis may thus reduce the nutritional vulnerability of newly hatched larvae to suboptimal conditions they may experience in the early stages of their planktonic life (Anger 2001). High levels of HUFA and PL improve the osmoregulation process by optimizing membranous lipid composition and/or structure of the gills (Tocher 1995), a key feature for larvae as the ones of *C. maenas* and *N. puber* that may hatch in estuarine systems prone to salinity shifts. Since HUFA are essential components in the nutrition of decapod larvae, the lipid composition of last embryonic stages may serve as an indicator of the physiological condition of larvae about to hatch. Additionally, lipid content in embryos can potentially determine early larval success and optimal development (Rosa et al. 2005). Higher lipid content in pre-hatching embryos is commonly interpreted as sign of superior tolerance by larvae to longer periods of starvation before first feeding (Nates & McKenney Jr 2000). It must be highlighted that to date no study has ever tried to link the performance of newly hatched larvae to the lipidome displayed by developing embryos. Therefore, until these studies are made available, any assumption on the superior/inferior quality of newly hatched larvae solely based on embryos lipidomics remains speculative and probably misleading. The unprecedented level of resolution achieved on polar lipids dynamics using lipidomics opens a new research window for studying maternal investment and resource partitioning in marine organisms.

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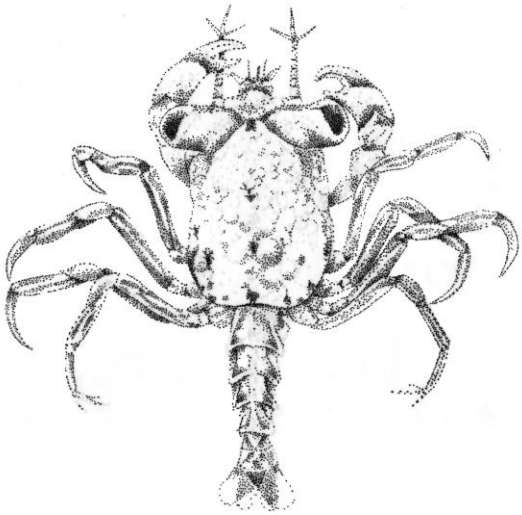

PART III

SETTLEMENT DYNAMICS AND POST-SETTLEMENT PERFORMANCE

3.1 CONTRASTING BENTHIC PERFORMANCE OVER CONSECUTIVE LARVAL SUPPLY EVENTS OF A MARINE INVERTEBRATE WITH BI-PHASIC LIFE

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Keywords

Complex life cycles

Megalopa

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Carry-over effects

Post-settlement performance

Upwelling

Carcinus maenas

3.1.1 ABSTRACT

The pelagic and benthic life of marine invertebrates with bi-phasic life cycles was often studied decoupled, assuming that metamorphosis would delete larval history. Presently, it is acknowledged that larval history can condition the post-metamorphic performance of benthic juveniles and ultimately contribute to the shaping of adult population dynamics. In the present study we evaluated late larval and early juvenile performance of the green crab *Carcinus maenas* in Ria de Aveiro (Portugal), over four consecutive larval supply events in 2013. Larvae collected from the plankton were kept in the laboratory under starvation and controlled conditions. Following metamorphosis juvenile crabs were haphazardly separated in two treatments: exposed to starvation and provided with food *ad libitum* (*Artemia* nauplii). Juveniles were stocked until they either died or reached the fifth juvenile instar. Results showed that larval size at metamorphosis shifted along supply events, with this being reflected in early benthic life. Overall, optimal grow-out conditions did not delete the larval history experienced by the megalopae (and their previous zoeal stages) collected from each supply event. We analysed the influence of upwelling index in larval and early juvenile performance, using general additive models (GAMs), with upwelling index explaining a significant percentage of the deviance recorded. Overall, contrasting trophic conditions experienced during larval life promoted a variation on larval size at settlement, which was carried over to early benthic life. A unifying integrated framework is therefore needed to better understand the population dynamics of marine invertebrates with bi-phasic life cycles and the role played by latent effects.

3.1.2 INTRODUCTION

The majority of marine invertebrates display complex life cycles that involve a pelagic larval phase and an adult benthic (benthopelagic) phase that are linked by larval hatching and metamorphosis. Benthic populations are conditioned by the success of their offspring in the plankton (Marshall & Morgan 2011) and favourable post-metamorphic conditions (Gebauer et al. 1999). Among other factors, larval survival in the plankton is determined by food supply, temperature and predation (Thorson 1950, Morgan 1995). Shifts in food quantity and quality experienced during larval life are paramount for larval growth (Rumrill 1990) and

can shape larval phenotypes in such a way that may condition their settlement success and early benthic life (Pechenik 2006).

Studies addressing the supply of marine invertebrate larval stages to infer settlement success have continued to be performed in recent years (Moksnes & Wennhage 2001, Queiroga et al. 2006, Olaguer-Feliu et al. 2010) and provided important insights on larval ecology and behavioural responses under particular oceanographic conditions (Giménez & Dick 2007, Domingues et al. 2011). Nevertheless, the study by Burgess & Marshall (2011) has revealed that variations in the larval phenotype can play a more important role in the regulation of marine populations than shifts in larval supply. In this sense, studies conducted in marine invertebrates have confirmed that larval history and nutritional status play a strong influence in survival and settlement performance, being propagated into early juvenile stages (Phillips 2002, Jarret 2003). As metamorphosis is an energetically expensive process, higher nutrient levels and energetic reserves at settlement can positively influence early juvenile fitness (Phillips 2002, 2004, Jarrett 2003). It is therefore legitimate to assume that the quality of settling larvae not only can condition juvenile performance post-metamorphosis, it can also affect the dynamics of populations and communities (Phillips 2002, Cowen & Sponaugle 2009, Giménez 2010). The comprehension of both pelagic and benthic life must therefore be addressed under an integrated framework that can allow us to unravel the complexity of the mechanisms regulating the population dynamics of marine invertebrates with bi-phasic life-cycles.

Upwelling is an important oceanographic phenomenon worldwide (Menge et al. 2003, Pfaff et al. 2011) and along the north-western coast of the Iberian Peninsula in particular (Villegas-Ríos et al. 2011, Álvarez et al. 2013). During the spring-summer months northerly winds prevail along the shelf allowing the upwelling of deep cold water (Fiuza et al. 1998). This water mass is rich in nutrients, which together with dissolved CO_2 and solar energy, is used by phytoplankton to produce organic compounds through photosynthesis. This process generates high levels of primary production, and, consequently leads to high biological diversity (Guisande & Harris 1995, Guisande et al. 2001, Huthnance et al. 2002, Santos et al. 2004). Spawning and hatching of many marine invertebrates occurs during the upwelling

season, suggesting that larval development may somehow benefit from such conditions during their pelagic life (Shanks & Eckert 2005, Morgan et al. 2009).

The green crab *Carcinus maenas* (L.), an invertebrate species with a bi-phasic life cycle, occupies both hard and soft bottoms, in intertidal and shallow subtidal habitats located in the shores, estuaries and coastal lagoons of the western margin of the Iberian Peninsula (D'Udekem d'Acoz 1999). Populations of *C. maenas* release their larvae during spring and early summer (Crothers 1967, Lyons et al. 2012), with newly hatched larvae being exported to shelf waters where they develop through four different larval stages (termed zoea) and moult to a fifth and last larval stage (the megalopa) that later reinvades estuarine or coastal waters, commonly from March to June (Domingues et al., 2011; Queiroga et al., 2006).

The relation between megalopa supply of *C. maenas* and oceanographic conditions has been analysed in detail over the years and in different locations (Queiroga et al. 2006, Giménez & Dick 2007, Domingues et al. 2011) revealing that larval supply mechanisms may vary within and among years. A modelling approach performed by Marta-Almeida et al. (2006), suggested that upwelling events can enhance the retention of larvae in the inner shelf and favour settlement. This scenario was later confirmed by a field study performed by Morgan et al. (2009). Nonetheless, few studies have yet explored the relation between contrasting oceanographic conditions experienced during larval development and success during early benthic life (Giménez 2010).

In the present study, we evaluated time to metamorphosis and size of *C. maenas* megalopae (using as a proxy the number of days required to perform their metamorphosis since they have invaded the estuary and carapace size at metamorphosis). We also evaluated the number of days that these specimens can survive prior to metamorphosis and after metamorphosis under starvation, thus solely relying in the catabolism of all energetic reserves accumulated prior to their collection from the plankton. The performance of juvenile crabs (using as a proxy the number of days required to reach the fifth juvenile crab instar (C5), as well as C5 size and weight) was also evaluated during four consecutive supply events (SEs). The following two null hypotheses were tested: 1) larval

size at metamorphosis, time to metamorphosis and survival time of the first crab instar under starvation do not shift over consecutive SEs; and 2) under standard laboratory conditions, the performance of early juvenile crab instars does not shift with the SE of the megalopa, neither with upwelling conditions experienced during planktonic larval development.

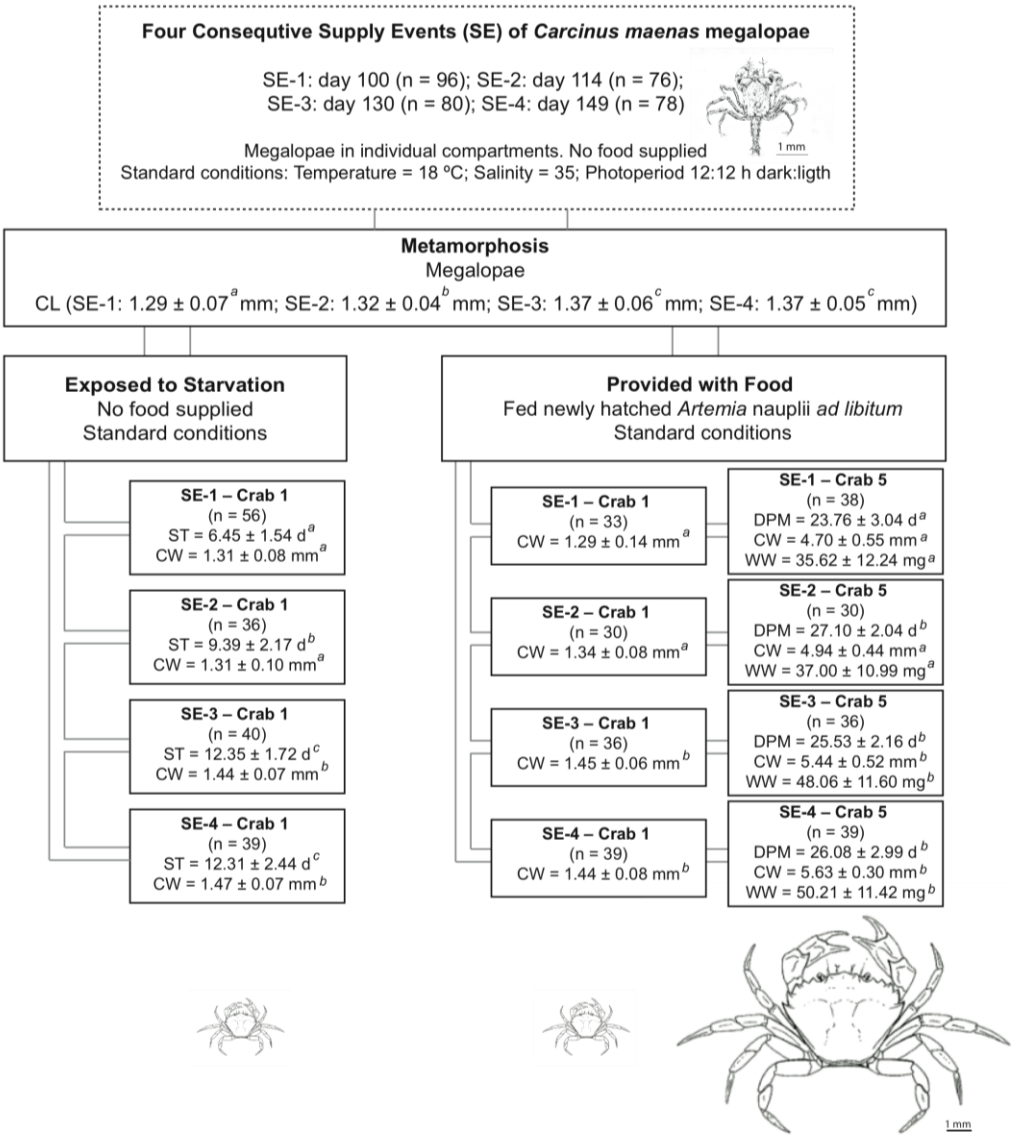

Figure 3.1.1 Schematic representation of the experimental design; values presented are averages (± SD). CL: carapace length; CW: carapace width; DPM: days post-metamorphosis; ST: survival time; WW: wet weight. The n in the CW of crab 1 and 5, provided with food *ad libitum*, indicates the number of shed exoskeletons that were possible to measure (not the number of surviving specimens per crab instar). Superscripts letters in values of the same variable represent significant differences among groups (Tukey HSD post hoc analysis, $P < 0.05$).

3.1.3 MATERIALS AND METHODS

3.1.3.1 COLLECTION OF WILD MEGALOPAE

Ria de Aveiro is a shallow coastal lagoon in the western margin of mainland Portugal, with a semidiurnal tidal circulation regime of ca. 12.42h (Pereira et al. 2000). This complex estuarine system, formed by 2 jetties, shallow channels and branches (with an average depth of 1 m), creates a favorable habitat for large populations of *C. maenas*. Wild megalopae of *C. maenas* were collected during four consecutive SEs at Costa Nova, Canal de Mira (40° 37' 17'' N, 8° 44' 56" W) in Ria de Aveiro, using two passive plankton nets as described by Queiroga et al. (2006). During each SE, the nets were deployed for 24 h at a constant depth of 0.1 m and with their entrances always facing the inlet of the estuary. Plankton held in the collector cup was gently rinsed and poured to a bucket filled with seawater and transported to the laboratory for analysis. The four SEs occurred between the months of April and May 2013. The dates of the different SEs surveyed were as follows: SE-1 10th of April; SE-2: 24th of April; SE-3: 10th of May and SE-4: 29th of May. Sampling days were numbered according to the Gregorian calendar as days of the year, in order to simplify data visualization and posterior analysis (SE-1: 100th day of the year; SE-2: 114th day of the year; SE-3: 130th day of the year; and SE-4: 149th day of the year).

3.1.3.2 EXPERIMENTAL SETUP

During each SE, *C. maenas* megalopae were individually sorted from the plankton sample collected in the laboratory using a light table and a modified plastic pipette. The number of megalopae sampled in each SE was as follows: SE-1: 96, SE-2: 76, SE-3: 80 and SE-4: 78. All sorted larvae were kept in individual PVC containers (with a volume of $\pm$ 300 mL) with artificial seawater (prepared by mixing Tropic Marin ProReef salt (Tropic Marine, Germany) with purified freshwater from a reverse osmosis unit) that was fully renewed daily. Controlled conditions of temperature (18 °C), salinity (35) and photoperiod (12:12 h dark:light) were employed for stocking sorted megalopae from the four SEs. No food was supplied to stocked megalopae until metamorphosis, with the rationale for this procedure being the assumption that megalopae entering estuaries have already reached the nutritional threshold required to perform metamorphosis (see Rey et al. 2015). Following

metamorphosis, all crabs in the first instar (C1) were haphazardly separated in two treatments: exposed to starvation (S) and provided with food *ad libitum* (F). In the F-treatment all crab specimens were supplied with newly hatched *Artemia* nauplii (provided daily *ad libitum*). Specimens from S and F treatments were visually inspected every day for exuviae or dead specimens until they have reached the crab 5 instar (C5). Routine full water changes were also performed after visual inspection and before supplying newly hatched *Artemia* nauplii (see Fig. 3.1.1 for a schematic representation of the experimental design). All shed exoskeletons were preserved individually in an 8:2 solution of ethyl alcohol (97%) and glycerine for posterior morphometric analysis. Carapace length (CL), measured from the tip of the rostrum to the posterior end of the cephalotorax, being determined for megalopae and carapace width (CW), measured between the first pair of lateral spines on the topside of the carapace, being determined for juvenile crab instars. CL and CW were determined to the nearest 0.01 mm using a stereomicroscope (SZX16, Olimpus) with a calibrated micrometer eyepiece. All specimens reaching C5 were sampled for recording their CW and wet weight (WW) (to the nearest 0.01 mg).

3.1.3.3 DATA ANALYSIS

One-way ANOVAs were performed to compare time to metamorphosis and size displayed by supplied megalopae (CL), using SE (four levels: SE-1, SE-2, SE-3, SE-4) as fixed factor. The effect of larval size (i.e., CL) on survival time of the first juvenile crab C1 starved of food (S-treatment) was tested over consecutive SEs using analysis of covariance (ANCOVA) where larval size was the covariate. Linear relationship between the covariate and the response and homogeneity of regression slopes was confirmed. Post hoc Tukey HSD test was used to test significant differences between SEs.

The early juvenile performance of specimens provided with food (F-treatment) was analysed using one-way ANOVAs. In this way, number of days post-metamorphosis required to reach C5, CW of C1, CW of C5 and WW displayed by C5 were compared over consecutive SEs, using SE (four levels: SE-1, SE-2, SE-3, SE-4) as fixed factor. Post hoc Tukey HSD test was used when ANOVA results revealed significant differences ($P < 0.05$). Assumptions of normality and homogeneity of variance were verified prior to analysis

through Shapiro-Wilks and Levene´s test, respectively. Whenever these assumptions were not verified we employed power transformations before performing statistical analysis. The level of statistical significance was $P < 0.05$.

General additive models (GAMs) allow the exploration of non-linear functional relationships between dependent and explanatory variables, fitting predictor variables by smooth functions (Guisan et al. 2002) rather than by linear or quadratic relationships (characteristics of the linear models). GAMs were applied based on the R-mgcv function (Zuur et al. 2007). Our models introduced the upwelling index from the Aveiro region (41° N, 10.5° W; 41.5° N, 10° W; 41° N, 9.5° W; 40.5° N, -10° W) as an explanatory variable (data from Instituto Español de Oceanografia, *www.indicedeafloramiento.ieo.es*, methodology used as described by González-Nuevo et al. (2014)). The rationale for this procedure was supported by available data from previous field (e.g., Queiroga 1996) and modelling studies (Marta-Almeida et al. 2006), which evidenced that upwelling favours the retention of larvae < 20 Km offshore in the study area. Settled megalopae in Ria de Aveiro can proceed from southern (< 60 Km) or northern estuaries (< 200 Km) (Domingues et al. 2012), thus their larval development occurs in the north-western coast of the Iberian Peninsula. Once upwelling index is quite homogeneous along this region (Haynes et al. 1993, Ramos et al. 2013), it is legitimate to assume that upwelling index from Aveiro region is a proxy of upwelling index experienced by settled megalopae during their larval development. In this way, daily values of the upwelling index were condensed to 60, 50, 40, 30, 20 and 10 d averages before each SE. The maximum period (60 d) was selected according to available estimates for the maximum duration of *C. maenas* planktonic larval development under water temperatures ranging between 12 and 15 °C (see Dawirs 1985). One GAM was built to each response variable in each time interval (60, 50, 40, 30, 20 and 10 d). All values of upwelling index were tested in the respective model to find the number of days that provided the best deviance explained by the response variables: CL, survival time, CW of C1, CW of C5 and WW of C5. Normality and outliers were checked following Zuur et al. (2007). The effects of upwelling index were tested using the following model:

$$E(Y_i) = \alpha + f_1(X_i) + \varepsilon$$

Where $E(Y_i)$ is the estimated value of the response variable (CL, survival time, CW of C1, CW of C5 or WW of C5), α is the intercept, X_i is the covariate (upwelling index), f_1 is the smooth function estimated for the covariate, ε is the error term and i represents the observed data (Wood 2006). The following model was used to test for the CL of megalopae: Y = CL (mm) and X_i = upwelling index ($m^3\ s^{-1}\ km^{-1}$). The following models were used to test for the different features recorded for juvenile crabs: Y = survival time for C1 (d); Y = CW for C1 (mm); Y = CW for C5 (mm) or Y = WW for C5 (mg) and X_i = upwelling index ($m^3\ s^{-1}\ km^{-1}$).

All data analysis were performed following the statistical package R version 2.13.2 (R Development Core Team 2011).

3.1.4 RESULTS

In all events, 100.0% of the megalopae were able to successfully metamorphose to C1, with the sole exception being recorded in SE-2, where survival reached 97.4% (Table 3.1.1). The differences in time to metamorphosis were not statistically significant (P = 0.1958) between events (Table 3.1.1), although specimens from SE-1 displayed a longer time to metamorphosis than those from other events. Moreover, significant differences were recorded in the CL of megalopae supplied during different events (P <0.0001), with those from SE-1 displaying the smallest CL (Fig. 3.1.2a).

When crabs were starved of food (S-treatment), survival time of first juvenile instars increased along the SEs (Table 3.1.1). ANCOVA revealed a significant interaction between survival time and larval size (i.e., CL) (R^2 = 0.59, $F_{4,94}$ = 33.77, P < 0.0001). Post hoc analysis showed significant differences (P < 0.001) between SEs (Table 3.1.1) (in other words, the larger the megalopae the longer the period endured under starvation by C1), with crabs originating from megalopae collected during SE-1 presenting the lowest size and survival time (Fig. 3.1.2 and Table 3.1.1, respectively). In the S-treatment, none of juvenile crabs C1 were able to moult to juvenile crab C2.

When crabs were supplied with food (F-treatment), at least 90.0% of all specimens from all events were able to reach C5, with the exception of those from SE-2 that presented a

survival of 78.9% (Table 3.1.1). The number of days post-metamorphosis required to reach C5 was significantly different between events ($P < 0.0001$), being the average days post-metamorphosis in SE-1 the lowest recorded in all events (Table 3.1.2). Statistical analysis of CW of C1 showed significant differences between SEs ($P < 0.0001$), with specimens from the two first events displaying smaller CW than those from the two last events (Fig. 3.1.2b). The analysis of CW of C5 showed significant differences between SEs ($P < 0.0001$). In the same way as C1, C5 from SE-1 and SE-2 displayed smaller CW than those from specimens originating from megalopae collected during the last events (Fig. 3.1.2c). Moreover, WW of C5 showed significant differences between events ($P < 0.0001$) (Fig. 3.1.2d). The trend recorded for WW was identical to that of CW, with C5 from the two first SEs displaying a lower WW than those from the two last events.

The juvenile crab instar that recorded the highest mortality was C1, with a number of cultured specimens being unable to reach C2 in all supply events, with the exception of SE-3. Moreover, the duration exhibited by each juvenile crab instar (Table 3.1.2) was significantly different between events (all at least $P < 0.0300$).

Upwelling regimens prevailing during larval development in the open ocean (Fig. 3.1.3) appeared to be significantly linked with the larval and juvenile performance recorded in the present study (Table 3.1.3). According to the GAM for the CL of megalopae, the additive model using upwelling index as explanatory variable indicated that CL presented a maximum with average upwelling regimens around 500 $m^3\ s^{-1}\ km^{-1}$, showing the highest deviance explained (26.2%) with an upwelling index average of 40 days before settlement (R^2 adjusted = 0.254, $P < 0.0001$) (Fig. 3.1.4a). Concerning GAMs for early juvenile performance, the smoothing function revealed that survival time displayed a positive correlation, showing the highest deviance explained (57.3%) with an upwelling index average of 30 days before settlement (R^2 adjusted = 0.564, $P < 0.0001$) (Fig. 3.1.4b). The maximum survival time was achieved with upwelling regimens around 600 $m^3\ s^{-1}\ km^{-1}$. Moreover, CW of C1 achieved a maximum level with upwelling regimen averaging around 400 $m^3\ s^{-1}\ km^{-1}$, showing the highest deviance explained (54.8%) with an upwelling index average of 40 days before settlement (R^2 adjusted = 0.536, $P < 0.0001$) (Fig. 3.1.4c). Regarding the F-treatment, the analysis of CW of C5 with smoothing function showed a

maximum level with upwelling regimen averaging around 300 m^3 s^{-1} km^{-1}, with the highest deviance explained (35.3%) referring to an upwelling index average of 50 days before settlement (R^2 adjusted = 0.335, $P < 0.0001$) (Fig. 3.1.4d). The GAM analysis for WW of C5 showed a maximum level with upwelling regimen averaging around 200 m^3 s^{-1} km^{-1}, showing the highest deviance explained (27.1%) with an upwelling index average of 50 days before the settlement (R^2 adjusted = 0.251, $P < 0.0001$) (Fig. 3.1.4e).

Table 3.1.1 Summary of the experimental design employed to survey wild megalopae (M) of *Carcinus maenas* and juvenile crab stages exposed to starvation (S-treatment) or provided with food (F-treatment). Data refer to four consecutive supply events (SE), with the following variables being monitored: time to metamorphosis (TM), survival from M to juvenile crab 1 (C1), survival time (ST) of C1 and survival from M to juvenile crab 5 (C5). Different superscripts letters represent significant differences among groups (Tukey HSD post hoc analysis, P < 0.05).

		Larval stage			Juvenile crab stages			
					S-treatment		F-treatment	
Supply Events	Day of the year	TM (d)	Survival (%) M-C1	n	ST (d) C1	n	Survival (%) M-C5	n
SE-1	100th	3.7 ± 1.1[a]	100.0	96	6.5 ± 1.5[a]	56	95.0	40
SE-2	114th	3.5 ± 1.6[a]	97.4	76	9.4 ± 2.2[b]	36	78.9	38
SE-3	130th	3.5 ± 1.0[a]	100.0	80	12.4 ± 1.7[c]	40	90.0	40
SE-4	149th	3.3 ± 1.9[a]	100.0	78	12.3 ± 2.4[c]	39	97.5	39

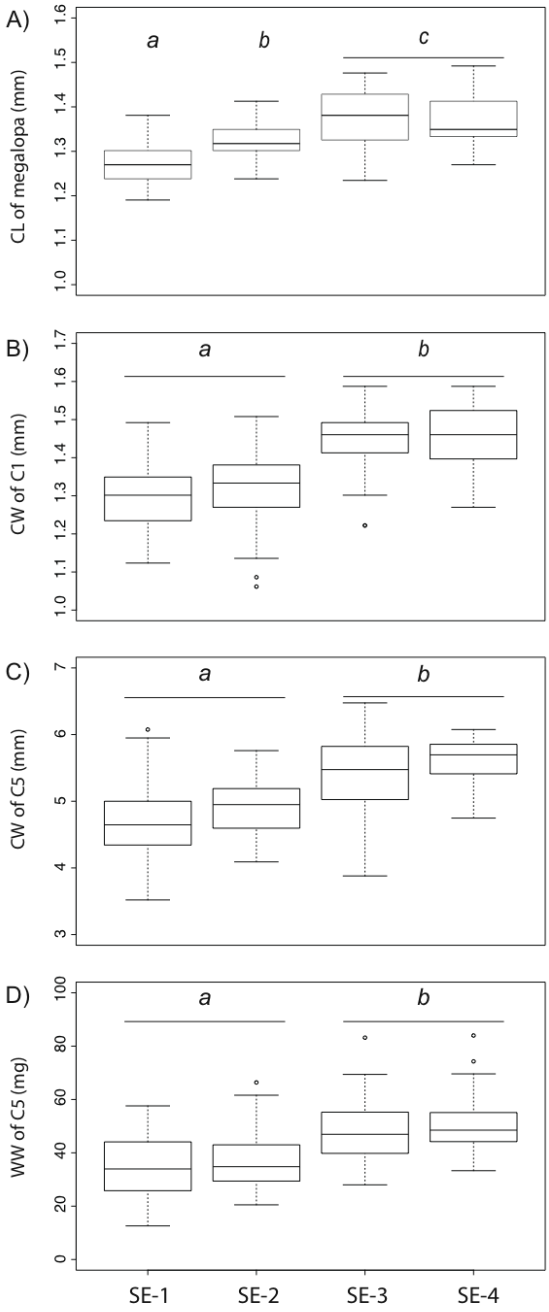

Figure 3.1.2 Larval size at metamorphosis and early juvenile performance of *Carcinus maenas* during four consecutive supply events (SE): SE-1: 100th, SE-2: 114th, SE-3: 130th, SE-4: 149th day of the year. A) Carapace length (CL) of the megalopa; B) Carapace width (CW) of first juvenile crab instar (C1) exposed to starvation (S-treatment) and provided with food *ad libitum* (F-treatment); C) CW of fifth juvenile crab instar (C5) in F-treatment; D) Wet weight (WW) of C5 in F-treatment. The line within each box indicates the median, and 50% of the values are within the box, while error bars represent range (from the minimum to the maximum value). Different letters represent significant differences among groups (Tukey HSD post hoc analysis, $P < 0.05$).

Table 3.1.2 Stage duration of juvenile crabs provided with food (Food-treatment) from juvenile crab 1 (C1) until juvenile crab 5 (C5), and days post-metamorphosis (DPM) from C1 to C5, during four consecutive supply events (SE). Significant differences between supply events are represented by different superscript letters within the same column (Tukey HSD post hoc analysis, $P < 0.05$).

		Stage duration (d)					
Supply Events	**Day of the year**	**C1**	**C2**	**C3**	**C4**	**DPM C1-C5**	**n**
SE-1	100th	4.2 ± 0.6[a]	4.2 ± 0.8[a]	5.2 ± 2.1[a]	9.2 ± 1.3[a]	23.8 ± 3.0[a]	40
SE-2	114th	5.4 ± 0.9[b]	6.0 ± 0.9[b]	6.6 ± 1.2[b]	8.2 ± 1.2[b]	27.1 ± 2.0[b]	38
SE-3	130th	5.2 ± 0.7[ab]	5.1 ± 0.4[a]	5.6 ± 1.4[ab]	8.6 ± 1.4[ab]	25.5 ± 2.2[b]	40
SE-4	149th	4.8 ± 0.8[ab]	5.5 ± 0.7[ab]	6.5 ± 0.9[b]	8.2 ± 2.0[ab]	26.1 ± 3.0[b]	39

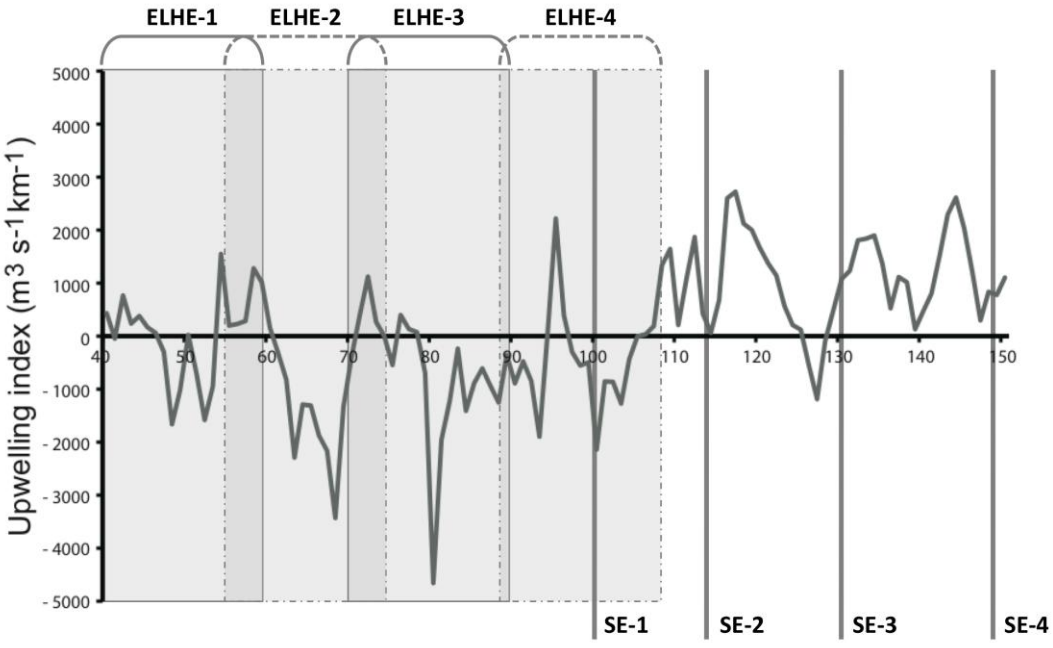

Figure 3.1.3 Time series of upwelling index in the coastal region of Aveiro, between the 40th and 150th day of year 2013. Shaded areas inside each rectangle indicate the range estimated for larval hatching events (ELHE) and vertical solid lines indicate the day of each supply event (SE) surveyed for *Carcinus maenas* megalopae performance in the present study: SE-1: 100th (ELHE-1: 40-60th); SE-2: 114th (ELHE-2: 54-74th); SE-3: 130th (ELHE-3: 70-90th); SE-4: 149th (ELHE-1: 89-109th).

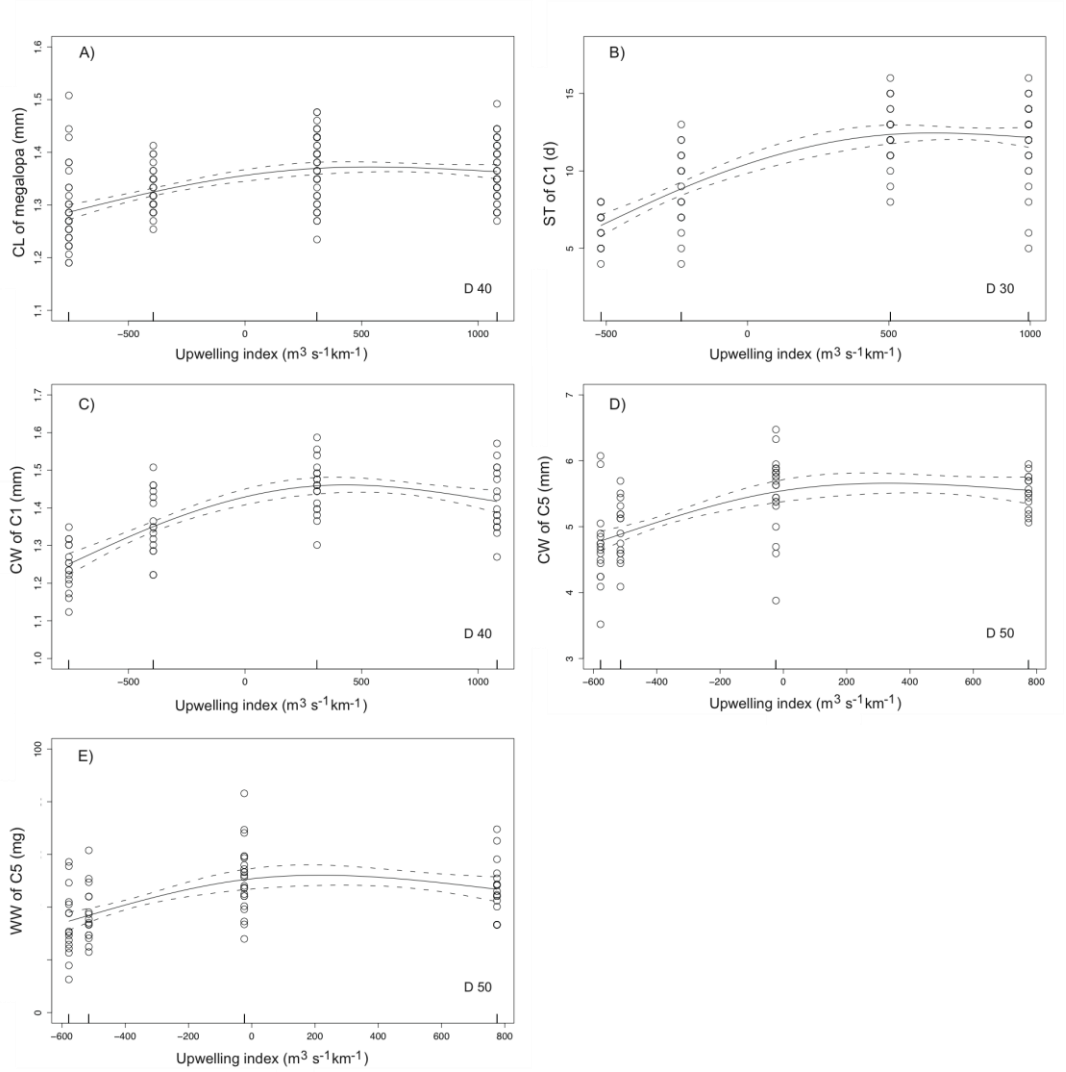

Figure 3.1.4 General additive models (GAMs) showing the partial effect of upwelling index on the larval size and juvenile crab performance of *Carcinus maenas.* A) Carapace length (CL) of the megalopa; B) Survival time (ST) of first juvenile crab instar (C1) exposed to starvation (S-treatment); C) Carapace width (CW) of C1 in S-treatment and CW of C1 provided with food *ad libitum* (F-treatment); D) CW of fifth juvenile crab instar (C5) in F-treatment; E) Wet weight (WW) of C5 in F-treatment. Dotted lines indicate 95% confidence intervals. Tick marks along the X-axis below each curve represent values of upwelling index average, condensed for different period (days) before each supply event. The number of days used to calculate upwelling index average for each analysis is presented in the right bottom corner of each graphic.

Table 3.1.3 Structure of the general addictive models (GAMs) selected to describe larval (carapace length (CL) of megalopa) and juvenile performance (survival time (ST), carapace width (CW) of crab instar 1 (C1), CW of crab instar 5 (C5) and wet weight (WW) of C5) of *Carcinus maenas* using the upwelling index (UI) (number of days of UI average that better explained the response variable) as explanatory variable. se: Standard error; edf: estimated degrees of freedom.

CL					
Parametric coefficients					
Parameter	Estimate	se	*t* value	*P* value	
Intercept	1.342	0.004	308.4	$< 2 \times 10^{-16}$	
Smooth terms (non-parametric)					
Parameter	edf	F	*P* value	R^2 adjusted	% Deviance explained
UI (D40)	1.939	27.92	3.63×10^{-11}	0.254	26.2
ST					
Parametric coefficients					
Parameter	Estimate	se	*t* value	*P* value	
Intercept	10.281	0.218	47.190	$< 2 \times 10^{-16}$	
Smooth terms (non-parametric)					
Parameter	edf	F	*P* value	R^2 adjusted	% Deviance explained
UI (D30)	1.946	60.44	$< 2 \times 10^{-16}$	0.564	57.3
CW – C1					
Parametric coefficients					
Parameter	Estimate	se	*t* value	*P* value	
Intercept	1.379	0.009	160.300	$< 2 \times 10^{-16}$	
Smooth terms (non-parametric)					
Parameter	edf	F	*P* value	R^2 adjusted	% Deviance explained
UI (D40)	1.971	41.17	1.52×10^{-12}	0.536	54.8
CW – C5					
Parametric coefficients					
Parameter	Estimate	se	*t* value	*P* value	
Intercept	5.227	0.058	89.610	$< 2 \times 10^{-16}$	
Smooth terms (non-parametric)					
Parameter	edf	F	*P* value	R^2 adjusted	% Deviance explained
UI (D50)	1.922	17.74	6.00×10^{-7}	0.335	35.3
WW – C5					
Parametric coefficients					
Parameter	Estimate	se	*t* value	*P* value	
Intercept	43.115	1.356	31.790	$< 2 \times 10^{-16}$	
Smooth terms (non-parametric)					
Parameter	edf	F	*P* value	R^2 adjusted	% Deviance explained
UI (D50)	1.931	11.92	3.55×10^{-5}	0.251	27.1

3.1.5 DISCUSSION

The results of the present study allow us to reject our first null hypothesis, as larval size at metamorphosis and survival time of the first crab instar under starvation significantly shifted over consecutive SEs, although the time to metamorphosis did not shift between events. The large number of megalopae collected from the plankton that were able to metamorphose to C1 under starvation conditions suggests that the last larval stage of *C. maenas* had already reached the Point of Reserve Saturation (PRS, *sensu* Anger & Dawirs (1981)) before entering the coastal lagoon. Upon reaching this critical point, larvae are able to endure periods of food shortage or even complete food deprivation and still undergo metamorphosis through the catabolism of nutritional reserves they have build-up prior to become competent (receptive to settlement cues) (Anger 2001). Furthermore, the short time frame recorded between the time of entrance in the coastal lagoon and metamorphosis suggests that the majority of megalopae collected was already in an advanced stage of their intermoult cycle, as already suggested by González-Gordillo et al. (2004) and Rey et al. (2015).

Megalopae sampled during the present study exhibited an increase in their CL along settlement events. Indeed, the larger size displayed by the last larval stage of *C. maenas* collected during the last two events (SE-3 and SE-4) suggests that these had developed under more favourable environmental and trophic conditions than conspecifics supplied during early events. Our data contrasts with that reported by Giménez (2010), who detected a decrease in the size of the megalopae supplied during the settlement season in Helgoland (North Sea, German Bight). Larger megalopae commonly give origin to larger sized juvenile crabs which display a superior performance during their early benthic life (Giménez 2010). These opposing trends in the megalopa size recorded by Giménez (2010) and the present study are most probably linked to the contrasting oceanographic regimes, and consequently trophic ecology, experienced by larvae developing in the eastern Atlantic upwelling system (Fiuza et al. 1998) and those developing in the German Bight (Giménez & Dick 2007).

Results recorded for C1 in the S-treatment confirmed that the first crab instar is unable to moult to C2 without the energetically provisioning secured through exogenous feeding, as already reported by Adelung (1971). Under starvation, megalopae that have reached their PRS consume their energetic reserves to undergo the dramatic shifts associated with metamorphosis; this energetic and nutritional drain inhibits subsequent moulting and ultimately leads to the death of the juvenile crab if deprived of food (Hartnoll 2001).

The differences in the survival time of juvenile C1 between SEs contribute to the idea presented above that suggests that larvae settled along the two last SEs (SE-3 and SE-4) may have spent their planktonic life under more favourable nutritional conditions than their conspecifics settling during SE-1 and SE-2. It must be highlighted that in our study a time frame of solely 30 days (the time elapsed between SE-1 and SE-3) was enough to duplicate the number of days that C1 were able to endure under starvation. The explanation for this contrasting performance may be related with the upwelling index recorded during the pelagic life of the zoeal stages that give origin to the megalopae collected during SE-1 and SE-3. Upwelling is an environmental index used to estimate coastal productivity (Tenore et al. 1995, Barth et al. 2007). Upwelling conditions along the Portuguese west coast give origin to nutrient-rich waters that promote the occurrence of high levels of phytoplankton (Oliveira et al. 2009), which triggers bottom up effects along the planktonic trophic webs in the pelagic environment and support high biological productivity. This oceanographic phenomena may lead to an increase in nutrients for planktonic organisms (e.g., plankonic larvae) and increase their settlement success (Blanton et al. 1987, Menge et al. 2003, Peteiro et al. 2011).

The time series of upwelling index (Fig. 3.1.3) shows that megalopae settled during SE-3 and SE-4 spent their larval life under positive and intermittent upwelling regimens. The succession of active upwelling (water fertilization) and relaxation periods (onshore transport) favours plankton blooms, as well as its coastal retention (Villegas-Ríos et al. 2011). As larval experience influences the post-settlement performance of marine invertebrates with complex life cycles (Pechenik 2006), the increase of survival time

during the SEs may most likely be related to the fertilization of coastal waters during upwelling events and the consequent enhancement of primary and secondary production in the Iberian region (Tenore et al. 1995, Guisande et al. 2001). In line with this rationale, the positive correlation recorded between CL and survival time may also be supported by the upwelling index. As time to metamorphosis did not differ significantly among events, it is legitimate to assume that megalopae from all these events may have made a similar energetic investment to metamorphose when stocked in the laboratory under identical conditions. In this way, the differences recorded in survival time may only be explained through the differential levels of energy accumulated by early stage megalopae (and previous zoeal stages) before re-invading their adult habitat. Marine invertebrate larvae are specialised in morphogenesis and growth, conditioning their feeding behaviour to maximize their performance (Calado & Leal 2015). Therefore, during planktonic life, larvae are well adapted to the patchy nature of the plankton, exploiting in a very efficient way even brief or intermittent periods of food availability (Harms et al. 1994, Giménez & Anger 2005, Calado & Leal 2015). Under favourable feeding conditions, developing larvae may easily accumulate and store high quality energetic reserves (Wang et al. 2014). Such energetic reserves will fuel subsequent periods of their life, namely energy demanding processes associated with metamorphosis and their early post-settlement life. Studies performed in other invertebrates, that also combined field and laboratory experiments, found a positive relationship between quality of settling larvae (e.g., larval nutritional conditions, larval size) and post-settlement performance (Phillips 2002, 2004, Jarret 2003, Marshall & Keough 2004).

The second null hypothesis tested in this study was also rejected, as under standard laboratory conditions, the performance of early juvenile crab instars significantly shifted with the SE of the megalopa and with upwelling conditions experienced during planktonic larval development. Juvenile crab traits surveyed during the present study (CW C1 and C5; and WW C5) kept a similar trend to that already described above for the megalopae, thus supporting the existence of carry-over effects from pelagic larval life to early benthic juvenile stages - as already highlighted by Pechenik (2006) “metamorphosis is not a new

beginning". Settlers originating from SE-1 and SE-2 presented a lower CW (at C1 and C5), as well as WW (C5), than those from SE-3 and SE-4, despite being stocked under identical culture conditions (e.g., food supplied *ad libitum*, constant temperature and salinity within optimal ranges for the species). This record evidences that early benthic life even under optimal conditions is not sufficient to erase the fingerprinting of larval life in the pelagic environment.

The analysis performed using GAMs showed that most of the deviance explained for megalopae size (i.e., CL) and juvenile crabs performance (i.e., CW of C1 and C5 and WW of C5) were obtained with upwelling index averaging between 40 to 50 days before the settlement event, with the exception of survival time where estimated upwelling index average was 30 days. The larval development of *C. maenas*, as in most marine invertebrates, is mainly controlled by temperature and food (Dawirs 1984, 1985, Calado & Leal 2015). The average temperature of the water in the months that preceded our sampling period commonly ranges between 12 and 15 °C (Domingues et al. 2011, Álvarez et al. 2013). Thus, according to Dawirs (1985), larval development of *C. maenas* is expected to range from 40 to 60 days from hatching to metamorphosis. In this way, it can be assumed that according to GAMs larval and juvenile traits recorded in the present study were determined by the nutritional conditions experienced by early larval stages (the zoea), as well as those experienced by the megalopae before reaching its PRS and becoming competent. The role played by the nutritional conditions experienced by megalopae at settlement will likely play a secondary role on its life post-metamorphosis. The positive influence of upwelling conditions in survival time (57.3% of deviance explained) suggests a relevant role of nutrient enrichment of the coastal waters during larval development. The low deviance explained (26.2%) in the CL analysis might be related to other factors which also condition larval phenotypes (e.g., physiological stress and maternal effects) (Marshall et al. 2008). Therefore, GAM results suggest an important role of larval history in juvenile performance, which can be explained by the occurrence of carry over effects (Pechenik 2006). Calado et al. (2005) and Calado (2008) showed that starving newly hatched larvae of a caridean shrimp and forcing them to catabolize their

reserves to moult to the second larval stage (Zoea II) only promoted detectable effects 20 to 40 days post-hatching (e.g., survival, larval stage duration and time of metamorphosis), and that these where carried over to early benthic life. Juvenile shrimp originating from larvae starved at hatching always displayed a poorer growth performance than conspecifics originating from larvae that never experienced a suboptimal feeding. In this way, environmental conditions prevailing during planktonic development (e.g., temperature, available nutrients) may condition larval performance (e.g., nutritional status, size) and these effects can persist during early benthic life.

Available data from long-term sampling of settlement events of marine invertebrates with bi-phasic life cycles have revealed the existence of a considerable variability in larval supply (Domingues et al. 2011, Peteiro et al. 2011, Pfaff et al. 2015), as well as in the quality of settling larvae (Phillips 2002, Jarret 2003, Giménez 2010, Cruz et al. 2010, Abdul Wahab et al. 2014). Our study showed that even within a reduced time frame (i.e., supply events with the highest number of megalope being recorded along the settlement season) the larval stages being supplied to undergo metamorphosis in the habitat occupied by their adult form displayed a variable post-metamorphosis performance. This natural variability appears to be associated with phenotypic plasticity, with GAM analysis exposing the relationship between larval and juvenile performance and the upwelling index prevailing during larval pelagic life. As larval survival and development are shaped by the quantity and quality of available food (Boidron-Métairon 1995), pelagic conditions experienced during larval life can determine the growth performance of juvenile specimens and their ability to recruit to the adult population (e.g., by reaching sexual maturity). Maternal investment is also known to play a key role in the success of early larval life (Marshall & Keough 2006, Marshall et al. 2008). It is now clear that researchers may only perceive the mechanisms ruling the population dynamics of marine invertebrates with bi-phasic life cycles by considering the driving force exerted by trait-mediated effects. It is therefore important to increase the number of studies that monitor not only the timing and quantitative supply of competent larvae, but also their quality in terms of survival and early benthic performance. Clarifying the origin of trait-mediated

effects, and being able to dissociate between maternal effects and environmental conditions as source of natural variability during early benthic life, will be a challenge for future studies. These topics are paramount to develop a unifying framework to better understand the population biology of marine invertebrates with bi-phasic life cycles.

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3.2 LABORATORY TRIALS REVEAL THAT EXPOSURE TO EXTREME RAINING EVENTS PRIOR TO METAMORPHOSIS AFFECTS THE POST-SETTLEMENT PERFORMANCE OF AN ESTUARINE CRAB

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Growth

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Phenotypic link

3.2.1 ABSTRACT

Meteorological forcing can impact planktonic communities, with extreme raining events promoting salinity decreases and triggering larval mortality in estuarine plankton. The present study evaluated how exposure to low salinities prior to metamorphosis of *Carcinus maenas* megalopae (last larval stage) may affect its ability to metamorphose and the post-metamorphosis performance of juvenile crabs. An extreme raining event that promoted a generalized decrease in salinity (from 25 to 10) in the whole water column of one of the main channels of a coastal lagoon was mimicked in the laboratory. Wild megalopae of *C. maenas* were collected and kept individually without any food at salinities of 10 or 25 (S10 or S25) until they either died or metamorphosed to the first crab instar (C1). Specimens metamorphosing in 5 days or less following their collection were labeled as early settlers (ES10 and ES25, from megalopae stocked at salinities of 10 and 25, respectively), while those taking more than 5 days were labeled as late settlers (LS10 and LS25, from megalopae stocked at salinities of 10 and 25, respectively). All newly metamorphosed crabs were kept individually until C5 at a salinity of 25 and fed *ad libitum*, with their intermolt periods and carapace width (CW) being recorded. Osmotic stress did not affect the survival or ability to metamorphose of *C. maenas* megalopae, with 89% of all larvae in both salinities being able to metamorphose. This result is supported by the ability of this larval stage to hyper-regulate. Nonetheless, an exposure of late settling megalopae to low salinities prior to metamorphosis promotes the occurrence of juvenile crabs with a smaller CW. The deleterious effects of exposing late settling megalopae to low salinities appears to be magnified during early benthic life, with C5 originating from treatment LS10 displaying a significantly smaller CW (4.87 ± 0.28 mm) and lower wet weight (WW) (28.95 ± 4.62 mg). On the other side, C5 originating from ES25 exhibited a significantly higher CW (5.90 ± 0.33 mm) and WW (50.89 ± 8.14 mg). The nutritional vulnerability experienced by megalopae starved for longer periods (late settlers) may have been magnified for specimens exposed to a lower salinity, with the energetic costs associated with hyper-regulation negatively affecting the growth performance of juvenile crabs. Osmotic stress experienced by late settling megalopae can shape adult populations of *C. maenas* by promoting the occurrence of smaller juveniles, thus more vulnerable to predation and cannibalism. Phenotypic links

must be incorporated in the study of marine invertebrates, namely when life stages are vulnerable to metereological forcing (e.g., extreme rainfall) at critical periods of their life cycle (e.g., metamorphosis).

3.2.2 INTRODUCTION

Salinity is a key ecological driver that shapes the patterns of growth and survival of marine larvae (Anger 2003). Large scale salinity decreases are recognized to be one of the major sources of larval mortality in estuarine plankton (Morgan 1995). Estuarine species with bi-phasic life cycles display a variable plasticity in their larval traits and environmental conditions can impact developing organisms in away that may structure adult populations (Giménez 2010). In other words, pre-settlement conditions experienced by developing larvae may affect their future benthic life through trait-mediated effects (Giménez 2006).

The European green crab *Carcinus maenas* (Linnaeus, 1758) is one of the best studied marine invertebrate species and is listed in the 100 "world's worst alien invasive species" (GISD 2009). This benthic brachyuran crab displays a bi-phasic life cycle with its first larval stage (zoea) hatching during the night at neap tides and being rapidly exported into coastal waters (Queiroga et al. 1997). Depending on water temperature, the pelagic zoeal stages of *C. maenas* take from 4 to 6 weeks in shelf waters to reach the last stage of its larval development – the megalopa (Dawirs 1985). It is the megalopa that reinvades the parental habitat, by traveling up the estuary during night flood tides through selective tidal stream transport, and latter metamorphoses into the first crab instar that assumes the benthic lifestyle of adult *C. maenas* (Queiroga et al. 1994, Zeng & Naylor 1996).

The present study aimed to identify how exposure to low salinities prior to metamorphosis of the estuarine crab *C. maenas* during its last larval stage (the megalopae) may affect its ability to metamorphose and the post-metamorphosis performance of juvenile crabs. The motivation for the present study was the occurrence of multiple and prolonged extreme raining events during late spring and early summer of 2013 that promoted a drop on the average salinity (from 25 to 10 and less) in the whole water column of the shallow

channels of Ria de Aveiro (Portugal) (see Section 3.2.3.1 *Collection of wild megalopae* for further details on this coastal ecosystem). While megalopae of *C. maenas* are able to osmoregulate when exposed to low salinities, their ability is inferior to that displayed by early crab instars (Cieluch 2004). In this way, one may assume that settling megalopae are likely to be affected by the sub-optimal salinities promoted by prolonged and extreme raining events. Moreover, it remains unknown if any potential deleterious effects resulting from this osmotic stress prior to metamorphosis can be carried-over to their early benthic life.

3.2.3 MATERIALS AND METHODS

3.2.3.1 COLLECTION OF WILD MEGALOPAE

Wild megalopae of *C. maenas* were collected in the same day during a supply event in June 2013 at Costa Nova, Canal de Mira (40º 37´ 17´´ N, 8º 44´ 56´´W) in Ria de Aveiro (Portugal) using passive plankton nets (described by Queiroga et al. 2006) deployed 0.1 m below the water surface. Ria de Aveiro is a shallow coastal lagoon in the western margin of mainland Portugal, with its inlet being artificially maintained by 2 jetties and its shallow channels and branches (with an average depth of 1 m) forming an intricate system that provides a favorable habitat for *C. maenas*.

3.2.3.2 EXPERIMENTAL SETUP

A total of 70 megalopae were randomly selected in the laboratory from the pool of sampled larvae and separated in two groups of 35. The first group was stocked at a salinity of 25 (S25) (the salinity was measured using the Practical Salinity Scale), a value identical to the one recorded at the collection site, and used as control. The second group was stocked at a salinity of 10 (S10), a value that mimicked a salinity drop promoted by extreme raining events at the collection site (Fig. 3.2.1). All larvae were placed in synthetic seawater (prepared by mixing Tropic Marin ProReef salt (Tropic Marine, Germany) with freshwater purified by a reverse osmosis unit), with megalopae in treatment S10 being acclimated over 5 h to a salinity of 10 through the addition of freshwater. Both treatments (S10 and

S25) were performed under controlled laboratory conditions of temperature (18 ºC) and photoperiod (12:12 h dark:light). All megalopae were kept in individual PVC containers (with a volume ~300 mL) and no food was provided until metamorphosis (the rationale for this procedure was the assumption that megalopae entering estuaries have already reached the nutritional threshold required to perform metamorphosis; see below). All newly metamorphosed crabs (crab instar 1, C1) were stocked under the same laboratory conditions previously described for the megalopae, with the exception of C1 from S10 treatment that were also stocked at a salinity of 25 (assuming that juvenile crabs will develop under a favorable salinity) (the acclimation protocol was similar to that previously described, with salinity being increased through the addition of water at a salinity of 25) (Fig. 3.2.1). Crabs originating from megalopae metamorphosing within 5 days after their sampling date were considered as early settlers (ES10 and ES25, from megalopae stocked at salinities of 10 and 25, respectively), while those taking more than 5 days to metamorphose were labeled as late settlers (LS10 and LS25, from megalopae stocked at salinities of 10 and 25, respectively). Our daily survey for over four consecutive months on the average time to metamorphosis displayed by *C. maenas* megalopae (following their capture from the plankton and stocking under starvation in the laboratory) was used as rationale to support this classification. Over 70% of all megalopae surveyed metamorphosed in 5 days or less following their collection from the wild. All specimens were cultured until C5 using newly hatched *Artemia* nauplii (provided daily *ad libitum*) (Fig. 3.2.1). The water from each individual container was fully renewed every day after a visual inspection for exuviae or dead specimens. All shed exoskeletons were preserved individually in an 8:2 solution of ethyl alcohol (97%) and glycerin for morphometric analysis. Carapace length (CL) solely for megalopae (measured from the anterior tip of the rostrum to the posterior end of the cephalothorax) and carapace width (CW) solely for crab instars (measured between the first pair of lateral spines of the carapace). CL and CW were determined to the nearest 0.01 mm using a stereomicroscope with a calibrated micrometer eye-piece (SZX16, Olimpus). All specimens reaching C5 were collected for analysis of CW and wet weight (WW) (to the nearest 0.01 mg).

3.2.3.3 STATISTICAL ANALYSIS

Time to metamorphosis (TM), CL, days post-metamorphosis (DPM) to reach C5, CW (for C1 and C5) and WW (for C5) were analysed using a two-way ANOVA with salinity (25 and 10) and settlement (early and late) being used as fixed factors. Duration of crab instars (from C1 to C4) was analysed using a three-way ANOVA with salinity (25 and 10), settlement (early and late) and crab instar (C1 to C4) being used as fixed factors. Assumptions of normality and homogeneity of variance were verified prior to analysis through Shapiro–Wilks and Levene's test, respectively. Whenever significance was accepted, at $P < 0.05$, Tukey's multiple comparison test was used for a pairwise comparison of the means. All data analysis was performed following the statistical package R version 2.13.2 (R Development Core Team 2011).

3.2.4 RESULTS

Mortality prior to metamorphosis was not affected by salinity, as 89% of all megalopae (at S10 or S25) were able to metamorphose. While specimens in treatment S10 displayed a higher time to metamorphosis (Fig. 3.2.1), the differences in average time to metamorphosis (± SD) for megalopae at S10 or S25 were not statistically significant (P = 0.987) (5.3 ± 1.2 and 4.9 ± 1.5 days, respectively). While no significant difference was recorded in the CL of megalopae collected from the field in the present study (P = 0.693; Fig. 3.2.1), newly metamorphosed crabs (C1) displayed a variable CW. No significant differences (P = 0.069) were recorded in CW of C1 originating from ES10 when compared to those from treatment LS10 (the same pattern was recorded for ES25 and LS25). Nonetheless, a significantly (P = 0.003) smaller CW was recorded for C1 originating from LS10 (1.37 ± 0.02 mm) when compared to those from treatment ES25 (1.45 ± 0.03 mm).

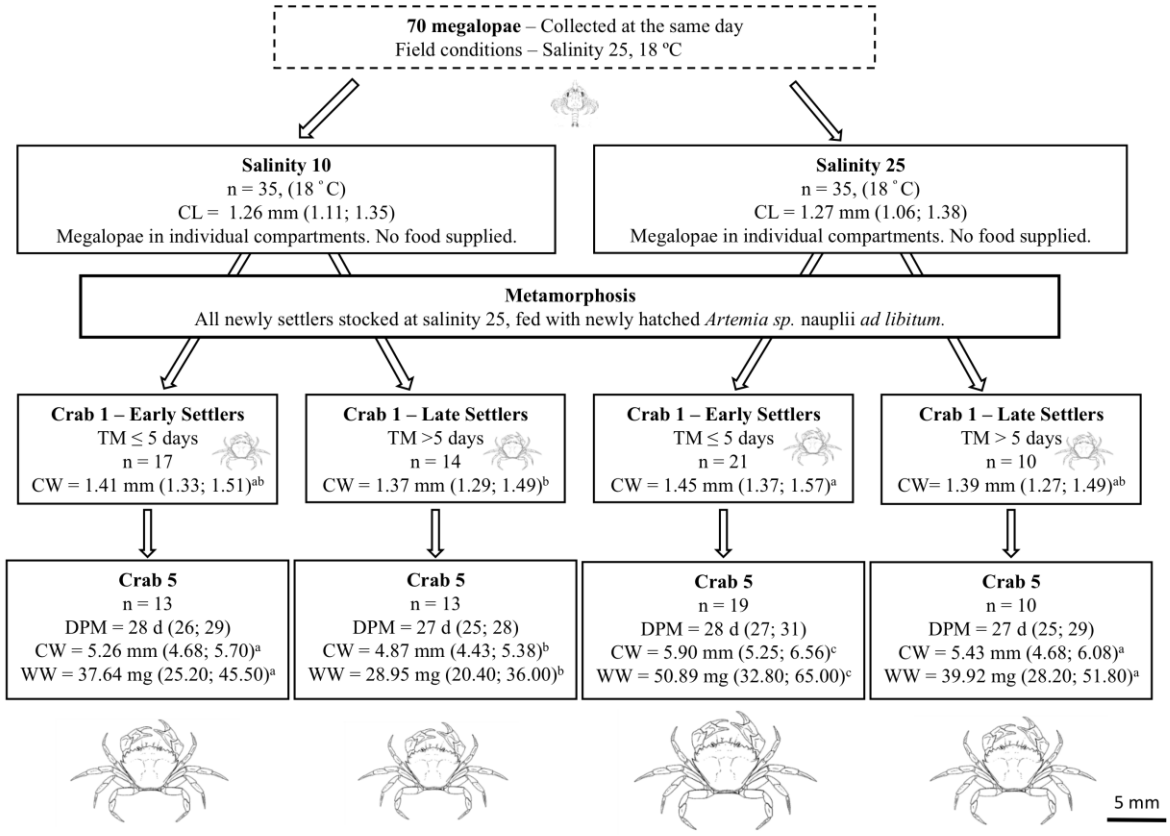

Figure 3.2.1 Schematic representation of the experimental design. Values presented are averages (minimum; maximum). CL: carapace length; CW: carapace width; WW: wet weight; TM: time to metamorphosis; DPM: day post-metamorphosis. Superscripts letters in the same row represent significant differences among groups $P < 0.05$.

The average time recorded to reach C5 for ES and LS was not significantly different (P = 0.958) (DPM averaging 27 to 28 in all treatments). However, the average duration of crab instars was different between ES and LS (Table 3.2.1). Indeed, the average intermolt period at C4 displayed by juvenile crabs originating from ES25 (8.3 ± 0.9 days) was significantly (P < 0.001) longer to that displayed by crabs at the same instar originating from all other treatments. This result is in line with the significantly larger (P < 0.003) CW (5.90 ± 0.33 mm) displayed by newly molted C5 originating from treatment ES25. The smallest CW recorded at C5 was that displayed by juveniles originating from treatment LS10 (4.87 ± 0.28 mm) (Fig. 3.2.1). The absence of significant differences in CW recorded at C1 between

specimens originating from ES and LS stocked at the same salinity, was no longer verified at C5, as crabs originating from ES megalopae always displayed a significantly (P = 0.002) wider CW (Fig. 3.2.1). The WW of C5 originating from ES and LS (initially stocked at the same salinity) was significantly different (P < 0.007), with C5 originating from ES always displaying a higher WW (when compared to LS from the same salinity treatment; 50.89 ± 8.15 and 37.64 ± 5.75 mg for ES25 and ES10, respectively) (Fig. 3.2.1). The lowest WW was that displayed by specimens originating from treatment LS10 (28.95 ± 4.62 mg) (Fig. 3.2.1).

Table 3.2.1 Survival (%) and average stage duration (days) (± SD) (as well as minimum and maximum stage duration) during early benthic life of *Carcinus maenas* initially exposed to a salinity of 10 or 25 at the larval stage of megalopae (M). C1: crab instar 1; C2: crab instar 2; C3: crab instar 3; C4: crab instar 4; C5: crab instar 5; ES: early settlers (time to metamorphosis ≤ 5 days); LS: late settlers (time to metamorphosis > 5 days).

Survival	*ES10*	*LS10*	*ES25*	*EL25*
M – C5	76.5	92.9	90.5	100.0
M – C1	100.0	100.0	100.0	100.0
C1 – C2	82.4	92.9	90.5	100.0
C2 – C3	100.0	100.0	100.0	100.0
C3 – C4	100.0	100.0	100.0	100.0
C4 – C5	92.9	100.0	100.0	100.0
Stage duration	*ES10*	*LS10*	*ES25*	*EL25*
C1	5.6± 0.6 (4, 6)	6.5 ± 0.5 (6, 7)	5.1 ± 0.2 (5, 6)	6.2 ± 0.8 (5, 7)
C2	6.1 ± 0.6 (5, 7)	5.5 ± 0.7 (5, 7)	6.3 ± 0.9 (5, 9)	5.7 ± 0.7 (5, 7)
C3	6.6 ± 0.8 (6, 9)	6.6 ± 1.3 (5, 9)	6.1± 0.6 (5, 7)	6.6 ± 0.5 (6, 7)
C4	8.1 ± 0.9 (6, 9)	7.5 ± 1.3 (6, 10)	9.3 ± 0.9 (8, 11)	7.5 ± 1.0 (6, 9)
C1 – C5	26.4 ± 1.0 (24, 28)	26.1 ± 1.0 (24, 27)	26.7 ± 1.0 (26, 30)	26.0 ± 1.2 (24, 28)

3.2.5 DISCUSSION

In the present study, the survival recorded for *C. maenas* megalopae collected from the wild confirms that the majority of specimens had already reached the D_0 threshold, a 'critical point in the larval development of decapod crustaceans' (Anger 1987). Briefly, if a decapod larva is exposed to suitable conditions of food availability until this critical point,

also termed as Point of Reserve Saturation (PRS) (Anger & Dawirs 1981), it will be able to complete its moulting cycle and advance to the next larval stage (or metamorphose), even if deprived of any exogenous food. Therefore it is legitimate to assume that food deprivation or suboptimal feeding will have a limited impact on the ability of larvae to metamorphose. However, it can be questioned if providing no food until metamorphosis to megalopae collected from the wild truly mimics conditions experienced *in situ*. It is possible that under osmotic stress megalopae that have already reached PRS may increase feeding to compensate for extra energetic expenditure in hyperregulation at lower salinities (see below). To date no study has addressed how suboptimal abiotic conditions (e.g., salinity, temperature…) may affect the feeding behavior of brachyuran megalaopae that have already reached their PRS. Indeed, it is still unknown how suboptimal feeding beyond PRS and until metamorphosis may condition the post-settlement performance of decapods. In this way, the suitability of starving larvae collected from the wild that have already reached their PRS and have already accumulated enough energetic reserves to undergo metamorphosis may need to be revised once the questions detailed above are addressed in future studies.

The megalopae of *C. maenas* already display the ability to hyperregulate at salinities lower than 25 (Cieluch 2004) and decreases in salinity have little to no effect on their survival and timing to metamorphosis (Zeng et al. 1997). Our data support these observations and confirm that even high levels of osmotic stress (salinity of 10 in the present study *vs.* salinity of 18 in Zeng et al. (1997)) plays little effect on the ability to undergo metamophsosis in *C. maenas* megalopae. As already reported by Zeng et al. (1997), *C. maenas* megalopae do not react to a number of cues known to condition metamorphosis in brachyuran crabs (e.g., salinity, light-dark regimes, odors from conspecifics, substratum…) (see Forward et al. 2001, Gebauer et al. 2003). Overall, this may be an overlooked feature that has allowed this species to display a remarkable distribution range, as well as become such a successful invader worldwide.

Under a scenario of an extreme raining event, such as the one simulated in the present

study, one may question whether emigration from estuaries could be a potential alternative for *C. maenas* megalopae to escape osmotic stress. Queiroga (1998) documented that during winter time, when salinity decreases promoted by raining events occur more frequently, *C. maenas* megalopae tend to avoid the upper layers of the water column. By staying near the bottom, megalopae can therefore avoid less favorable salinities. Nonetheless, this behavior will also avoid larvae to be exported from the estuary, thus impairing megalopae emigration as a pathway to avoid osmotic stress. The emigration pathway is also unlikely to be possible for *C. maenas* megalopae, as Zeng and Naylor (1996) reported that this larval stage 'swarmed in surface waters mainly during flood tides'. This tidal-influenced swimming behavior would therefore favor the invasion of the estuarine habitat by the megalopae, rather than their emigration. It is worth referring that larval behaviors in *C. maenas* can be locally adapted (Moksnes et al. 2014). Therefore, given the extreme salinity decrease recorded in the study area, as well as the shallow depth of most water channels in Ria de Aveiro (that likely impair the occurrence of well-marked salinity clines) further studies are necessary to exclude emigration from the estuary as a potential pathway for *C. maenas* megalopae to avoid osmotic stress.

Megalopae delaying their metamorphosis are fueled energetically through the consumption of their internal reserves and commonly give origin to smaller sized C1 (Gebauer et al. 1999). Moreover, size variability displayed by megalopae in the field at the time of metamorphosis may also affect the size of newly settled recruits (Giménez 2010). Nonetheless, in the present study, no significant differences were recorded in the CL of megalopae used in the experimental trials. The differences recorded in CW at C1 for LS10 are most likely the result of the energetic costs associated with hyper-regulation (a consequence of their exposure to a low salinity), rather than promoted by a larger CL at the stage of megalopae.

It could be predicted that with longer culture periods juvenile crabs originating from megalopae exposed to osmotic stress would be able to compensate from nutritional deficits associated with the energetic expenses of hyper-regulation at low salinities. As

crabs from all experimental treatments were fed *ad libitum*, it was somehow unexpected to record such significant differences in CW at C5 (in comparison to C1). These results may be explained by CW increment being significantly higher in the first four crab stages in *C. maenas* (Shen 1935). Over the culture period, size differences would tend to be magnified, with larger specimens at C1 becoming even larger at latter juvenile stages (e.g., C5), regardless of being fed *ad libitum*. Overall, under identical feeding conditions, juvenile *C. maenas* originating from megalopae exposed to osmotic stress and taking a longer period to metamorphose (LS10) appear to be unable to undergo compensatory growth (a feature exhibited by several decapod crustaceans; see Wickins and Lee, 2002) and reach similar sizes to those of conspecifics. It has already been shown for other decapod crustaceans that prolonged periods of starvation may negatively affect food conversion efficiencies upon re-feeding (Wu et al. 2000). This finding agrees with our results, as it explains why juveniles originating from LS megalopae (starved for longer periods) were always outperformed by ES conspecifics. The poorer growth performance displayed by juveniles from treatment LS10 as late as at C5 may therefore be a result of lack of compensatory growth and/or an inferior food conversion efficiency.

The longer intermolt periods displayed by older crab instars originating from treatment ES25 agree with a well-established premise for decapods: larger specimens commonly display longer intermolt periods (Hartnoll 1982). As at the end of the experiment C5 from treatment ES25 displayed the widest average CW (5.90 ± 0.33 mm) (Fig. 3.2.1) the differences recorded for the duration of intermolt periods are somehow expected.

In the present study, juveniles originating from LS megalopae have most likely allocated a higher proportion of the energy derived from the food provided post-metamorphosis to compensate the nutritional deficiency prompted by the longer periods of food deprivation endured prior to metamorphosis. Larval growth displayed by euryhaline crustaceans, such as *C. maenas*, appears to be unaffected by the energetic costs associated with osmoregulation (Torres et al. 2011). Nonetheless, the nutritional vulnerability experienced by starved megalopae in the present work may have been magnified when these

specimens were exposed to a lower salinity (S10), with the energetic costs associated with hyperregulation negatively affecting the growth performance of juveniles. The present study revealed that *C. maenas* megalopae invading the estuary exhibit variable levels of competence to metamorphosis. It is possible that at times megalopae may be supplied to estuaries 'before time' (prior to or only a few days after reaching the thresholds of energetic reserves that may allow the larvae to metamorphose), as larval supply in the area is often episodic, especially when driven by highly energetic SW wind events, which are also associated with strong precipitation and decreases in salinity (Domingues et al. 2011). Coastal zones in temperate regions can be exposed to episodic meteorological forcings (e.g., extreme raining events) that are known to promote dramatic disturbances (*sensu* Pickett et al. (1989) – discrete events that change the structure of a community) in the plankton (Nogueira et al. 2000). While phytoplankton and bacterioplankton are more readily affected from such disturbances (Guadayol et al. 2009), zooplankton that may tolerate the osmotic stress promoted by salinity decreases is likely to be negatively impacted through the occurrence of unfavorable trophic conditions (the most dramatic being intermittent or prolonged starvation). In this way, not only the scenarios tested in our study recreating a variable exposure (from 1 to 8 days) to food deprivation and osmotic stress prior to metamorphosis are likely to occur in the wild in the presence of prolonged events of extreme rainfall, they also show that such conditions can negatively impact early benthic life. Predation on *C. maenas* juveniles is strongly regulated by CW (Almeida et al. 2011) and decreases dramatically from 90% on newly metamorphosed juveniles to 10% on juveniles with a CW between 5 and 9 mm (Moksnes et al. 1998). Therefore, osmotic stress experienced by megalopae that take longer than 5 days to metamorphose has the potential to shape adult populations of *C. maenas* by promoting the occurrence of smaller juvenile crabs, thus more vulnerable to predation and cannibalism.

Overall, salinity decreases due to extreme raining events appear to have little to no effect on the survival and ability to metamorphose of *C. maenas* megalopae invading estuarine habitats. Nonetheless, and despite their ability to hyper-regulate, the present study shows

that the exposure of *C. maenas* megalopae to low salinities prior to metamorphosis can promote deleterious effects that cascade over their early benthic life and negatively affect the growth performance of young recruits. It is therefore especially important to incorporate phenotypic links in the study of marine invertebrates with bi-phasic life cycles, namely when life stages are vulnerable to metereological forcing (e.g., extreme rainfall) at critical periods of their life cycle (e.g., metamorphosis).

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PART IV

CONCLUDING REMARKS AND FUTURE STUDIES

4.1 CONCLUDING REMARKS

This thesis integrated two topics, maternal effects (Part II) and post-settlement performance (Part III), to achieve a deeper understanding of the natural variability in marine invertebrate larvae during their pelagic life and post-metamorphosis when they undergo dramatic behavioural, morphological and physiological changes and shift to a benthic lifestyle. The combination of field samples and laboratorial experiments, using the model species *Carcinus maenas,* provided new insights that helped us advance the state of the art and which will likely also be used in other research fields than that of marine biology/ecology.

4.1.1 MATERNAL PROVISIONING

Embryogenesis is a dynamic process where lipids play a central role as structural and energetic components (Lee et al. 2006, Rosa et al. 2007). The reserves transferred by the females to the yolk fuel embryonic development and define early larval quality (Anger 2001). The majority of female decapod crustacean brood their embryos to provide a favourable environment and protect them during embryogenesis. Within the brooding chamber, the environmental conditions can differ between embryos located in inner or outer regions of the brood, which may be reflected in their catabolism during embryonic development (Fernández et al. 2002). Nonetheless, in *C. maenas*, the position of embryos within female's brooding chamber was not a source of variability. The fatty acid analysis of embryos from four different areas of the brooding chamber showed no significant differences in either newly extruded embryos or embryos about to hatch. The lack of significant differences in their fatty acid profile, in both embryonic stages, allowed to outline these main findings:

- ✓ There are no differences in maternal investment during oogenesis in *C. maenas*.
- ✓ Embryo location in females brooding chamber does not affect the metabolic pathways of embryogenesis during the incubation period.

- ✓ *Carcinus maenas* females exert an effective maternal care while brooding its embryos, since no significant differences were recorded in the lipid catabolism between different brooding areas (namely in inner and outer regions of the brood).

The second and third studies of Part II analysed the maternal investment and fatty acid catabolism at two temporal scales. The second study investigated the interannual variability in maternal investment and embryogenesis of *C. maenas* in the same period of the reproductive season, as well as the effect of female size. The main findings were:

- ✓ There are no significant differences in maternal investment between small and large females of *C. maenas*. This suggests that nutritional resources available for both female sizes are similar and not limited.
- ✓ There are no significant differences in lipid catabolism of embryos from small and large females of *C. maenas*. This suggests that the efficiency of maternal care during incubation does not depend of maternal size.
- ✓ There are no significant differences in maternal investment of *C. maenas* between two consecutive years. This suggests that nutritional resources available are similar quantitatively and/or qualitatively over these two consecutive years.
- ✓ There are significant differences in lipid catabolism of *C. maenas* embryos incubated during two consecutive years. This result suggests that environmental conditions prevailing during embryogenesis can influence the quality newly hatched larvae.

The third study analysed the effect of season in embryonic quality during the reproductive period of *C. maenas*. The effect of maternal size was also analysed in this study and the main findings recorded agree with those from the study described above. The main findings were:

- ✓ The lipid composition of the yolk is conditioned by the period within the reproductive season in which the ovarian maturation occurs, suggesting an influence of food quality/availability during this process.
- ✓ The reproductive season acts as source of variability in offspring phenotype, suggesting a relationship with the nutritional status of females rather than their size.

The last study of Part II is a high-resolution study of the polar lipid dynamics during the embryogenesis of *C. maenas* and *Neroca puber*. This study presented a new perspective to analyse lipid catabolism in marine invertebrate embryos. The combination of several biochemical tools provided a detailed analysis, which identified and quantified phospholipids classes, their molecular species and fatty acid composition during the embryonic development of both crab species. The main conclusions were:

- ✓ There are no significant differences in maternal investment between *C. maenas* and *N. puber*.
- ✓ There are no significant differences in lipid catabolism between *C. maenas* and *N. puber* during embryogenesis.
- ✓ The occurrence of phospholipids classes during embryogenesis suggests a specialization of their functions in embryonic development.
- ✓ Both crab species present a positive retention of highly unsaturated fatty acid during embryogenesis, which suggests a preparation for early planktonic life.

4.1.2 SETTLEMENT DYNAMICS AND POST-SETTLEMENT PERFORMANCE

The larval background and the quality of the settlers exert a strong influence in the population dynamics of organisms with complex life cycle (Giménez 2010). In this regard, the larval competence and juvenile performance of *C. maenas* were evaluated during four consecutive larval supply events in Ria de Aveiro. The main findings were:

- ✓ Larval quality is not homogeneous along supply events.

- ✓ Larval history affects juvenile crab performance, at least until instar 5.
- ✓ Optimal grow-out conditions do not delete the larval history.
- ✓ Upwelling index prevailing during larval development has a strong influence in larval quality and early juvenile crab performance, at least until instar 5.

The last study of Part III demonstrated that the exposure to unfavourable environmental conditions prior to metamorphosis (e.g., extreme salinity) can significantly affect the juvenile performance of *C. maenas*. The main findings were:

- ✓ Megalopa competence and survival is not affected by osmotic stress.
- ✓ Exposure to low salinities prior to metamorphosis promotes a reduction of carapace width and wet weight of juvenile instars, at least until instar 5.
- ✓ Under the same environmental conditions, early settlers display a better performance than late settlers.

4.2 FUTURE STUDIES

The full understanding of organisms with complex life cycle will only be achieve when the different phases of their life history are examined from a holistic point of view. This thesis confirmed that maternal effects should not be overlooked in marine ecology studies. Previous studies addressing decapod crustacean confirmed the existence of within brood variability in the fatty acid profile of newly extruded and developing embryos (Pochelon et al. 2011, Leal et al. 2013). Nonetheless, while the high level of embryonic packing present in brachyuran crabs is known to limit oxygen availability in the centre of the brooding chamber (Naylor et al. 1999, Fernández et al. 2000, Baeza & Fernández 2002), no within-brood variability was recorded in the fatty acid analysis of *C. maenas*. In this way, additional studies on within-brood variability should be performed in brachyuran crabs with similar body sizes and highly packed embryos to determine if the findings recorded for *Carcinus maenas* are a potential exception to a generalized rule in this group of decapods.

Taking advantage of the invasive profile of *C. maenas* and its spreading along distant locations (Argentina to the USA, South Africa to Australia) outside its home range, future studies should also address large scale spatial variability in maternal provisioning and embryonic lipid catabolism. The analysis of fatty acid profiles of a number of marine invertebrates already allowed researchers to identificy zoogeographical patterns, even at a small spatial scale (e.g., Grahl-Nielsen et al. 2010, Ricardo et al. 2015). The knowledge of maternal provisioning and embryonic dynamics in invaded habitats may contribute to a better understanding of the success of this invader and help to control *C. maenas* populations in these sites.

The conditions prevailing during larval development play an important role in population dynamics (Kirby et al. 2007, Marshall & Morgan 2011). Until the present, it is impossible to monitor in the field larval life forms from hatching to settlement, but we can gain additional knowledge on larval life by surveying the performance of young settlers (Giménez 2010). The analysis of natural diets using and/or biochemical tools (Dalsgaard et al. 2003, Wang et al. 2014) provides long term information on the nutritional environment experienced during larval development and a deeper understanding of natural diets will be paramount to advance the state of the art of larval ecology. Moreover, it will also be an integrative instrument to understand complex life cycles.

Finally, the phenotypic links across different life stages of bi-phasic life cycles must be incorporated to truly understand the dynamics of marine invertebrates population. Environmental and trophic conditions influence each stage of the life history of these organisms (González-Ortegón & Giménez 2014, D'Urban Jackson et al. 2014) and their effects can be carried-over to the following stages (also known as "latent effects") (Pechenik 2006, Marshall & Morgan 2011). Therefore, more studies in phenotypic plasticity and their consequences on larval, juvenile and adult fitness must be performed to understand their ecological and evolutionary consequences.

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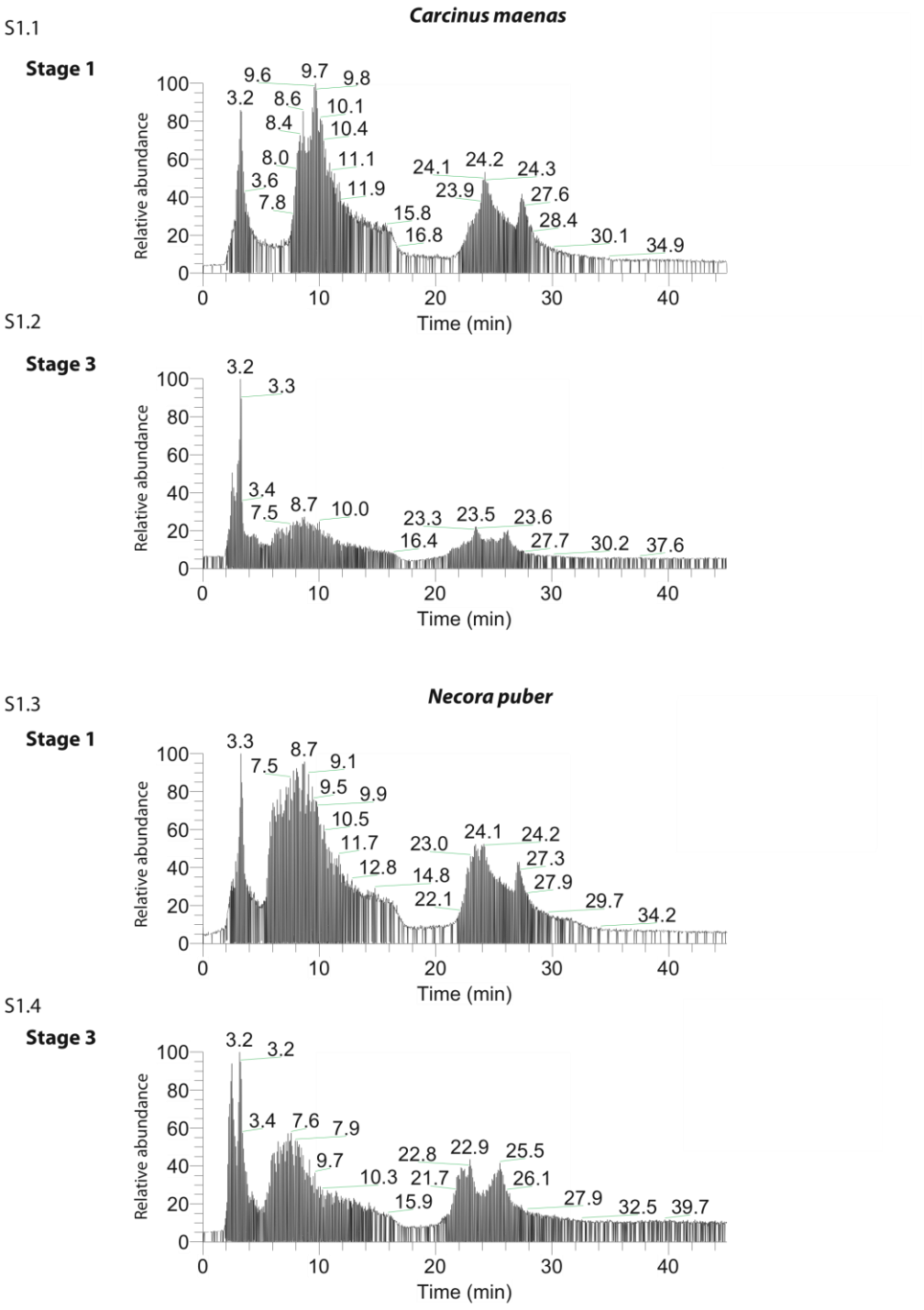
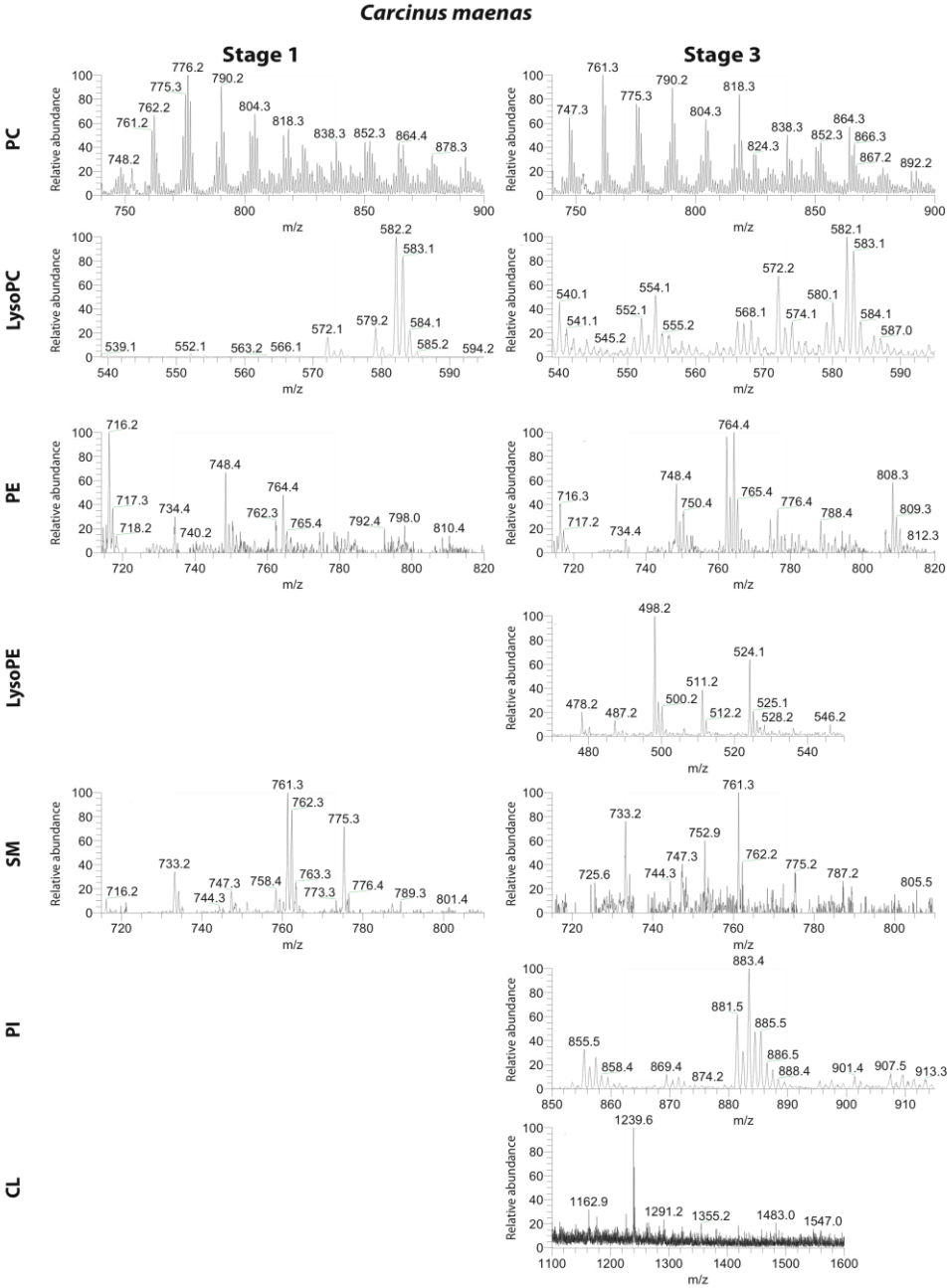
Supplementary Material

Supplementary Figure S1: (S1.1) LC-MS chromatograms of *Carcinus maenas* embryos at stage 1 and (S1.2) stage 3, (S1.3) LC-MS chromatograms of *Necora puber* embryos at stage 1 and (S1.4) stage 3.

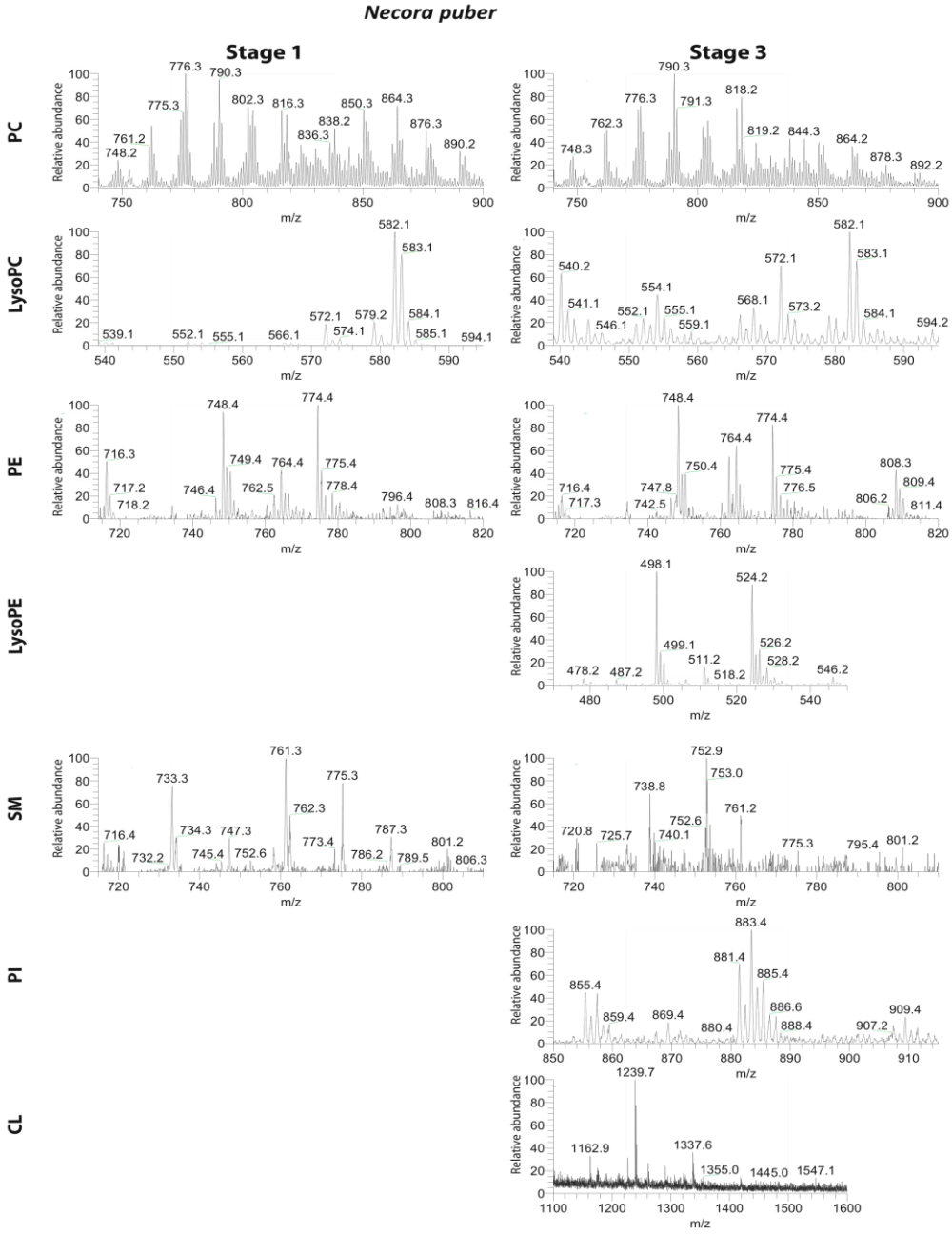


Supplementary Figure S2: MS spectra of *Carcinus maenas* embryos at stage 1 and stage 3. Abbreviations: PC – phosphatidylcholine; LysoPC – lysophosphatidylcholine; PE – phosphatidylethanolamine; LysoPE – lysophosphatidylethanolamine; SM – sphingomyelin; PI – phosphatidylinositol; CL – cardiolipin



Supplementary Figure S3: MS spectra of *Necora puber* embryos at stage 1 and stage 3.

Abbreviations: PC – phosphatidylcholine; LysoPC – lysophosphatidylcholine; PE – phosphatidylethanolamine; LysoPE – lysophosphatidylethanolamine; SM – sphingomyelin; PI – phosphatidylinositol; CL – cardiolipin



Supplementary Table S4: Total molecular species identified by LC-MS in negative-ion mode.

m/z	m/z	Phospholipid Class	Molecular species (C:N)	Fatty acyl composition					
478.2	[M-H]⁻	LysoPE	18:1						
498.2	[M-H]⁻	LysoPE	20:5						
500.2	[M-H]⁻	LysoPE	20:4						
506.2	[M-H]⁻	LysoPE	20:1						
524.2	[M-H]⁻	LysoPE	22:6						
526.2	[M-H]⁻	LysoPE	22:5						
528.2	[M-H]⁻	LysoPE	22:4						
530.2	[M-H]⁻	LysoPE	22:3						
540.1	[M+CH3COO]⁻	LysoPC	15:0 or O-16:0						
546.2	[M-H]⁻	LysoPE	22:2+O-*						
552.1	[M+CH3COO]⁻	LysoPC	16:1						
554.1	[M+CH3COO]⁻	LysoPC	16:0						
566.1	[M+CH3COO]⁻	LysoPC	17:1 or O-18:1						
574.1	[M+CH3COO]⁻	LysoPC	18:4						
580.1	[M+CH3COO]⁻	LysoPC	18:1						
582.1	[M+CH3COO]⁻	LysoPC	18:0						
594.1	[M+CH3COO]⁻	LysoPC	19:1						
716.4	[M-H]⁻	PE	34:1	14:0/20:1;	16:0/18:1;	16:1/18:0			
718.4	[M-H]⁻	PE	34:0	16:0/18:0;	17:0/17:0				
719.3	[M+CH3COO]⁻	SM	31:1	d18:1/13:0					
720.4	[M-H]⁻	PE	35:6 and O-36:6	15:1/20:5;	O-16:1/20:5				
722.4	[M-H]⁻	PE	35:5 and O-36:5	15:0/20:5;	17:1/18:4				
733.3	[M+CH3COO]⁻	SM	32:1	d18:1/14:0					
734.4	[M-H]⁻	PE	36:6	14:1/22:5;	16:1/20:5;	O-16:1/20:4;	O-16:0/20:5;	O-18:1/18:4	
736.4	[M-H]⁻	PE	36:5	14:0/22:5;	14:1/22:4;	16:0/20:5;	16:1/20:4;	18:1/18:4	
738.4	[M-H]⁻	PE	36:4	16:0/20:4;	16:1/20:3;	18:3/18:1			
742.4	[M-H]⁻	PE	36:2	16:0/20:2;	16:1/20:1;	17:2/19:0;	18:0/18:2;	18:1/18:1	
746.4	[M-H]⁻	PE	36:0 and 37:7 and O-38:7	16:0/20:0;	17:0/19:0;	18:0/18:0;	15:1/22:6;	17:2/20:5;	O-16:1/22:6
747.3	[M+CH3COO]⁻	SM	33:1	d18:1/15:0					
748.2	[M+CH3COO]⁻	PC	29:1 or O-30:1						

m/z	m/z	Phospholipid Class	Molecular species (C:N)	Fatty acyl composition							
748.4	[M-H]$^-$	PE	37:6 and O-38:6	15:0/22:6;	17:1/20:5;	17:2/20:4;	22:5/15:1;	O-16:0/22:6;	O-16:1/22:5	O-18:1/20:5	
750.4	[M-H]$^-$	PE	37:5 and O-38:5	17:0/20:5;	17:1/20:4;	15:0/22:5;	15:1/22:4;	O-16:0/22:5;	O-16:1/22:4	O-18:0/20:5	O-18:1/20:4
761.3	[M+CH3COO]$^-$	SM	34:1	d18:1/16:0							
762.3	[M+CH3COO]$^-$	PC	30:1 or O-31:1								
762.4	[M-H]$^-$	PE	38:6	16:0/22:6;	16:1/22:5;	18:1/20:5;	18:2/20:4				
763.3	[M+CH3COO]$^-$	SM	34:0	d18:0/16:0							
764.4	[M-H]$^-$	PE	38:5	16:0/22:5;	16:1/22:4;	18:0/20:5;	18:1/20:4;	18:2/20:3			
766.4	[M-H]$^-$	PE	38:4	16:0/22:4;	18:0/20:4;	18:1/20:3;	18:2/20:2;	18:3/20:1;	18:4/20:0		
773.3	[M+CH3COO]$^-$	SM	35:2	d18:1/17:1							
774.4	[M-H]$^-$	PE	38:0 and 39:7 and O-39:0 and O-40:7	17:0/21:0;	17:1/22:6;	17:2/22:5;	18:0/20:0;	O-18:0/21:0;	O-18:1/22:6		
776.3	[M+CH3COO]$^-$	PC	31:1 or O-32:1								
776.4	[M-H]$^-$	PE	39:6 and O-40:6	17:0/22:6;	17:1/22:5;	O-18:0/22:6;	O-18:1/22:5				
778.3	[M+CH3COO]$^-$	PC	31:0 or O-32:0								
778.4	[M-H]$^-$	PE	39:5 and O-40:5	17:0/22:5;	17:1/22:4;	19:0/20:5;	O-18:0/22:5;	O-18:1/22:4			
780.4	[M-H]$^-$	PE	39:4 and 40:4	17:0/22:4;	17:2/22:2;	19:0/20:4;	O-18:0/22:4				
782.4	[M-H]$^-$	PE	39:3 and 40:10 and O-40:3	22:2/17:1;	22:3/17:0;	20:5/20:5;	O-18:1/22:2				
787.3	[M+CH3COO]$^-$	SM	36:2	d18:0/18:2							
788.3	[M+CH3COO]$^-$	PC	32:2 or O-33:2								
788.4	[M-H]$^-$	PE	40:7 and 39:0	19:0/20:0;	20:5/20:2;	22:6/18:1					
789.3	[M+CH3COO]$^-$	SM	36:1	d18:1/18:0							
790.3	[M+CH3COO]$^-$	PC	32:1 or O-33:1								
790.4	[M-H]$^-$	PE	40:6	18:0/22:6;	18:1/22:5;	20:1/20:5;	20:2/20:4				
792.4	[M-H]$^-$	PE	40:5	18:0/22:5;	18:1/22:4;	20:4/20:1;	20:5/20:0				
794.4	[M-H]$^-$	PE	40:4	18:0/22:4;	20:2/20:2;	20:4/20:0					
796.4	[M-H]$^-$	PE	40:3	18:1/22:2;	20:1/20:2						
798.2	[M-H]$^-$	PE	40:2	18:0/22:2;	18:1/22:1;	20:1/20:1;	20:2/20:0				
801.3	[M+CH3COO]$^-$	SM	37:2	d18:1/19:1							
802.3	[M+CH3COO]$^-$	PC	33:2 or O-34:2								
804.3	[M+CH3COO]$^-$	PC	33:1 or O-34:1								
806.4	[M-H]$^-$	PE	41:5	19:0/22:5;	21:0/20:5						
808.4	[M-H]$^-$	PE	42:11	20:5/22:6							
816.3	[M+CH3COO]$^-$	PC	34:2 or O-35:2								
816.4	[M-H]$^-$	PE	42:7	20:1/22:6;	20:2/22:5						

m/z	m/z	Phospholipid Class	Molecular species (C:N)	Fatty acyl composition				
818.3	$[M+CH_3COO]^-$	PC	34:1					
818.4	$[M-H]^-$	PE	42:6	20:0/22:6;	20:1/22:5;	20:2/22:4;	20:4/22:2	
824.3	$[M+CH_3COO]^-$	PC	35:5 or O-36:5					
836.3	$[M+CH_3COO]^-$	PC	36:6					
838.3	$[M+CH_3COO]^-$	PC	36:5					
844.4	$[M+CH_3COO]^-$	PC	36:2					
850.3	$[M+CH_3COO]^-$	PC	37:6 or O-38:6					
852.3	$[M+CH_3COO]^-$	PC	37:5 or O-38:5					
855.4	$[M-H]^-$	PI	36:5	16:0/20:5;	16:1/20:4			
857.4	$[M-H]^-$	PI	36:4	16:0/20:4;	16:1/20:3			
859.4	$[M-H]^-$	PI	36:3	16:0/20:3;	16:1/20:2;	18:0/18:3;	18:1/18:2	
864.3	$[M+CH_3COO]^-$	PC	38:6					
866.3	$[M+CH_3COO]^-$	PC	38:5					
869.4	$[M-H]^-$	PI	37:5	17:0/20:5;	17:1/20:4			
876.3	$[M+CH_3COO]^-$	PC	38:7					
878.3	$[M+CH_3COO]^-$	PC	39:6 or O-40:6					
881.4	$[M-H]^-$	PI	38:6	18:1/20:5;	18:2/20:4			
883.4	$[M-H]^-$	PI	38:5	16:0/22:5;	18:0/20:5;	18:1/20:4		
885.4	$[M-H]^-$	PI	38:4	16:0/22:4;	18:1/20:3;	18:2/20:2		
887.4	$[M-H]^-$	PI	38:3	16:1/22:2;	18:0/20:3;	18:1/20:2		
889.4	$[M-H]^-$	PI	38:2	16:1/22:1;	18:0/20:2;	18:1/20:1;	18:2/20:0	
890.3	$[M+CH_3COO]^-$	PC	30:9 or O-40:0 and 40:7					
892.3	$[M+CH_3COO]^-$	PC	40:6					
897.5	$[M-H]^-$	PI	39:5 or O-40:5	17:0/22:5;	17:1/22:4;	19:0/20:5;	O-18:0/22:5;	O-18:1/22:4
901.4	$[M-H]^-$	PI	39:3 or O-40:3 and 40:10	17:1/22:2;	19:0/20:3;	20:5/20:5;	O-18:1/22:2	
907.5	$[M-H]^-$	PI	40:7	18:1/22:6;	18:2/22:5;	20:2/20:5;	20:4/20:3	
909.5	$[M-H]^-$	PI	40:6	18:0/22:6;	18:1/22:5			
911.4	$[M-H]^-$	PI	40:5	18:0/22:5;	18:1/22:4;	20:0/20:5;	20:1/20:4;	20:2/20:3
913.4	$[M-H]^-$	PI	40:4	18:0/22:4;	18:2/22:2;	20:0/20:4;	20:1/20:3;	20:2/20:2
1321.6	$[M-H]^-$	CL	62:1					
1337.6	$[M-H]^-$	CL	64:0					
1355.2	$[M-H]^-$	CL	65:1					
1487.6	$[M-H]^-$	CL	74:3					

m/z	m/z	Phospholipid Class	Molecular species (C:N)	Fatty acyl composition
1547.2	$[M-H]^-$	CL	80:14	
1557.1	$[M-H]^-$	CL	80:9	

*12,15-epoxy-13,14-dimethyl-eicosadienoate

Supplementary Table S5.1: Fatty acid (FA) composition (expressed as % of total pool of FAs) identified by GC-MS on *Carcinus maenas* embryos at stage 1 and stage 3. Values are average (± SD) of embryos from three different females (n=3).

Carcinus maenas	**Stage 1**		**Stage3**	
	Mean	**SD**	**Mean**	**SD**
12:0	0.0018	0.0032	0.0000	0.0000
14:0	0.9546	0.1694	0.6218	0.1858
15:0	0.8372	0.3694	0.6676	0.2066
16:0	16.3137	0.3904	18.5953	1.2780
17:0	0.7944	0.1647	0.9922	0.1467
18:0	4.2530	0.5350	6.2146	0.3764
19:0	0.0922	0.0466	0.1066	0.0686
20:0	0.1450	0.0904	0.1174	0.0636
22:0	0.0554	0.0203	0.0290	0.0215
Σ SFA	**23.4473**	**1.7548**	**27.3444**	**1.2684**
14:1*n*-5	0.0649	0.0662	0.0282	0.0489
15:1*n*-1	0.1455	0.0473	0.0486	0.0575
16:1*n*-7	15.3720	3.2407	10.6776	1.0733
16:1*n*-5	0.3500	0.0681	0.3912	0.1578
7-methyl-hexadec-6-enoate	0.6405	0.1650	0.4649	0.1176
17:1*n*-9	0.1110	0.0552	0.1017	0.0583
17:1*n*-8	0.1257	0.1058	0.0466	0.0808
18:1*n*-9*c*	10.8554	1.8536	12.2672	0.5400
18:1*n*-9*t*	0.2086	0.0631	0.2062	0.0657
18:1*n*-7	4.8435	0.4660	5.8258	0.4798
18:1*n*-5	0.5127	0.8880	0.0292	0.0506
19:1*n*-9*c*	0.1868	0.0516	0.0899	0.1358
19:1*n*-8	0.0946	0.1625	0.0184	0.0318
20:1*n*-9	1.7158	0.6807	1.2598	0.4449
20:1*n*-7	2.9278	0.3009	2.4961	0.6690
22:1*n*-11	0.1454	0.2296	0.0000	0.0000
22:1*n*-9	0.2092	0.2743	0.0266	0.0233
Σ MUFA	**38.5095**	**4.4452**	**33.9780**	**1.0500**
18:2*n*-6	0.8628	0.3923	0.6536	0.0613
18:2*n*-3	0.6744	0.2569	0.1908	0.1034
18:3*n*-3	0.0000	0.0000	0.0000	0.0000
18:3*n*-3*	1.1166	0.1162	0.7562	0.0679
19:2*n*-7	0.0777	0.0218	0.0309	0.0351
20:2*n*-9	0.8645	0.4562	0.9147	0.1629
20:2*n*-7	0.6329	0.1645	0.7722	0.1259
20:2*n*-6	0.4334	0.2893	0.0271	0.0470
20:3*n*-4	0.2682	0.1309	0.3485	0.1010
22:2*n*-9	0.9436	0.4752	0.3441	0.1632
22:3*n*-6	0.5003	0.1641	0.2518	0.1388
Σ PUFA	**6.3745**	**1.5274**	**4.2899**	**0.4996**
18:4*n*-3	0.0000	0.0000	0.0000	0.0000
20:4*n*-6	2.0076	0.3205	2.0455	0.3135
20:5*n*-3	9.0156	2.0613	14.3223	0.7481
21:5*n*-3	0.3621	0.1208	0.1312	0.0818
21:6	0.0393	0.0601	0.0000	0.0000
22:4*n*-6	0.7832	0.1241	0.7127	0.1425
22:5*n*-6	0.3221	0.0840	0.0554	0.0870
22:5*n*-3	2.0572	0.2039	1.3284	0.2404
22:6*n*-3	9.3595	4.0247	9.6308	2.0175
Σ HUFA	**23.9466**	**3.9574**	**28.2263**	**2.7588**

Carcinus maenas	Stage 1		Stage3	
	Mean	**SD**	**Mean**	**SD**
4,8,12-trimethyl-tridecanoate	0.3519	0.2421	0.2758	0.0802
9-methyl-tetradecanoate	0.0000	0.0000	0.0449	0.0777
13-methyl-tetradecanoate (iso)	0.1764	0.0214	0.1984	0.1127
12-methyl-tetradecanoate (anteiso)	0.1384	0.0780	0.1805	0.0804
14-methyl-pentadecanoate (iso)	0.3929	0.1164	0.2715	0.0971
10-Methyl hexadecanoate	0.1014	0.0903	0.1469	0.0338
15-methyl-hexadecanoate (iso)	1.5827	0.1911	1.4026	0.1356
14-methyl-hexadecanoate (anteiso)	1.8503	0.1485	1.5504	0.2375
16-methyl-heptadecanoate (iso)	0.3576	0.0532	0.3795	0.0433
17-methyl-octadecanoate (iso)	0.0963	0.0385	0.0725	0.0523
16-methyl-octadecanoate (anteiso)	0.4271	0.0981	0.4307	0.1750
Σ BrFA	**5.4750**	**0.8166**	**4.9537**	**0.6533**
9,10-methylene-octadecanoate	0.1326	0.0576	0.0741	0.0660
11,12-methylene-octadecanoate	0.0442	0.0766	0.0140	0.0242
11,12-methylene-eicosanoate (1)	0.0624	0.0823	0.0000	0.0000
11,12-methylene-eicosanoate (2)	0.0695	0.0916	0.0285	0.0378
Σ CyFA	**0.3087**	**0.2772**	**0.1166**	**0.1200**
10,13-epoxy-11,12-dimethyl-octadecadienoate	0.1939	0.0928	0.0477	0.0538
12,15-epoxy-13,14-dimethyl-eicosadienoate	1.5876	0.2807	0.9994	0.1964
Σ EpFA	**1.7814**	**0.2139**	**1.0470**	**0.2427**
F1	0.1570	0.1190	0.0440	0.0394
Σ UnFA	**0.1570**	**0.1190**	**0.0440**	**0.0394**
TOTAL	**100.0000**		**100.0000**	

* 9c,11t,15c-octadecatrienoate (9,11,15-18:3)

Abbreviations:

SFA – Saturated FA

MUFA – Monounsaturated FA

PUFA – Polyunsaturated FA

HUFA – Highly-polyunsaturated FA

BrFA – Branched FA

CyFA – Cyclic FA

EpFA – Epoxy FA

UnFA – Unknown FA

Supplementary Table S5.2: Fatty acid (FA) composition (expressed as % of total pool of FAs) identified by GC-MS on *Necora puber* embryos at stage 1 and stage 3. Values are average (± SD) of embryos from three different females (n=3).

Necora puber	Stage 1		Stage 3	
	Mean	SD	Mean	SD
12:0	0.0000	0.0000	0.0000	0.0000
14:0	1.9321	0.1778	1.4130	0.1845
15:0	1.2154	0.1761	0.9156	0.0478
16:0	18.8602	0.3308	19.5742	0.3369
17:0	0.6671	0.0597	0.8404	0.1721
18:0	3.4659	0.3148	4.7023	1.0062
19:0	0.0754	0.0108	0.0741	0.0214
20:0	0.0874	0.0079	0.0941	0.0215
22:0	0.0314	0.0544	0.0000	0.0000
Σ SFA	**26.3348**	**0.6824**	**27.6137**	**1.2912**
14:1*n*-5	0.0000	0.0000	0.0000	0.0000
15:1*n*-1	0.1769	0.0360	0.1198	0.0335
16:1*n*-7	17.2082	1.1380	12.8234	2.9087
16:1*n*-5	0.6520	0.1004	0.7784	0.1313
7-methyl-hexadec-6-enoate	0.5824	0.0422	0.5111	0.0748
17:1*n*-9	0.0000	0.0000	0.0000	0.0000
17:1*n*-8	0.1336	0.0348	0.1275	0.0190
18:1*n*-9*c*	10.6265	0.8736	10.6764	0.4332
18:1*n*-9*t*	0.3532	0.0121	0.4668	0.0706
18:1*n*-7	4.1246	0.3687	4.9527	0.3056
18:1*n*-5	0.0000	0.0000	0.0000	0.0000
19:1*n*-9*c*	0.1134	0.0197	0.1192	0.0431
19:1*n*-8	0.1371	0.0151	0.0983	0.0068
20:1*n*-9	1.1618	0.1231	1.1579	0.0803
20:1*n*-7	2.8793	0.5247	2.8029	0.1651
22:1*n*-11	0.1111	0.0967	0.0720	0.0628
22:1*n*-9	0.0000	0.0000	0.0000	0.0000
Σ MUFA	**38.2599**	**0.4576**	**34.7063**	**3.4697**
18:2*n*-6	1.3927	0.0678	0.7847	0.1645
18:3*n*-3	0.5412	0.0360	0.4167	0.0321
18:3*n*-3*	1.4856	0.2306	1.0884	0.2682
19:2*n*-7	0.0000	0.0000	0.0000	0.0000
20:2*n*-9	1.0318	0.3495	1.0416	0.2075
20:2*n*-7	0.5436	0.0363	0.6439	0.1743
20:2*n*-6	1.0140	0.1824	1.0155	0.1283
20:3*n*-4	0.6056	0.0876	0.8593	0.0230
22:2*n*-9	1.4200	0.1300	1.1333	0.1426
22:3*n*-6	0.4469	0.0536	0.4488	0.0371
Σ PUFA	**8.4814**	**0.9190**	**7.4321**	**0.5837**
18:4*n*-3	0.7409	0.0635	0.5231	0.1392
20:4*n*-6	1.5305	0.1752	2.1904	0.6280
20:5*n*-3	8.1688	0.5888	10.1091	1.0991
21:5*n*-3	0.1551	0.0248	0.1098	0.0246
21:6	0.0000	0.0000	0.0000	0.0000
22:4*n*-6	0.8003	0.0808	0.9012	0.0470
22:5*n*-6	0.1510	0.0258	0.1759	0.0573
22:5*n*-3	1.4824	0.2600	1.7100	0.1071
22:6*n*-3	7.5820	0.8328	8.0057	1.4822
Σ HUFA	**20.6109**	**1.3967**	**23.7252**	**2.1594**

Necora puber	Stage 1		Stage 3	
	Mean	SD	Mean	SD
4,8,12-trimethyl-tridecanoate	0.4653	0.0862	0.2330	0.0837
9-methyl-tetradecanoate	0.0000	0.0000	0.0000	0.0000
13-methyl-tetradecanoate (iso)	0.2339	0.0343	0.2659	0.0993
12-methyl-tetradecanoate (anteiso)	0.1284	0.0937	0.0973	0.0621
14-methyl-pentadecanoate (iso)	0.5466	0.0112	0.4556	0.0146
10-methyl-hexadecanoate	0.2211	0.0445	0.2364	0.0511
15-methyl-hexadecanoate (iso)	1.6652	0.0512	1.6187	0.0605
14-methyl-hexadecanoate (anteiso)	1.9076	0.1141	1.9154	0.2122
16-methyl-heptadecanoate (iso)	0.0000	0.0000	0.5556	0.1416
17-methyl-octadecanoate (iso)	0.0498	0.0164	0.0482	0.0064
16-methyl-octadecanoate (anteiso)	0.2789	0.0230	0.3338	0.0606
Σ BrFA	**5.4969**	**0.0590**	**5.7600**	**0.1961**
9,10-methylene-octadecanoate	0.1091	0.0036	0.1018	0.0128
11,12-methylene-octadecanoate	0.0934	0.0068	0.0746	0.0127
11,12-methylene-eicosanoate (1)	0.0406	0.0052	0.0381	0.0317
11,12-methylene-eicosanoate (2)	0.0000	0.0000	0.0000	0.0000
Σ CyFA	**0.2430**	**0.0096**	**0.2146**	**0.0542**
10,13-epoxy-11,12-dimethyl-octadecadienoate	0.0000	0.0000	0.0000	0.0000
12,15-epoxy-13,14-dimethyl-eicosadienoate	0.5351	0.1412	0.5365	0.0890
Σ EpFA	**0.5351**	**0.1412**	**0.5365**	**0.0890**
F1	0.0380	0.0119	0.0115	0.0200
Σ UnFA	**0.0380**	**0.0119**	**0.0115**	**0.0200**
Total	**100.0000**		**100.0000**	

* 9c,11t,15c-octadecatrienoate (9,11,15-18:3)

Abbreviations:

SFA – Saturated FA

MUFA – Monounsaturated FA

PUFA – Polyunsaturated FA

HUFA – Highly-polyunsaturated FA

BrFA – Branched FA

CyFA – Cyclic FA

EpFA – Epoxy FA

UnFA – Unknown FA