UNIVERSITY OF SOUTHAMPTON

FACULTY OF ENGINEERING, SCIENCE AND MATHEMATICS

SCHOOL OF OCEAN AND EARTH SCIENCE

REPRODUCTIVE PATTERNS LINKING DEEP-SEA AND SHALLOW-WATER INVERTEBRATE PHYLOGENIES

by

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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Doctor of Philosophy

REPRODUCTIVE PATTERNS LINKING DEEP-SEA AND SHALLOW-WATER INVERTEBRATE PHYLOGENIES

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The patterns of evolution and speciation derive mostly from species' ability to adapt to variable environmental conditions. Morphological and genetic variability results mainly from natural selection acting independently on individual life-history components or traits, promoting an independent evolution of each other. This study aims to elucidate the possible evolutionary pathways of deep-sea hydrothermal vent species and their shallow-water relatives. To understand the evolution of the deep-sea fauna and their physiological adaptations to high-pressure, it is important to know the effects of pressure in their shallow-water relatives. *Mytilus edulis* and *Palaemonetes varians* adult populations are only found in shallow-water environments, but have a close phylogenetic relationship with deep-sea hydrothermal vent species, belonging to Bathymodiolinae and Alvinocarididae, respectively. Laboratory-based experiments on the physiological tolerances to temperature and pressure of the early ontogenies of *M. edulis* and *P. varians* (defined in terms of fertilization success, survival, developmental rates, and changes in biomass), showed that both species have the ability to tolerate a wide range of temperatures and pressures, far beyond conditions encountered in their natural shallow-water habitat. In *M. edulis*, successful fertilization under pressure is possible up to 500 atm (50.66 MPa) and larvae are able to develop under pressures of 200 atm. *P. varians* larvae are able to survive and develop at pressures equivalent to at least 1000 meters water depth. In both species, with increasing pressure a slower development is observed; principally, pressure narrows the physiological tolerance window in the different ontogenetic stages and slows down metabolism. Asynchronous gametogenesis was observed in the hydrothermal vent shrimps *Mirocaris fortunata* and *Rimicaris exoculata*, from the Mid-Atlantic Ridge. The carapace lengths in females of *M. fortunata* collected from Lucky Strike are significantly larger than those from the Rainbow vent field, which may reflect different habitat conditions between sites. Adaptive maternal effects in response to different environmental conditions are likely to induce changes in offspring phenotype. This process could be occurring among populations of different sites. The fact that the early ontogenies of *M. edulis* and *P. varians* tolerate deep-sea conditions in terms of high-pressures, presumes that they had eurybathyal ancestors. Ancestors may have lived when an isothermal water column was present (~65 Mya). The subsequent re-establishment of deep-water circulation pattern (~49Mya) may have caused the geographical and reproduction isolation of ancestor population, leading to the development of separate shallow-water and vent faunas. Furthermore, the present results challenge previous hypothesis of a wood/bone to seep/vent colonization pathway proposed for the Bathymodiolinae, for excluding the cold-eurythermal character of both hydrothermal vent fauna and their shallow-water relatives.

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Declaration of Authorship

I, Nélia Cristina da Costa Mestre, declare that the thesis entitled "**REPRODUCTIVE PATTERNS LINKING DEEP-SEA AND SHALLOW-WATER INVERTEBRATE PHYLOGENIES**" and the work presented therein are my own. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;

- where any part of this work has been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

- where I have consulted the published work of others, this is always clearly attributed;

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- I have acknowledged all main sources of help;

- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

- parts of this work have been published as:

Mestre, N. C., S. Thatje, and P. A. Tyler. 2008. The ocean is not deep enough: pressure tolerances during early ontogeny of the blue mussel *Mytilus edulis*. Proceedings of the Royal Society B: Biological Sciences (doi:10.1098/rspb.2008.1376) (online first).

Signed:

Date:

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Chapter 1. Introduction

1.1. General Introduction

Reproduction and growth patterns help to determinate the success of individuals and populations, and the evolution of species. It is assumed that natural selection acts independently on individual life-history components or traits, promoting an independent evolution of each. Adaptive patterns in different environments are optimized by selection (Darwin, 1859). Therefore, our understanding of the biology and ecology of living organisms, terrestrial or aquatic, requires knowledge of the environmental factors that govern their life history. More than 50% of the Earth's surface is 3000 m below the mean sea level, making the deep sea the largest ecosystem on Earth (Gage & Tyler, 1991). Our knowledge of the physical, chemical, and biological processes occurring in the deep sea is constrained by its dimensions and inaccessibility. Ultimately, most of our understanding of deep-sea biology and ecology is merely indirect and is dependent on the human ability to develop new methods to observe, measure, and sample the marine organisms living there (Herring, 2002).

Deep-sea hydrothermal vents are commonly found along the mid-ocean ridges, back-arc spreading centres and are also associated with island arc volcanism. The hot and chemically altered seawater that emerges from vents is rich in reduced ions, which are used by autotrophic bacteria to convert carbon dioxide, water, and nitrate into essential organic substances (Jannash, 1995). In these ephemeral chemosynthetic ecosystems, bacteria and their symbiotic hosts rely on chemical energy as their primary energy source. At hydrothermal vents, most organisms are sessile or highly sedentary as adults, and thus mostly rely on the capability of the early ontogeny to disperse and colonize new vent sites (Tyler & Young, 1999; Van Dover, 2000). However, it as been suggested that alvinellid dispersal stages attach to brachyuran or anomuran crabs for transport (Tunnicliffe & Jensen 1987; Zal et al. 1995; Chevaldonné & Olu 1996). *In situ* collection of early life stages from the deep sea has been attempted (Berg & Van Dover, 1987; Kim et al., 1994; Mullineaux et al., 1995; Kim & Mullineaux, 1998; Khripounoff et al., 2000, 2008) but they are often

very difficult to identify due to lack of taxonomic descriptions. Their origin therefore is mostly obscure. *In vitro* fertilization methods have only been applied to a few early life history stages of vent organisms (Marsh et al., 2001; Pradillon et al., 2001, 2005). This method is commonly used to study developmental patterns in the early ontogeny of shallow-water species, reared under controlled lab conditions (e.g. Sprung, 1984; Young et al., 1997; Tyler & Young, 1998; Tyler et al., 2000; Thatje et al., 2001, 2004a, b; Anger et al., 2003, 2004; Benitez Villalobos et al., 2006; Aquino-Souza et al., 2008; Mestre et al., 2008).

Recent studies have revealed a close phylogenetic relationship between some deep-sea hydrothermal vent and shallow-water species. Hydrothermal vent shrimps belonging to the family Alvinocarididae have a monophyletic relationship with the shallow-water family Palaemonidae (Tokuda et al., 2006). The subfamily Mytilinae is the taxonomically closest shallow-water group to the hydrothermal vent and seep mussels of the subfamily Bathymodiolinae (Distel et al., 2000; Jones et al., 2006; Samadi et al., 2007). The depth limits of species are intimately linked with environmental factors such as temperature, pressure, sunlight penetration, and food availability that affect both their physiology and reproduction. More studies on pressure and temperature tolerances of the dispersal stages of related deep-sea and shallow-water species, together with knowledge of past changes in deep-ocean circulation, are required to understand better the origins and antiquity of deep-sea species (Tokuda et al., 2006; Pradillon & Gaill, 2007).

Current hypotheses regarding the origins of deep-sea faunas are based on the assumption that deep-sea species have a narrow and conservative thermal tolerance. One hypothesis suggests that high latitude, cold-isothermal water columns served as a route for shallow-water species to colonize the deep sea, as species from these areas are thermally adapted to the cold deep sea (Kussakin, 1973; Menzies et al., 1973; Hessler & Thistle, 1975; Hessler & Wilson, 1983; Tyler & Young, 1998; Thatje et al., 2005). This is supported by evidence from shallow-water species, a sea urchin *Echinus acutus* (Tyler & Young, 1998) and a seastar *Stichastrella* spp. (Howell et al., 2002), which have recently extended their depth distributions. Another hypothesis proposes that the low latitude warm-isothermal water column, which existed during the late Mesozoic and early Cenozoic, provided a route for

shallow-water fauna to migrate to the deep sea (Menzies et al., 1973; Benson, 1975; Berger, 1979; Schopf, 1980; Hessler & Wilson, 1983; Young et al., 1997). Over time, these warm-stenothermal adapted species would have adapted to the cold temperatures now encountered in the deep sea. In addition, larvae of shallow-water echinoids from the warm-isothermal Mediterranean Sea, tolerate relatively high pressures suggesting that increased pressure is not necessarily a barrier to the penetration of shallow-water species, at least to bathyal depths (Young et al., 1997). According to both hypotheses, once organisms reached the deep sea, species diversity increased through specialization, adaptive radiation, and geographic or reproductive isolation (Tyler & Young, 1998). However, neither of the two currently accepted hypotheses involving deep-sea colonization by shallow-water species considers the cold-eurythermal character of some organisms living at hydrothermal vents. These habitats demand a fundamentally different set of physiological adaptations than organisms adapted to cold-stenothermal conditions on the vast abyssal plains (Mestre et al., 2008).

There are some hypotheses on the evolutionary pathways and origins of deep-sea hydrothermal vent species:

1) some congenerics of hydrothermal vent species may be immigrants from the surrounding non-vent deep sea (e.g. galatheid crabs, pycnogonids, echinoderms, zoarcid and bythitid fish);

2) others may be immigrants that evolved from closely related shallow-water species (e.g. eurybathyal small polychaete worms);

3) vent taxa shared with other chemosynthetic ecosystems point to a radiation from cold-seep to vent and/or the opposite (e.g. bathymodiolid mussels, tubeworms, vesicomyid clams, alvinocarid shrimp);

4) vent taxa are shared with other chemosynthetic ecosystems as well as non-chemosynthetic habitats; with speciation or genetically distinct populations of the same species reflecting geographic isolation (e.g. polychaete family Polynoidae);

5) specialized taxa confined to vents have their closest shallow-water relatives only at the order level (e.g. alvinellid polychaete of the order Terebellida);

6) other taxa found at vents constitute the most primitive living forms of their group (e.g. ancient barnacles, molluscs).

The high endemism of some vent taxa either suggests a long evolutionary history or a high rate of evolution, i.e. high rate of genetic divergence (Cohen & Haedrich, 1983; Hickman, 1984; McLean, 1985; Newman, 1985; Van Dover, 2000).

1.2. Deep-sea research history in a nutshell

Knowledge of deep-sea ecosystem functioning has advanced with the development of new methods and tools for its study. Over the last two centuries, oceanographic expeditions have enabled the development of deep-sea research. The explorer Ferdinand Magellan made the first, although unsuccessful, attempt at deep-sea sounding, during the first world circumnavigation. He tried to determine the depth of the Pacific Ocean using sounding lines only about 300 m long and as he failed to touch the bottom he concluded that he had reached the deepest part of the world ocean (Murray & Hjort, 1912). Edward Forbes in 1845 introduced the concept of the "azoic zone" for ocean depths below 0.6 km, after his participation in the H.M.S. Beacon cruise to the Aegean Sea (1841-1842), one of the first deep-sea biology cruises (Forbes, 1844; Thomson, 1873; Murray & Hjort, 1912; Menzies et al., 1973; Mills, 1983; Rice, 1986). It is now known that the "azoic theory" was based on evidence from one of the faunistically poorest regions of the deep sea. The deep sea was considered as monotonous cold and dark environment given the relative constancy of the physical properties of the deep-water masses and thus species poor with slow sink of material from the surface and absence of primary production at the deep sea (Thomson, 1873; Menzies, 1965; Tyler, 2003).

Before Forbes, however Sir John Ross had explored the Northwest Passage in 1818, and recovered a basket star from depth greater than 1.6 km (Menzies et al., 1973; Tyler, 1980). Michael Sars published a list of 19 deep-sea species in 1850 that

was extended to 92 species in collaboration with his son George Ossian Sars (Murray & Hjort, 1912). Fleming Jenkin in 1860 found a solitary coral from 2184 m (Murray & Hjort, 1912). H.M.S. Lightning cruise in 1868 to the NE Atlantic, promoted by Charles Wyville Thomson and W.B. Carpenter, focused on the deep-water fauna and clearly demonstrated the existence of life at great depths. They also found negative bottom temperatures north of 60ºN and positive temperatures south of 50ºN. This cruise was followed by short cruises on board H.M.S. Porcupine to NW Atlantic and Mediterranean Sea, where several dredges at different depths retrieved diverse fauna (Jeffreys, 1869; Thomson, 1873; Murray & Hjort, 1912; Menzies et al., 1973; Mills, 1983; Rice, 1986). A topographic survey cruise lead by Sir John Murray, on the H.M.S. Knight Errant in 1880, confirmed the existence of the Wyville Thomson ridge between 50ºN and 60ºN in the NE Atlantic (Murray & Hjort, 1912).

H.M.S. Challenger started a scientific circumnavigation expedition of three and half years on the 7th December 1872, led by C.W. Thomson. This expedition is regarded as the starting point of modern oceanography (Murray & Hjort, 1912; Menzies et al., 1973; Mills, 1983; Rice, 1986). During this cruise, widespread and diverse fauna was collected from the deep sea (Murray, 1875). Cruises promoted by Alexander Agassiz off the East Coast of North America and Gulf of Mexico aboard the Blake (1877-1880), and off Central America aboard the Albatross (1891) foccused on biological sampling in the NE Atlantic. On the European side, Alphonse Milne-Edwards and others led deep-sea cruises to the Mediterranean Sea, Azores and Sargasso Sea aboard Travailleur (1880-1882) and Talisman (1883). Between 1885 and 1914, several oceanographic cruises in the Mediterranean and NE Atlantic were organized, financed and directed by Prince Albert I of Monaco, on board of Hirondelle, Prince Alice I, Prince Alice II and Hirondelle II (Menzies et al., 1973; Mills, 1983). The era of great national oceanographic expeditions ended with the Swedish Albatross (1947-1948) and the Danish Galathea (1950-1952) campaigns. During the latter, scientists recovered fauna from the Philippines trench at 10190 m depth (Gage & Tyler, 1991).

Beebe's Bathysphere (1930) was the first deep-water vehicle that made it possible for humans to observe the seabed (Beebe, 1935; Sweeney, 1970; Busby, 1976; Ballard, 2000). The Bathysphere was lowered on the end of a cable, as were

some later vehicles, including "Trieste" (Piccard & Dietz, 1956) which reached the deepest point in the ocean in 1960. The 1960's saw the development of manned submersibles and unmanned ROV's (Remotely Operated Vehicles), which have enhanced the possibilities for in situ experimentation and observation of the deep sea (Heirtzler & Grassle, 1976; Geyer, 1977; Gage & Tyler, 1991). Some countries have developed this technology and nowadays the manned submersibles available for research include: Nautile and Cyana (France); Alvin, Johnson-Sea-Link I and II (USA); Mir I and II (Russia); Shinkai 6500 (Japan). The ROVs in use to explore the deep sea include: Angus and Jason (USA); Victor 6000 and Epaulard (France); Dolphin-3K and Kaiko (Japan); Isis (UK); Luso (Portugal). In the course of technological advancement, research efforts have switched from a descriptive to a more ecological and process oriented approach to deep-sea biology. This approach was pioneered by Robert R. Hessler, Howard L. Sanders and Frederick Grassle (Hessler & Sanders, 1967; Sanders & Hessler, 1969; Grassle & Sanders, 1973; Grassle, 1977; Sanders, 1979). Current knowledge of the deep-sea environment reveals high species diversity and, in some areas, the existence of periodic benthic storms, seasonal input of surface-derived energy, as well as primary production at hydrothermal vents and cold-seeps (Tyler, 2003).

1.3. The deep-sea environment

Around 70% of the Earth surface is covered by the world's ocean containing seawater that represents about 97% of the water on Earth. Half of the seafloor is deep ocean floor (>200 m) with a mean depth of about 3800 m. This makes the deep sea the largest ecosystem on Earth. It is a food-limited environment (except at hydrothermal vents and cold seeps) and the sunlight penetration is restricted to the first hundred meters of the water column. Hydrostatic pressure increases by 1 atm (atmosphere; 10^5 Pascal) every 10 meters of water depth. In addition, temperature decreases with depth, being less than 4ºC below 2000 m, except in the Mediterrenean and in the Red Sea (Gage & Tyler, 1991; Tyler, 2003).

The ocean floor comprises different areas characterized by their depth, relief and/or distance to the continents. These areas are visible in a cross section of a

typical ocean (Fig. 1.1.). The gradual slope where the continent meets the ocean at a depth of ~200 m (about 400-500 m in case of polar shelves) is termed the continental shelf. It is followed by the steeper continental slope, which merges into the continental rise, a zone with a more gradual slope. The three zones together constitute the continental margin, which is incised in some regions by deep-sea canyons. Central oceanic basins are occupied by the abyssal plains that are interrupted by ocean ridges and trenches. Other deep-sea features include hydrothermal vents, cold seeps, seamounts, and back-arc basins (Leeder, 1985; Gage & Tyler, 1991). The deep sea starts at the edge of the continental shelf or, according to hydrographic criteria, below the permanent thermocline (Gage & Tyler, 1991). The ocean floor is also divided into the following depth zones: sublittoral – low water mark at 200 m; bathyal – 200 m to 2 km; abyssal – 2 km to 6 km; hadal – more than 6 km.

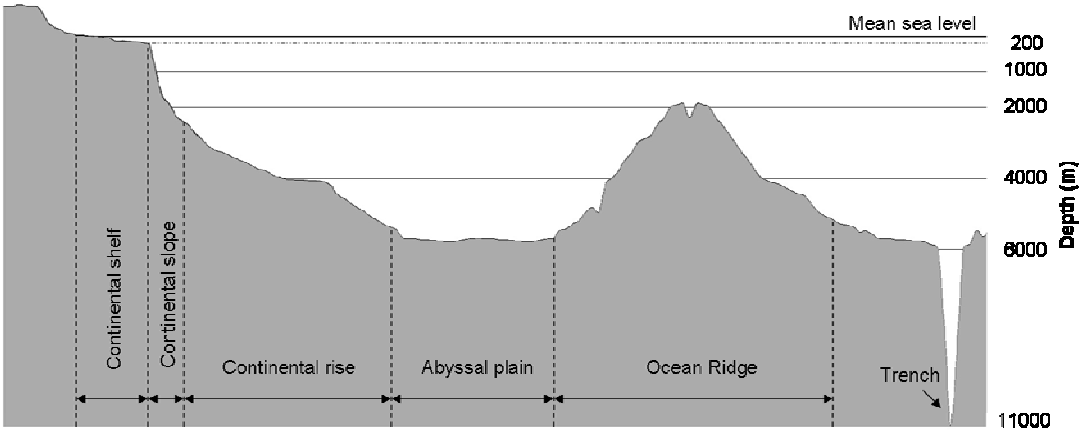

Fig. 1.1. Cross section through a typical ocean (x-axis not to scale; adapted from: Marshall, 1971; Anikouchine & Sternberg, 1973; Gage & Tyler, 1991; Herring, 2002).

The epipelagic realm constitutes the upper pelagic zone and reaches from the surface to about 200 m water depth, and corresponds to the photic zone. Below this zone, the mesopelagic zone begins, characterized by low light levels. The deep sea starts below the depth has reached full darkness (200 to 1000 m). The deep sea is subdivided into the bathypelagic zone (1000 to 4000 m), the abyssopelagic zone (4000 to 6000 m) and the hadopelagic zone (6000 m to the sea floor, as deep as 11000 m; Gage & Tyler, 1991; Madin & Madin, 1995). Horizontally, the pelagic

zone is also divided into the neritic zone (a stripe area close to the continent, ending in the edge of the continental shelf) and the oceanic zone.

1.3.1. Hydrothermal vents and other deep-sea chemosynthetic environments

1.3.1.1. *Hydrothermal vents*

The discovery of hydrothermal vents in the late seventies was foreseen, but not the nature of the associated fauna. However, the possibility of life being fuelled by alternative forms of energy, like the geothermal energy, had been raised 12 years earlier to the findings of hydrothermal vents (Hutchinson, 1965). It was in the Galapagos Rift, off the coast of Ecuador in 1976, that deep-sea hydrothermal vent communities were first observed directly in photographs taken of clumps of clams by the Deep Tow (Lonsdale, 1977). In winter 1977, Alvin dives revealed the full extent of these communities (Corliss & Ballard, 1977; Corliss et al., 1979). Because of their implications concerning microbial biology, biochemistry, physiology, systematics, and ecology, these discoveries probably rank among the most important made in biology during the 20th century (Corliss et al., 1979; Tunnicliffe, 1991; Hessler & Kaharl, 1995).

Individual hydrothermal vent fields are complex structures. They vary in size from a few to ten and even hundreds of meters across. In most vent systems, high-temperature fluids reach the seafloor at point sources, producing the 'black smoker' vents in which hot (~350°C) acidic fluids mix with cold (~2°C) seawater and generate plumes of particulate sulphide (Cann et al., 1994). Compared to seawater, black-smoker fluids have a low pH (3-5) and are especially rich in sulphide (H_2S), hydrogen (H_2), methane (CH_4), manganese (Mn), and other transition metals (iron, zinc, copper, lead, cobalt, aluminium). Where subsurface mixing has moderated the temperature (100-250°C) and chemistry of hydrothermal fluids, 'white smokers' occur. In these systems, the hydrothermal fluids have cooled below the surface of the seabed through mixing with entrained cold seawater and some of the sulphide has precipitated before the fluid exits the hydrothermal mound. Much of the area of vent fields is usually dotted with areas of diffuse, low temperature venting. These emit shimmering clear water at temperatures of near ambient to about 100°C, from cracks

and fissures in the rock, which have precipitated all of their metal sulphides below the ocean floor, though they still contain dissolved sulphide species (i.e. H_2S, HS^-; Rona & Trivett, 1992; Schultz et al., 1992; Cann et al., 1994; Van Dover, 2000). Different styles of mineralization and evolution of massive sulphide deposits are found at seafloor hydrothermal vents, ranging from sub-seafloor deposition within conduits to simple columnar chimneys, or to larger complex structures such as the ore-body formations (Van Dover, 2000).

Hydrothermal vents support taxonomically unique invertebrate communities. Exotic life forms like giant tubeworms and other less strange forms that look like common mussels, crabs and shrimps, are found living in these hydrothermal structures that are an oasis of life in the middle of the surrounding deep-sea "desert". Hydrothermal vent communities live in the interfacial zone where hydrothermal fluid mixes with bottom seawater (Fig. 1.2.; Fisher, 1990; Tunnicliffe, 1991; Childress & Fisher, 1992; Von Damm, 1995).

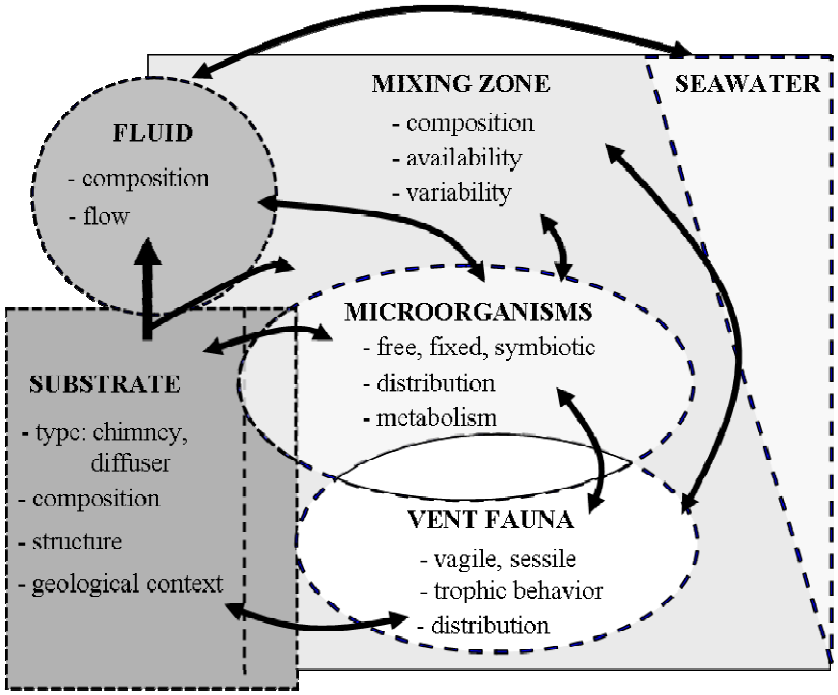

Fig. 1.2. Abiotic and biotic components to the functioning of the hydrothermal vent ecosystem (adapted from Sarradin, 2001).

The hugely successful associations between chemoautotrophic symbiotic microorganisms and their macroinvertebrate hosts characterize deep-sea vent ecosystems. The chemically altered seawater that emerges from beneath the seafloor is rich in reduced ions (principally sulphide- S^{2-}), which are used by autotrophic bacteria to convert carbon dioxide (CO_2), water (H_2O) and nitrate (NO_3^-) into essential organic substances (chemosynthesis) (Jannash, 1995). It is hypothesized that these autotrophic bacteria live in the subsurface plumbing system of vents, on the rocky or sedimentary surfaces that surround the vent openings, on the surfaces of vent animals, and resuspended within the effluent itself. Some of these microbes are symbiotic, and they live within certain vent animals in a mutualistic relationship. Both host and bacteria profit from the union: the hosts provide the bacteria with essential inorganic nutrients, and the bacteria provide the host with their food (Fisher, 1990; Childress & Fisher, 1992). Two modes of symbiont acquisition by hosts are distinguished. In vertical transmission, the symbionts are passed from one generation to the next, through the direct transmission of symbionts from the parent to the egg or embryo. In horizontal transmission, the symbionts are either taken up from the environment or from co-occurring hosts. However, strict vertical transmission can be disrupted when symbionts are exchanged between host species or when new symbionts are acquired from the environment (reviewed by Cavanaugh et al., 2006 and Dubilier et al., 2008).

In addition to primary producers, there are a variety of consumer types among invertebrates and fish (i.e., grazers, suspension-feeders, deposit-feeders, predators, parasites, and commensals; Fig. 1.3.; Tunnicliffe, 1991; Van Dover, 2000). Despite the chemosynthetic base of hydrothermal vent ecosystems, their trophic structure is comparable in many ways to food webs of shallow-water ecosystems.

One of the major contrasts within the ridge megahabitats is between sediment covered, infaunal dominated habitats and bare rock, dominated by epifaunal communities. The habitat variability at microscale (turbulent mixing gradient) becomes of crucial importance for the epifaunal community microtopography in terms of local and large-scale variations in water chemistry (Cann et al., 1994).

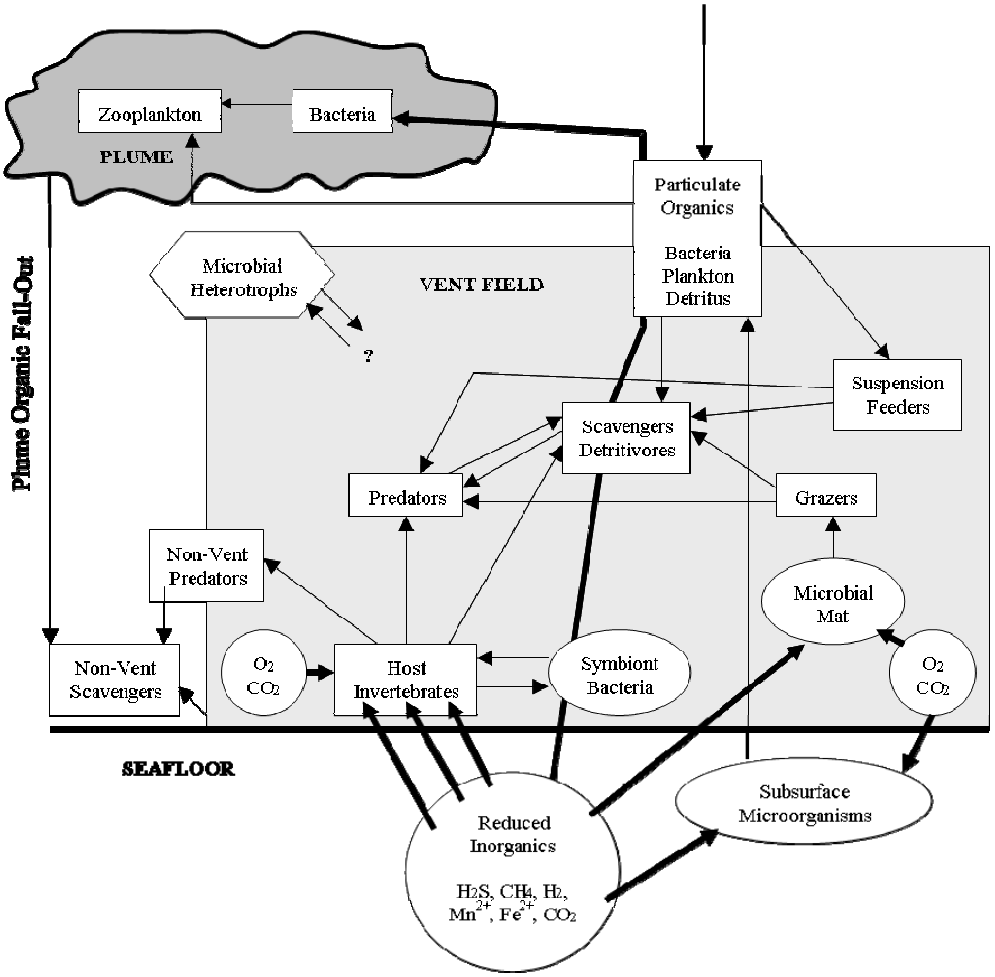

Fig. 1.3. Hydrothermal vent ecosystem food web (adapted from Tunnicliffe, 1991).

More than 500 new species and over a dozen new families have been described from hydrothermal vents, with greater than 90% of the species known only from vents (Tunnicliffe & Fowler, 1996; Desbruyères et al., 2006). Molluscs, arthropods and annelids comprise more than 90% of the invertebrate fauna recorded from vents (Tunnicliffe et al., 1998). Invertebrate species are clearly distributed in zones and microhabitats within vent environments, with some taxa adapted to warm, sulphide-rich waters and other taxa limited to cooler, low-sulphide peripheral regions (Dahloff & Somero, 1991; Dahloff et al., 1992). Many deep-sea organisms also exploit these food-rich vent habitats (Van Dover, 2000).

When vent communities were first discovered, geologists and biologists appreciated their transitory nature and puzzled over the hydrothermal cycle through

which venting and fauna became established, evolved, and declined (Van Dover, 2000). After a volcanic eruption in 1991, on the East Pacific Rise, time-series observations of community development and associated geochemical conditions in many areas of nascent low-temperature hydrothermal venting were conducted over a period of four years. Shank and colleagues (1998a) reported the existence of a correlation between patterns of faunal succession and changing geochemical conditions at hydrothermal vents along intermediate and fast-spreading mid-ocean ridges. The authors suggested that future models of faunal succession should consider the interplay of species-specific life-history strategies, trophic interactions and physical oceanographic processes, and the effect of changing geochemical conditions acting on the sequential colonization of macrofaunal species. In addition, the potential for high microbial production and associated abiotic conditions to facilitate the recruitment of larvae and/or preclude the recruitment of sessile organisms should also be considered in evaluating the underlying controls of community development (Shank et al., 1998a). In contrast, at the hydrothermal vents from the slow-spreading region of the mid-Atlantic ridge near the Azores plateau, the variability of the microenvironment and the possible overlap between the spatial distributions of individual species, suggests that the inter-specific competition is of greater importance for the structure of vent assemblages (Desbruyères et al., 2001).

Rates of crust construction vary considerably along the mid-ocean ridge systems. Slow spreading rates characterize the Atlantic Ocean, where the plates separate at 15-30 mm per year, whereas fast spreading rates are typical of Pacific Ocean ridge (50-180 mm of separation per year). Globally, about 3 km^2 of new ocean floor is produced each year, so that the whole floor of the ocean is renewed on average each 100 million years (Cann et al., 1994). Hydrothermal vents are found on active spreading ridges, which are generally disposed in a linear fashion around the globe (Fig. 1.4.). On the fast-spreading East Pacific Rise, high-temperature vents are confined to the narrow axial zone, typically within the existing small rift valley. On the slow-spreading mid-Atlantic ridge, vent fields can occur towards the margin of the large, deep median valley as well as in the centre.

One interesting observation was to find different fauna between hydrothermal vent fields, within the same ridge. This is related to different seafloor spreading

velocities at a regional scale, and may be explained by the different geological structures that form the ridges, and the life-time and spatial distribution of individual hydrothermal vent fields. Mid-ocean ridges are laterally offset and segmented by transverse features known as axial discontinuities that occur at various scales and have subtle differences between slow- and fast-spreading ridges. The types of ridge axis discontinuities are the transform faults, the non-transform offsets and overlapping spreading centre, and the zero-offset discontinuities. For organisms migrating along the seafloor or dependent on uninterrupted vent-to-vent transport pathways, transform faults may represent major biogeographic barriers. The connectivity along the mid-ocean ridges is quite high compared to the back-arc basins of the western Pacific. In such regions, where the ridges are short, barriers include subduction zones, islands, and interposing plates. Speciation occurs as a result of isolation, and plate tectonics provide an obvious mechanism for such isolation (Cann et al., 1994; Tunnicliffe et al., 1998).

On the fast spreading ridge system of the East Pacific Rise only active or very recent hydrothermal fields occur along the ridge axis, but on the slow-spreading ridges like the mid-Atlantic ridge fossil and active fields co-occur. Radiochronological data for the sulphide deposits of the TAG vent field on the mid-Atlantic ridge estimate that the field is 125 000 years old with the present episode of venting starting 50 years ago (Lalou et al., 1990, 1993). This episodic activity is also suggested for Snake Pit and Lucky Strike vent fields, also along the mid-Atlantic ridge (Van Dover, 2000). The spatial frequency of high-temperature venting on the mid-Atlantic ridge is on the order of one site every 100 to 350 km, while on the East Pacific Rise it is possible to find 1 high-temperature venting site every 5 km of ridge axis (Haymon et al., 1991; Murton et al., 1994; German et al., 1996; Van Dover, 2000).

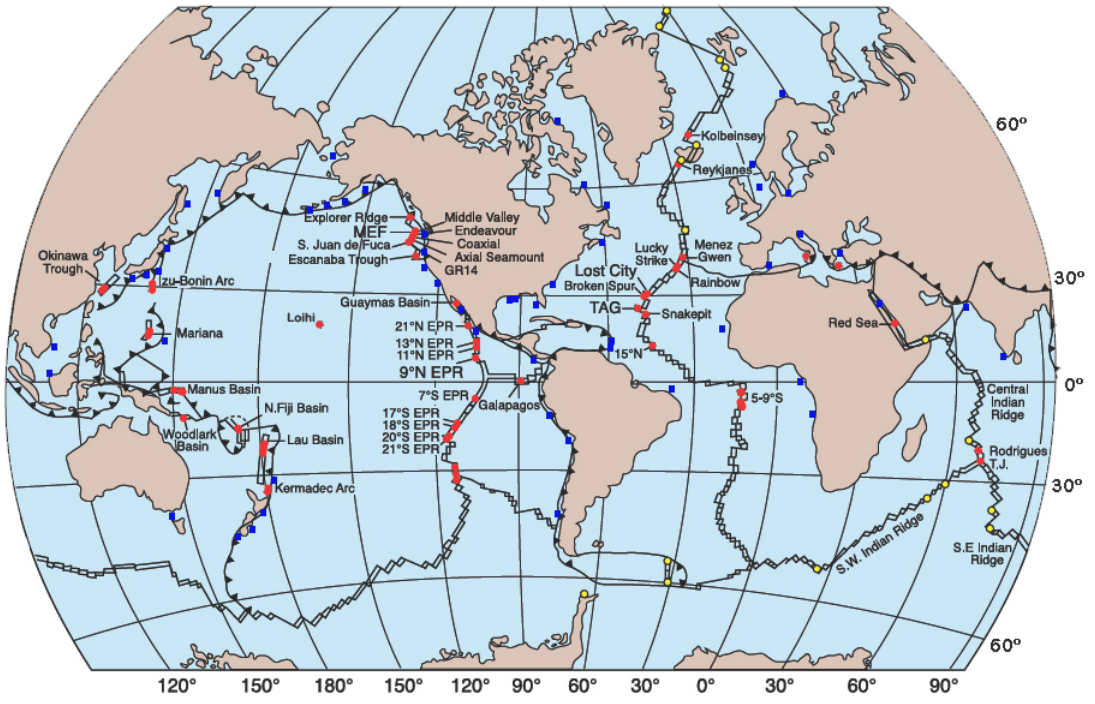

Fig. 1.4. Global distribution of known hydrothermal vents (red circles), areas of activity as indicated by mid-water chemical anomalies (yellow circles) and known cold seeps (blue squares; adapted from Tivey, 2007; with additions from Sibuet & Olu, 1998; Tyler et al., 2003; Levin, 2005; Olu-Le Roy et al., 2007).

When vents were first discovered on the mid-Atlantic ridge in the mid-1980s, a striking absence of the polychaete families Siboglinidae and Alvinellidae familiar from the vents of the Pacific was noted (Mevel et al., 1989; Murton et al., 1995; Van Dover, 1995). The mid-Atlantic ridge fauna is, at the present time, the most isolated based on the taxonomic uniqueness of its species and genera (Juniper et al., 1990). At a regional scale, vent habitats among the mid-Atlantic ridge fields differ mainly in their fluid chemistry (including particulate content) and mineral deposit pattern. These groups of factors are deeply influenced by water depth and the geology of source rocks and hydrothermal deposits (Desbruyères et al., 2000). The most widely distributed species found at hydrothermal vents from the mid-Atlantic ridge known from Menez Gwen (37ºN) to Logatchev (14ºN) (Martin & Haney, 2005) is considered to be the hydrothermal vent shrimp *Mirocaris fortunata* Martin & Christiansen, 1995. Another vent shrimp *Rimicaris exoculata* Williams & Rona, 1986 is present in high-density aggregations (>1500 individuals m^{-2}) around high-temperature vents (Van Dover et al., 1988; Copley et al., 1997) and occurs in the

mid-Atlantic ridge from the Lucky Strike vent field southwards (Martin & Haney, 2005). The mussel *Bathymodiolus azoricus* von Cosel, Comtet & Krylova, 1999 occurs in high-density aggregates around vent structures and dominates the fauna in the mid-Atlantic ridge at Menez Gwen (37°50'N, 850 m), Lucky Strike (31°17'N, 1700 m) and Rainbow (26°13'N, 2300 m).

Vents are continuously being found in mid-ocean ridges and are also present at back-arc spreading centres and associated with island-arc volcanism (Cronan et al., 1982; Van Dover et al., 2002; Tyler et al., 2003). Fossil analogues of vent communities are found in geological records from the Tertiary, Cretaceous, Carboniferous and Silurian (Haymon et al., 1984; Banks, 1985; Oudin et al., 1985; von Bitter et al., 1990; Campbell & Bottjer, 1995; Little et al., 1997).

1.3.1.2. *Cold seeps*

Following the discovery of hydrothermal vents, other chemosynthetic environments were found on passive and active continental margins. Emission of reducing substances from localized areas of the seafloor fuels the chemosynthesis-based benthic communities. The geological setting determines the diverse properties of the fluid emissions, including the temperature, but shares biological characteristics. The first brine seeps – hypersaline cold sulphide seeps, were found in the Gulf of Mexico, housing large epifaunal organisms at the base of the Florida escarpment (Paull et al., 1984), followed by the discovery of chemosynthetic mussels on hydrocarbon seeps on the Louisiana slope, also in the Gulf of Mexico (Kennicutt et al., 1985). Many of the cold seep invertebrate taxa are shared with hydrothermal vents. On active margins, subduction-zone cold seeps were found on the Oregon continental margin (Suess et al., 1985; Kulm et al., 1986), in the Japan Trench region (Laubier et al., 1986; Sibuet et al., 1988), on the Barbados prism (Faugères et al., 1987), in the Peru Trench (Fiala-Médioni et al., 1992) in the Aleutian trench (Suess et al., 1998), in the Eastern Mediterranean (Corselli & Basso, 1996), just to name a few (Sibuet et al., 1988; Sibuet & Olu, 1998; Fig. 1.4.).

1.3.1.3. *Other chemosynthetic environments*

Chemosynthetic based communities are found in other areas of the deep sea, not only vents and cold-seeps. Large food falls like whale carcasses or accumulations of sunken wood and organic matter, as well as some oxygen minimum zones create highly reduced habitats. The release of sulphide sustains the sulphide-oxidizing bacteria and the chemosynthetic invertebrate communities develop on these reducing habitats and share a similar structure/composition with that of vents and cold-seeps (Smith et al., 1989; Levin, 2003; Smith & Baco, 2003). Sunken whale skeletons can last for decades on the deep-sea floor and provide habitat for endemic fauna (e.g. mytilid mussels *Idas pelagica*, *Adipicola pelagica*; polychaetes; gastropods; vesicomyid clams; etc) and also for conspecific species that occur at cold-seeps and hydrothermal vents (e.g. mytilid mussels *Idas washingtonia*; vesicomyid clams *Vesicomya gigas*; gastropods; isopods; etc; reviewed by Smith & Baco, 2003). Along with these findings, hypothesis arose that whale falls serve as sulphide-rich "stepping stones" for the dispersal of chemosynthesis-dependent deep-sea animals (Smith et al., 1989). Furthermore, molecular evidence on the phylogenetic relatedness of bathymodiolin mussels from these different habitats, suggested that whale- and wood-falls provided evolutionary stepping stones for the radiation of mytilids moving down the continental slope and into deep-sea vent and seep habitats (Distel et al., 2000). This hypothesis, however, is being challenged as this evolutionary pathway would imply that bathymodiolin mussels regained cold eurythermy to colonize hydrothermal vents following the colonization of cold-stenothermal chemosynthetic environments; an evolutionary step that is generally assumed to be highly unlikely (Mestre et al., 2008).

1.4. Reproductive patterns and the early ontogeny of marine invertebrates

The life history of a species is constrained by the energy resources available to it. The way the energy is allocated between growth, reproduction, activity and metabolic maintenance will determine the life history of the individual (e.g. Clarke, 1980; Herring, 2002). Deep-sea organisms, like those in shallow water, show a variety of reproductive patterns including asynchronous (continuous) and

synchronous (seasonal) life histories (Lutz et al., 1984; Gage & Tyler, 1991; Tyler & Young, 1999; Young, 2003). There is evidence that the reproductive patterns of some hydrothermal vent organisms are phylogenetically conservative (Van Dover et al., 1985; Tyler & Young, 1999) and the main adaptations to this environment appear to be related to nutritional and respiratory physiology. Nonetheless, the still poorly understood processes by which new vents are colonized might involve the adaptation of some life history characteristics that enables the reproductive propagule to find and colonize a hydrothermal vent (Tyler & Young, 1999).

In general, a marine invertebrate life cycle, composed of different life stages, includes processes of gametogenesis, spawning and/or copulation, larval development, dispersal, settlement, and recruitment (Fig 1.5.; Tyler & Young, 1999). For the understanding of these processes, the following questions should be addressed: what is the growth rate in adult specimens; what is the sexual maturity size/age; what is the energy input, reproductive effort and periodicity of gametogenesis; which is the fertilization success; what are the features of the early development; are larval stages able to disperse, and if so, where do they disperse to; and what is the behaviour associated to the settlement/recruitment of the offspring? (Fig. 1.5.).

Gametogenesis is the process of production of male and female gametes from germ cells. The germ cells are diploid and proliferate by mitosis. Following mitosis, meiosis occurs and reduces the number of chromosomes by half; these haploid cells will differentiate, grow and mature into sperm and eggs. Spermatogenesis consists in the formation of sperm cells, in the testes (male gonad) and the production of eggs, called oogenesis, occurs in the ovaries (female gonad). However, in the Porifera, which have a primitive cellular level of organization, gametogenesis occurs in the mesohyl. It is possible to make some predictions on the possible larval type, by studying the gametogenesis, *via* histological examination of field-collected samples (Gage & Tyler, 1991; Van Dover, 2000; Herring, 2002).

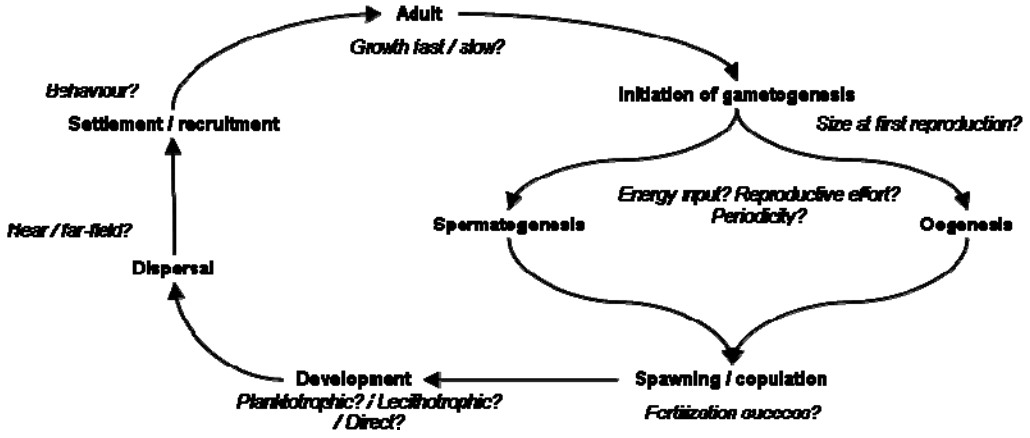

Fig. 1.5. Generalized marine invertebrate complex life cycle (adapted from Tyler & Young, 1999).

The egg is a single, large, highly specialized cell that, following activation initiates the process of embryogenesis (e.g. Eckelbarger, 1994), transmits genetic information to the zygote in sexually reproducing species and also provides energetic reserves for the developing embryo (Jaeckle, 1995). These energy reserves (yolk) are formed and stored during vitellogenesis. In invertebrates this process can be autosynthetic (oocyte slow growth rate), heterosynthetic (oocyte fast growth rate) or mixed (Eckelbarger, 1994; Jaeckle, 1995). The patterns of production of energetic reserves in an organism are phylogenetically constrained and will affect the rate of egg production (the fecundity), as well as larval development (Van Dover & Williams, 1991; Eckelbarger, 1994; Eckelbarger & Watling, 1995). Yet, the final reproductive output of a species can be affected by habitat variability and by slowing down vitellogenesis or by changing the amount of energy allocated to reproduction. Low food levels/quality can induce a decrease or arrest the synthesis of yolk, slowing down the egg production rate as well as reducing fecundity (Qian & Chia, 1991; Bridges et al., 1994; Eckelbarger, 1994; Levin et al., 1994, Marshall et al., 2008).

Thorson (1936, 1950) postulated that the low temperatures typically found at high latitudes and in the deep sea should favour low fecundity and the production of large eggs, which he assumed were typical of direct developers or morphologically advanced offspring. In non-planktotrophs, which have direct and lecithotrophic

development, there is a larger maternal investment in energy per egg, with the production of few and large eggs per female, than in planktotrophic development. Larger, more energy-rich eggs are often indicative of a higher endotrophy in larvae that hatch at a larger size and at a more advanced stage of development than smaller planktotrophic larvae (Thatje, 2004). A slow metabolism at low temperatures (Clarke, 1983, 1992) would result in prolonged larval developmental periods which, in planktotrophic larvae, could fail to synchronize with the short season of food availability (primary production) at high latitudes. Planktonic larval development and some other intermediate states for larval development have also been observed at high latitudes and in the deep sea (Anger, 2001; Thatje, 2004; Thatje et al., 2005). Larger eggs would be expected in most deep-sea benthic species.This is the case in some genera of galatheid crabs (Van Dover & Williams, 1991), although, there is no obvious relationship between egg-size and depth distribution in bivalves (Scheltema, 1994) or in pandalid shrimps (Company & Sardà, 1997). In some deep-sea benthic species small-sized eggs are produced, leading to planktotrophic larval development, allowing these larvae to escape from the cold deep sea and vertically migrate towards the euphotic zone where food is available (e.g. pandalid shrimp *Plesionika* spp., Rosa et al., 2007). In addition, in some taxa, density-dependent energy contents in eggs may complicate a simple egg-size comparison as an energetic measure across latitudes or depths (Anger et al., 2002; Thatje et al., 2005).

The sperm cells' morphology is highly related to the biology of fertilization, and the possible fertilization mechanisms can be predicted with knowledge of the ultrastructure of these cells. For example, an unusually large spermatozoon is necessary to fertilize a large egg, which likely will be related to a lecithotrophic larval development (Franzén, 1983; Beninger & Le Pennec, 1997). Yet, in the ostracod species *Mytilocypris praenuncia* and *Pseudocandona marchica* giant spermatozoa and small-sized eggs are reported (Matzke-Karasz, 2005). The morphology of the sperm cells will be also appropriate for the environment where fertilization occurs, e.g. internal or seawater. Based on the assumption that reproductive patterns are phylogenetically conservative, comparative studies of the ultrastructure of the sperm between closely related taxa has been widely used in systematics and phylogeny of several taxa (e.g. Chia et al., 1975; Eckelbarger & Young, 2002; Healy et al., 2008).

In general, species can be classified according to their sex type: dioecious - male and female members; monoecious or hermaphroditic - one individual having both sexes; simultaneous hermaphrodite - one individual having both sexes, but mating occurs; sequential (protandric) hermaphrodites - both sexes at different times in the life cycle. Dioecious species may have sexual dimorphism, i.e. the male is morphologically distinct from the female. Variations can occur among closely related species or within the same species. For example, the shallow water mussel *Mytilus edulis* is dioecious and, although rare, the occurrence of hermaphrodites has also been reported (Seed, 1976; Micallef & Tyler, 1988). It is also known that many polychaetes are hermaphroditic but the majority of the deep-sea forms appear to have both sexes (Young, 2003). On the other hand, most of the prosobranch gastropods are dioecious though hermaphroditic species, more particularly protandric hermaphrodites, are known from the deep sea. Deep-sea scaphopods, aplacophorans, and bivalves (with very few exceptions) are dioecious (Young, 2003). Within the Crustacea, hermaphroditism has been found in tanaids from hadal depths (Wolff, 1956) and facultative hermaphroditism has been reported in a deep-sea isopod (Wilson, 1981). Most species of pandalid shrimp in shallow water are protandrous hermaphrodites, changing from male to female as they grow, which has been discussed as an energetic adaptation to food constrained environments. In contrast, there is evidence that many species of deep-water tropical pandalids are fully dioecious and never undergo sex reversal (King & Moffitt, 1984; Young, 2003).

Fertilization can be external by free mass spawning or internal, if copulation occurs. For mass spawning to occur, the gametes must reach maturity at the same time, both within individuals and within populations. Reproductive synchronization in mass spawning will increase fertilization success. In some species, gametogenesis can be cyclic with endogenous rhythms controlled by hormones cued by extrinsic factors such as temperature, food supply, phase of the moon or the amount of daylight (Giese & Kanatani, 1987), which is an important feature of shallow water benthic ecosystems. Deep-ocean current systems connect the abyssal plains of the different oceans at the global scale (Menzies, 1965) and it has been found that the cold dense water close to the bottom has semi-diurnal and annual tidal variations (Gould & McKee, 1973; Tyler, 1988), which could be used as a cue for synchronized spawning (Tyler & Gage, 1984; Tyler, 1988). However, the gametogenic condition

may vary between populations of the same species exposed to different extrinsic factors. In asynchronous gametogenesis, only part of the population, and/or part of the gametes within individuals of the population, is mature at any time. Orton (1920) predicted a continuous reproduction for deep-sea benthic species based on the assumption that the deep sea was seasonless and received a constant input of organic matter (Menzel, 1974). However, this hypothesis was dismissed after the seasonal arrival of aggregated phytodetritus on the seabed was discovered (Billett et al., 1983; Lampitt, 1985; Rice et al., 1986). This is now accepted as a widespread phenomenon under areas of high surface productivity (Gooday, 2002). Annual periodicity and synchrony in the reproductive cycles in deep-sea benthic species was first reported for echinoderms (Tyler & Gage, 1980; Tyler et al., 1982). It is now known to occur in different deep-sea benthic taxa including some of those living at hydrothermal vents and cold seeps (reviewed by Young, 2003).

The patterns of cleavage during the early embryonic development reflect differences in the egg cytoplasm organization. The early developmental process involves progressive change and the embryo takes on the forms of the various stages until it reaches the larval stage, or a miniature form of the adult. Development is often conservative and reveals evolutionary links among organisms. In the metazoans, excluding the Porifera, Cnidaria and Ctenophora (Fig. 1.6.), which do not possess three embryonic tissue layers, there are two major lineages based on the early developmental patterns: the protostomes and the deuterostomes. The main differences between protostomes and deuterostomes are the pattern of cleavage, the fate of the blastopore of the blastula stage, the mode of coelom formation, the way body division occurs and the type of larva (e.g. Purves et al., 1995; Table 1.1.).

Fertilization happens when a sperm cell penetrates an egg and gives rise to a zygote. The resulting zygote nucleus is activated and initiates DNA replication and mitosis. The process of cleavage starts with mitotic divisions of the zygote and within hours, hundreds to eventually thousands of cells arise. In most embryos, the first divisions occur at the same time. Throughout cleavage, the embryo retains roughly the same external, spherical form until it becomes a hollow structure called blastula. The fluid-filled space in the centre is the blastocoel and it is surrounded by the blastomeres, and all together forms the blastoderm. In the subsequent

developmental stage - the gastrula, the cells and material undergo a massive rearrangement. Cells from the surface of the blastula move to the interior to form layers. This redistribution of cells forms an embryo with a gut connected to the outside world by either an anus or a mouth. The blastoderm invaginates to form a pocket of an inner germ layer - the endoderm, leaving an outer germ layer - the ectoderm. Between these two layers is formed a third germ layer - the mesoderm, giving rise to the three-layered embryo that is characteristic of most species (e.g. Purves et al., 1995).

Table 1.1. Developmental differences between Protostomes and Deuterostomes (adapted from Purves et al., 1995).

PROTOSTOMES	DEUTEROSTOMES
• Determinate cleavage	• Indeterminate cleavage
• Spiral cleavage	• Radial cleavage
• Blastopore becomes mouth	• Blastopore becomes the anus
• Schizocoelomic formation – mesoderm derives from cells on lip of blastopore; mesoderm splits to form coelom.	• Enterocoelomic formation – mesoderm derives from walls of developing gut; mesoderm usually outpockets to form coelom.
• Body division – never tripart division	• Body division – tripart division
• Larvae – trochophore	• Larvae – dipleurula larvae

The development continues with cell divisions, growth, cell movement and commitment followed by differentiation and increasing embryo complexity. The subsequent life history pattern will then involve either direct development or hatching as an immature and morphologically primitive form of the adult, the larva. There are many definitions of “larva” as this term has been used in different ways (reviewed by Young, 2002). One major problem is the detection of the exact moment at which the embryonic development ends and the larva “hatches”. For this reason it has been proposed to use the term “embryo” only for those stages that are common to the Metazoa (cleavage, blastula, and gastrula; McEdward & Janies, 1993).

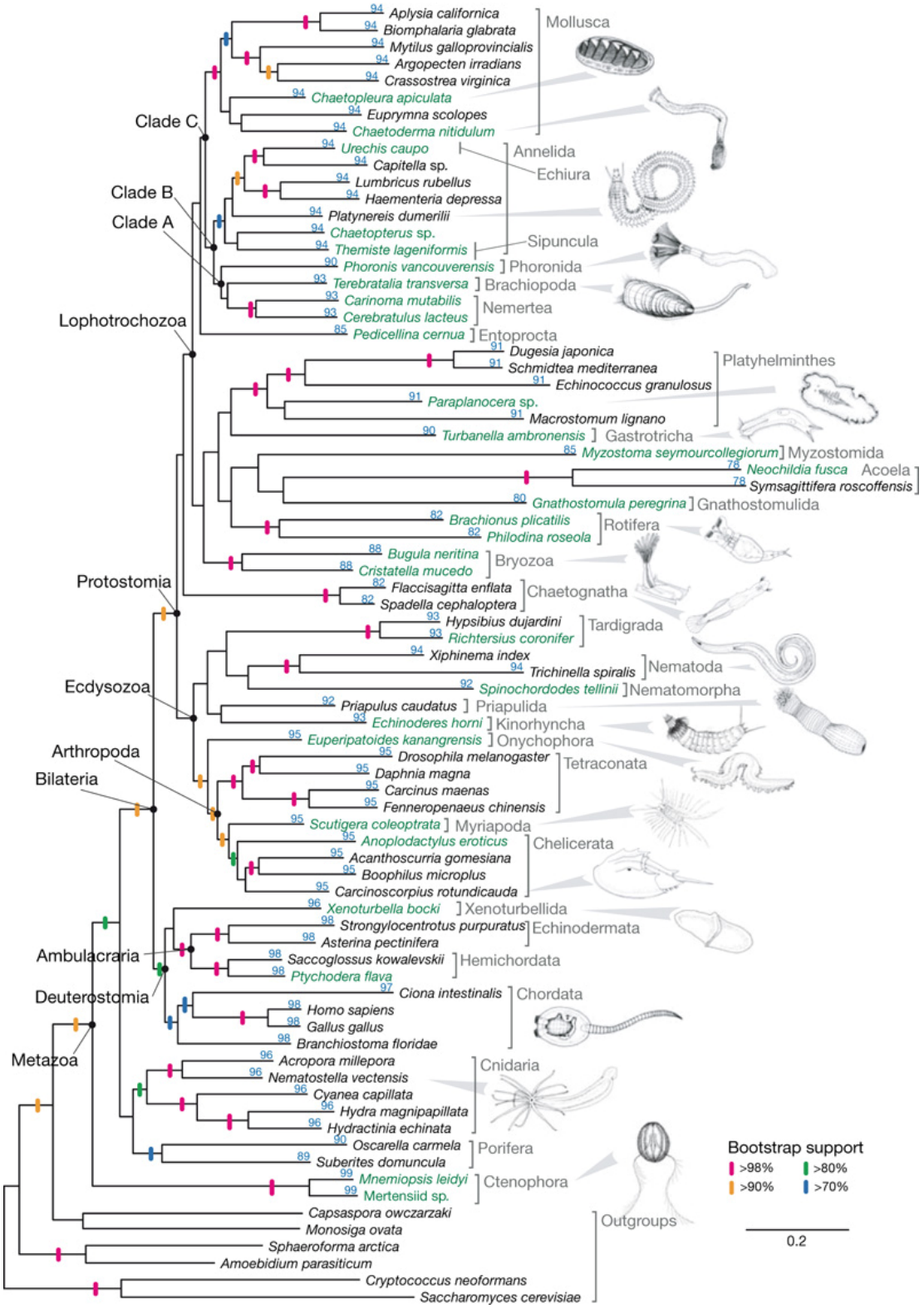

Fig. 1.6. Phylogram of the Animal Kingdom. Note the clades for Deuterostomia and Protostomia (77-taxon RaxML maximum likelihood analyses conducted under the WAG model; the figured topology and branch lengths are for the sampled tree with the highest likelihood; support values are derived from 1,000 bootstrap replicates; leaf stabilities are shown in blue above each branch; taxa with new data are shown in green; adapted from Dunn et al., 2008).

Most larvae originate via sexual reproduction: oviparity – no parental care (e.g. broadcast spawners); ovoviviparity – brooding of embryos, and the larva is the first free-living stage (e.g. most of crustaceans); viviparity – nutrients are transferred from the mother to the embryo through a direct tissue connection (e.g. some ascidians and echinoderms); mixed development – eggs are deposited in capsules or gelatinous egg masses, hatching as free-living larvae (e.g. molluscs and polychaetes). Nevertheless, there are some exceptions, like the polyembryony in some bryozoans (asexual multiplication of embryos; Ryland, 1970) or the occurrence of budding in oceanic larvae of some asteroids (Jaeckle, 1994). Cilia are present in most of larvae, like the planula larva in cnidarians, the trochophores in annelids and molluscs, or the dipleurula larva in echinoderms. In the unciliated crustacean larvae, movement is achieved by muscular locomotion. Different names are given for the different developmental stages of the larvae. For example, the mollusc trochophores become veligers with the development of the larval shell, while in crustaceans the instars are separated by moulting events, as in the brachyuran crabs that start with the zoeal stages and end with a megalopa stage (Young, 2002).

The processes associated with larval development, dispersal, settlement and recruitment are the least known but possibly the most crucial ones in the life history of most marine invertebrates (Tyler & Young, 1999). Knowledge of the processes affecting larval development mainly has been obtained in laboratory-based studies under controlled conditions (Anger, 2001), as direct observation or collection from their natural habitat is often quite difficult. The larval dispersal potential of benthic species with direct development will be non-existent or limited, contrasting with the existence of an actively swimming or free-floating larval stage in indirect developers. The actual dispersal distance for either type of larval development is a complex function influenced by a combination of temperature, abundance of food, hydrographic regimes, larval behaviour and time spent in the water column (e.g. Thorson, 1950; Strathmann, 1974; Lutz et al., 1984; Scheltema, 1986; Gage & Tyler, 1991).

According to the mode of nutrition, indirect developers can be of two principal types: lecithotrophs or planktotrophs. Lecithotrophs possess free-swimming and non-feeding larvae that rely on maternal reserves for nutrition. Planktotrophs have free-

swimming larvae, which are able to feed on small particles available in the plankton. Intermediate types of larval development are in general characterized by an abbreviation in development and/or the first larval stages being lecithotrophic and the later stages planktotrophic. This pattern is known from many caridean shrimps and can be related to the larval developmental plasticity allowing for energy saving traits, as an adaptation to variable natural conditions (Anger, 2001; Thatje et al., 2001, 2005; Thatje, 2004). For example, in the caridean shrimp genera *Campylonotus* and *Chorismus,* the low temperature and seasonally limited primary productivity at high latitudes has selected for strongly abbreviated larval developments combined with larval resistance to starvation in the first larval stages (Thatje et al., 2001, 2004a, b; Thatje & Lovrich, 2003; Thatje, 2004). This adaptation has enhanced synchronization with short periods of food availability and reduced the time of larval dependence on planktonic food sources (Clarke, 1988; Anger et al., 2003; Thatje, 2004).

During the planktonic phase, marine invertebrate larvae are subject to variations in many ecological factors that determine their survival, development, dispersal and recruitment. Dispersal largely depends on the duration of larval life and on oceanographic currents, prevailing wind directions and seabed/coastline morphology (or season, in case of seasonally reproducing species; Tyler &Young, 1999). These constrain the genetic exchange between geographically separated populations as they are subject to a differential recruitment (Bunch et al., 1998; Anger, 2001; Young, 2003). For hydrothermal vent organisms, the dispersal potential has been inferred from the genetic structure of populations (e.g. Won et al., 2003; Johnson et al., 2006; Tokuda et al., 2006). Larval abundance patterns near deep-sea hydrothermal vents as been investigated, to determine how physical transport processes and larval behavior may interact to influence larval dispersal from, and supply to, vent populations (e.g. Mullineaux & France, 1995; Mullineaux et al., 2005). Studies on biochemical markers (e.g. Pond et al., 1997a, c; Allen et al., 2001) and physiological tolerances of larval stages of vent organisms (e.g. Tyler & Dixon, 2000; Marsh et al., 2001) have also been used to estimate the potential for migration/drift to the upper water column.

Metamorphosis into a juvenile stage mostly happens prior to or during settlement of a planktonic form into a benthic habitat, contributing to recruitment and maintenance of adult benthic populations. In most marine benthic invertebrates, a specialized settlement stage will respond to environmental cues presumably indicative of a suitable location for survival and successful reproduction (e.g. adult habitat or shelter from predation). In many species, metamorphosis is induced by specific physical or chemical cues associated with the settlement habitat, but in the absence of these, settling larvae may delay their metamorphosis (within some limits) to search for a more suitable substratum (e.g. Crisp, 1976; Chia & Rice, 1978; Pechenik, 1990; Zimmer-Faust & Tamburri, 1994; Anger, 2001). A settlement response to habitat-specific cues has been observed especially in competent larvae of sessile animals such as cirripedes, ascidians, or sessile polychaetes (Anger, 2001; Lau & Qian, 2001). Potential settlement cues are the physical and chemical properties of the settlement substrates or habitats (e.g. estuaries or hydrothermal vent field) or chemical substances released by conspecific adults (e.g. pheromones in marine gastropods; Painter et al., 1998).

Despite the body of literature referred to above, little is known about the biochemical, physiological and anatomical aspects of larval biology, as well as about the intraspecific variability within or between separate populations. For the understanding of evolutionary adaptation and speciation, emphasis should be given to studies of the phenotypic plasticity and genetic divergence (e.g. in larval morphology or stress tolerance). Early ontogenetic adaptations to extreme or unpredictable ecological conditions are important in the evolutionary transitions between different marine habitats (Van Dover, 2000; Anger, 2001; Young, 2003).

1.5. Adaptations to the deep sea and ecological role of high-pressure

Temperature is an ecological factor of great importance in defining the distribution of species. It decreases with ocean depth, being less than 4ºC below 2000 m (Gage & Tyler, 1991; Herring, 2002). The geographic distribution of a species is usually related to its temperature tolerance range. A stenothermal response is generally associated with species from the tropics, high latitudes, or from the deep

sea, while those adapted to pronounced seasonal and regional temperature variations are typical of intermediate climatic zones and show an eurythermal response (Anger, 2001, and references therein). Deep-sea hydrothermal vents can be considered as areas of temperature variation resulting from the interaction between the hot fluid emissions and the cold surrounding deep seawater. Organisms living there are exposed to these fluctuations and most likely will have a eurythermal response. Temperature accelerates or delays the rates of metabolism and thus affects larval growth, development, and survival as well as seasonal variation in the occurrence of larvae in the plankton. In general, physiological adaptations to low temperatures are usually linked with slow metabolism, which is associated with low fecundity and long developmental periods (Anger, 2001; Clarke, 2003). The temperature tolerances for each life stage of a marine invertebrate can vary in a single species and affect the survival and the ability of a species to colonize new habitats.

The availability of food, which is related to depth, interacts with temperature, affecting the physiology and reproductive patterns of species. The quantity of food arriving at the deep-sea floor is dependent on the surface productivity (except for deep-sea chemosynthetic environments) as photosynthesis is limited to a thin layer of the ocean's surface. Depending on the area of the World Ocean being considered, this surface-derived material can be insignificant or seasonally driven. Occasionally, large food-falls arrive at the seafloor, such as carcasses of whales, seals, dolphins and larger fishes (Stockton & DeLaca, 1982), and even seagrass or terrestrial plant debris (Wolff, 1976, 1979). Hence, most of the deep-sea macrofauna is composed of sediment feeders, scavengers or carnivores, and also a few suspensions feeders (Hessler & Sanders, 1967; Gage & Tyler, 1991; Herring, 2002; Gage, 2003).

Life inhabits the deepest parts of the ocean. Hydrostatic pressure increases by 1 atmosphere every 10 meters of water depth. Pressure is the single variable that has a continuous relation with depth and is largely unaffected by other factors. It is one of the less studied physical properties but known to affect all organisms living in aquatic environments. Pressure influences chemical reactions (Le Chatelier's principle[1]), molecular interactions, and at all levels of biological organisation. For

[1] "if a chemical system at equilibrium experiences a change in concentration, temperature, volume, or total pressure, the equilibrium will shift in order to counter-act the imposed change"

instance, all biochemical and physiological processes involved in increasing or decreasing volume will be conditioned by the effect of pressure. Other physico-chemical factors affecting biological processes, like temperature (affecting both volume and energy), pH, salinity, and oxygen tension will intensify or reduce the pressure tolerance of an organism. Furthermore, it is sometimes difficult to determine the exact effects of pressure as these are caused by concomitant changes in, for example, pH, dissolved gases, density, viscosity, electrical conductivity, ionization of water and other substances, volume changes and basic chemical reactions (e.g. Kinne, 1972; Flügel, 1972; Somero, 1992; MacDonald, 1997; Pradillon & Gaill, 2007).

Aquatic species are typically found within certain depth limits, and their tolerance for depth changes will reflect a variety of molecular adaptations that tend to counteract the perturbations of the biochemical and physiological processes. Pressure sensitivities of enzymes, structural proteins, and membrane-based systems differ markedly between shallow-water and deep-sea species (Somero, 1992; Pradillon & Gaill, 2007). Morphological anomalies, growth inhibition, or even cell death, can be expected in organisms from shallow water exposed to high-pressures, while organisms retrieved to the surface from the deep sea require high-pressure for normal functioning and ultimately survival (e.g. Zimmerman & Marsland, 1964; Marsland, 1970; Yayanos, 1978; Bourns et al., 1988; Theron & Sébert, 2003).

Several studies analysed the effect of pressure on the lipid bilayers of biological membranes, which are considered one of the more pressure sensitive molecular assemblages (Somero, 1992; MacDonald, 1997; Pradillon & Gaill, 2007). An increase in pressure tends to reduce the membrane fluidity in 1 atm adapted organisms (Behan et al., 1992), but deep-sea invertebrates exhibit changes in the lipid composition of membranes in order to maintain fluidity under pressure (Cossins & MacDonald, 1984; DeLong & Yayanos, 1985). In organisms exposed to pressures different from those they are naturally adapted to, cellular processes like osmoregulation or “action potential transmission” in nervous cells will be affected (Wann & MacDonald, 1980; Sébert et al., 1997; Siebenaller & Garrett, 2002). The latter is reflected in a motor impairment, spasm or even paralysis (high pressure neurological syndrome; Yayanos, 1981; Treude et al., 2002; Pradillon & Gaill,

2007). Pressure and temperature act in synergy promoting protein denaturation and loss of function (Balny et al., 1997; Pradillon & Gaill, 2007). An increase in the expression of heat shock proteins (HSP) and a regulation of gene expression as a stress response to an increase in pressure in 1-atm adapted organisms was frequently demonstrated (e.g. Symington et al., 1991; Elo et al., 2000; Sironen et al., 2002; Fernandes et al., 2004). Many other high-pressure effects on biological processes have been described (reviewed by Flügel, 1972; Somero, 1992; MacDonald, 1997; Pradillon & Gaill, 2007).

Pressure has also been considered a potentially limiting factor for faunal exchange to occur between different depth habitats, and special attention has been given to dispersal limits of developing embryos and larvae (e.g. Young & Tyler, 1993; Tyler & Young, 1998). Field sampling of these early life forms is very difficult and thus most attempts to determine potential dispersal limits are based on laboratory studies of tolerance to pressure combined with temperature. A slower rate of cell division, abnormal forms of embryo development, reduced swimming activity of larvae, often accompanied with spasm and ultimately death, are among the most common effects of elevated pressure (or exposure to pressures out of the tolerance range). It has also been observed that, in general, embryos and larvae have higher pressure tolerance ranges than the observed adult depth distribution (Marsland, 1950; Young & Tyler, 1993; Young et al., 1997; Tyler & Young, 1998; Benitez Villalobos et al., 2006; Aquino-Souza et al., 2008). For instance, evidence from some shallow-water echinoid larvae, which are sufficiently tolerant to high pressures, suggests that these larvae could potentially follow an isothermal layer into the deep sea. This would imply that the species could theoretically send colonists to deeper waters within a single generation (Tyler & Young, 1998). In addition, it has been shown that the pressure tolerances in embryos of bathyal *Echinus acutus* are broader than those of shallow-water conspecifics suggesting that this species may presently be invading the deep sea by slowly adapting to increased pressure (Tyler & Young, 1998). Furthermore, the vertically overlapping distribution of species of *Echinus*, from the North Atlantic, that have retained both planktotrophy and seasonal reproduction at all depths, supports the hypothesis of an evolutionary adaptative radiation from shallow water to the deep sea (Tyler et al., 1996; Tyler et al., 2000).

1.5.1. Experimental pressure work - history and state of the art

Research on the biological effects of high hydrostatic pressure was inspired by the discovery of organisms living in the deep sea during the expedition aboard the Talisman at the end of the nineteenth century. Certes (1884) and Regnard (1884, 1891) conducted the first laboratory studies analysing the effect of high pressure (up to 600 atm; using a simple hydraulic pump connected to a pressure vessel) in a variety of unicellular plants, animals, and of fish and other higher forms (Johnson & Eyring, 1970; Marsland, 1970; Kinne, 1972). In the early experiments, temperature was not considered as an important factor to interact with the effects of pressure and most experiments were performed at the most suitable temperature for the organisms (Johnson & Eyring, 1970; Marsland, 1970). The first studies on the effect of pressure on embryonic development investigated the properties of the mitotic spindle and cell membranes (Marsland, 1970). It was early observed that pressure would retard/inhibit cell division of zygotes from shallow-water sea urchins (Marsland, 1938, 1950, 1970; Zimmermann & Marsland, 1964). For these microscopic studies, a pressure chamber with a total capacity of 400 mL was developed, allowing direct observations of cultures at magnifications of up to 600 × and pressures of up to 1360 atm (Marsland, 1938).

In laboratory experiments on marine organisms, the intensity of pressure can be regulated by: varying the weight on a piston; moving columns of mercury or water; or by employing compressed air or vacuum systems (Kinne, 1972). A variety of pressure equipment has been developed for the purpose of experimental work (e.g. Morita, 1970; Zobell & Oppenheimer, 1950; Kinne, 1972). Nowadays, a few large volume vessels are available, where collected adult specimens from the deep sea can be maintained under continuously controlled pressure and temperature (e.g. Brauer et al., 1980; MacDonald & Gilchrist, 1980; Quetin & Childress, 1980; Kochevar et al., 1992; Goffredi et al., 1997; Girguis et al., 2000; Shillito et al., 2001, 2004, 2006; Koyama et al., 2002; Toullec et al., 2002; Ravaux et al., 2003; Pradillon & Gaill, 2007). For example, the IPOCAMP (Incubateur Pressurisé pour l'Observation et la Culture d'Animaux Marines Profonds) has imaging facilities allowing the study of the behaviour of organisms (Shillito et al., 2001). Other systems allow the study of embryonic and larval development of deep-sea organisms (Marsh et al., 2001;

Pradillon et al., 2001, 2004; Pradillon & Gaill, 2007). However, *in vivo* studies of deep-sea animals are limited by decompression effects following sampling, which often cause the death of the organism (MacDonald, 1997). This circumstance and major obstacle in the study of deep-sea metazoan physiology inspired the development of isobaric *in situ* collection devices (MacDonald & Gilchrist, 1978, 1982; Yayanos, 1978, 1981; Koyama et al., 2002; Shillito et al., 2008).

Despite these advances, high-pressure and temperature tolerance studies on early life-history stages of shallow-water marine invertebrates are scarce and have concerned only a few species of sea urchins (*Arbacia punctulata*, *A. lixula, Paracentrotus lividus*, *Sphaerechinus granularis*, *Echinus esculentus*, *E. acutus*, *Sterechinus neumayeri* and *Psammechinus miliaris*) and seastars (*Asterias rubens* and *Marthasterias glacialis*; Marsland, 1950; Young & Tyler, 1993; Young et al., 1996, 1997; Tyler & Young, 1998; Tyler et al., 2000; Benitez Villalobos et al., 2006; Aquino-Souza et al., 2008). Even fewer pressure and temperature studies are available for deep-sea species, because of the difficulty of isobaric sampling, which affects survival of adults and in the likelihood of obtaining viable gametes. Even so, temperature and pressure studies are available for some deep-sea species and at least some stages of their early ontogeny; these include *Plutonaster bifrons* (asteroid) and *Echinus affinis* (echinoid), as well as the deep-sea hydrothermal vent species *Mirocaris fortunata* (shrimp), *Alvinella pompejana* (polychaetes) and *Riftia pachyptila* (tubeworm; Young et al., 1996; Tyler & Young, 1998; Tyler & Dixon, 2000; Marsh et al., 2001; Pradillon et al., 2001, 2005). The lack of such studies for the Mollusca, from both shallow water and the deep sea, is remarkable, given the importance of this group both in terms of abundance and diversity (Sanders, 1977).

1.6. Aims and Objectives

The patterns of evolution and speciation derive mostly from the ability of a species to adapt to variable environmental conditions. Morphological and genetic variability results mainly from natural selection acting independently on individual life-history components or traits, promoting an independent evolution of each. The non-genetic effects, i.e. environment induced effects, on the reproductive output of a species are likely to cause changes in offspring phenotype. Hence, different reproductive traits between populations exposed to different environmental conditions are likely to occur.

This study aims to elucidate the possible evolutionary pathways of deep-sea hydrothermal vent species and their shallow-water relatives.

The underlying hypotheses of this study are:

- The physiological limitations expressed by the adult may differ from those that characterize the early ontogeny;
- Past evolutionary adaptations to changes in the environment required a wide physiological tolerance that can be retained in the early ontogeny of extant species;
- The early ontogeny of extant shallow-water species with a close phylogenetic relationship to deep-sea species can tolerate high pressure.

The specific objectives of this study are:

- To study the temperature and pressure effects on the fertilization success, embryonic and larval development of the shallow-water mussel *Mytilus edulis*, which expresses a close phylogenetic relationship with the deep-sea hydrothermal vent mussels of the Bathymodiolinae, and to assess its potential to colonize the deep sea (Chapter 2);

- To study the temperature and pressure effects on the survival, development and energetic contents of the first larval stage of the shallow-water shrimp *Palaemonetes varians*, which is phylogenetically closely related to the deep-sea hydrothermal vent shrimps of the Alvinocarididae, and assess its potential to colonize the deep sea (Chapter 3);

- To study the reproductive biology of two deep-sea hydrothermal vent shrimps *Mirocaris fortunata* and *Rimicaris exoculata* and assess the reproductive variability between contrasting vent habitats (Chapter 4);

- To explore an evolutionary link between deep-sea hydrothermal vent invertebrate species and their shallow-water relatives.

Chapter 2. The Ocean is not deep enough: pressure tolerances during early ontogeny of the blue mussel *Mytilus edulis*

2.1. Summary

Early ontogenetic adaptations reflect the evolutionary history of a species. To understand the evolution of deep-sea fauna and its adaptation to high pressure, it is important to know the effects of pressure on their shallow-water relatives. In this study, I analyse the temperature and pressure tolerances of early life-history stages of the shallow-water species *Mytilus edulis*. This species expresses a close phylogenetic relationship with hydrothermal vent mussels of the subfamily Bathymodiolinae.

Tolerances to pressure and temperature are defined in terms of fertilization success and embryo developmental rates in laboratory-based experiments. In *M. edulis*, successful fertilization under pressure is possible up to 500 atm (50.66 MPa), at 10, 15 and 20ºC. A slower embryonic development is observed with decreasing temperature and with increasing pressure; principally, pressure narrows the thermal tolerance window in different ontogenetic stages of *M. edulis*, and slows down metabolism.

This study provides important clues on possible evolutionary pathways of hydrothermal vent and cold-seep bivalve species and their shallow-water relatives. Evolution and speciation patterns of species derive mostly from their ability to adapt to variable environmental conditions, within environmental constraints, which promote morphological and genetic variability, often differently for each life-history stage. The present results support the view that a direct colonization of deep-water hydrothermal vent environments by a cold eurythermal shallow-water ancestor is indeed a possible scenario for the Mytilinae, challenging previous hypothesis of a wood/bone to seep/vent colonization pathway.

Keywords: pressure, temperature, early-ontogeny, Mytilidae, shallow water, deep sea, evolution.

2.2. Introduction

Hypotheses on the origins of the deep-sea fauna say that extant deep-sea organisms dispersed for long distances through isothermal water masses in the past glacial periods (Tyler et al., 2000; Thatje et al., 2005). Therefore, today's faunal exchange between deep-sea and shallow-water animals could occur via cold-adapted species living at high latitudes, where the water column is isothermal (Kussakin, 1973; Menzies et al., 1973; Hessler & Thistle, 1975; Hessler & Wilson, 1983; Tyler & Young, 1998; Thatje et al., 2005). At lower latitudes, during the Mesozoic and early Cenozoic the deep sea was warmer than at present, and could have allowed shallow-water species to invade deeper ocean habitats (Menzies et al., 1973; Benson, 1975; Berger, 1979; Schopf, 1980; Hessler & Wilson, 1983; Young et al., 1997). To further elucidate this hypothesis, increasing relevance has recently been given to studies on pressure and thermal tolerance of the early life stages of both shallow-water and deep-sea invertebrates, together with studies on past changes in deep-sea hydrography (Tyler et al., 2000; Tokuda et al., 2006; Pradillon & Gaill, 2007).

The temperature tolerances for each life stage of a marine invertebrate can vary in a single species and affect the survival and the ability of a species to colonize new habitats. Temperature accelerates or delays the rates of metabolism and thus affects larval growth, development, and survival as well as seasonal variation in the occurrence of larvae in the plankton (Anger, 2001; for review see Clarke, 2003). Marine species' habitats are often also defined in relation to upper and lower depth limits and these limits are ultimately related to pressure tolerances of the organisms. Ideally, these habitat boundaries should be assessed for each life stage, as the depth tolerance range in each stage is different in some species (Anger, 2001; Aquino-Souza et al., 2008). The early life stages of marine benthic invertebrates, for instance, are the most likely opportunities they have to disperse over long distances and to colonize new habitats (Tyler, 1995; Young et al., 1996; MacDonald, 1997; Tyler & Young, 1998).

In order to understand the evolution/adaptation of the deep-sea fauna, in terms of its sensitivity to pressure, it is important to know how their biological structures and processes differ from their shallow-water relatives (Childress &

Fisher, 1992; Somero, 1992). In biological systems, the effect of pressure causes a compression of the system, conditioning physiological and biochemical processes involved in increasing or decreasing cell volume. A combination of the pressure effect with other physico-chemical factors affecting biological processes, e.g. temperature, pH, and salinity, will intensify or reduce the effect of pressure alone. Pressure sensitivities of enzymes, structural proteins, and membrane-based systems differ markedly between shallow-water and deep-sea species (Somero, 1992; Pradillon & Gaill, 2007).

The subfamily Mytilinae is the shallow-water closest taxonomic group to the hydrothermal vent and seep mussels of the Bathymodiolinae (Distel et al., 2000). Jones et al. (2006) established that vent species evolved multiple times, a process that involved habitat shifts, but evidence is that there was a progressive evolution from shallow to deep habitats. Mid-ocean hydrothermal vent species may represent a monophyletic group with one noticeable reversal, and this is in agreement with previous hypotheses regarding evolution from wood/bone to seeps/vents (Jones et al., 2006).

The blue mussel *Mytilus edulis* Linné, 1758 is a widespread semi-sessile epibenthic bivalve found on rocky shores, shallow sublittoral zones, and estuaries (Newell, 1989). It is very common in northern Europe and parts of the Atlantic coast off Canada, but also colonizes temperate zones in the Southern Hemisphere (Gosling, 1992). The upper vertical distribution limit of *M. edulis* populations on rocky shores is determined by its tolerance to temperature and desiccation (Seed & Suchanek, 1992). This species is relatively tolerant to extreme cold and freezing and it can survive occasional short frost events, but may not be resistant to persistent very low temperatures (Bourget, 1983). The lower depth limit of distribution of *M. edulis* is presumably mainly influenced by predation (Seed, 1969). In addition, burial and abrasion by shifting sands is also of importance (Daly & Mathieson, 1977; Holt et al., 1998). The maximum depth distribution recorded for this species in the Baltic Sea is 40 m, and its depth limit is associated with the presence/absence of a hard bottom substratum (Bubinas & Vaitonis, 2003). Subtidal populations have been reported on seamounts, dock pilings and offshore oil platforms, where they grow to a larger size, probably because of a lack of predators (Seed & Suchanek, 1992).

Occasionally, it has been found in deeper and cooler waters (100 to 499 m, Theroux & Wigley, 1983) because of lack of shallower habitat and/or presence of hard substratum at greater depths. The genus *Mytilus* is also an important invasive species (Carlton, 1999).

Mytilus edulis is dioecious with rare occasions of hermaphroditism (Seed, 1976; Micallef & Tyler, 1988), and has a large number of small-sized eggs and a planktotrophic larva (Bayne, 1976). Gametogenesis is synchronous and spawning occurs when eggs and sperm are fully ripe and are released through the exhalent siphon into the water column where fertilization takes place (Bayne, 1976; Newell, 1989). The embryonic development takes a few days and comprises cleavages, the formation of cilia, vellum and shell gland (the trochophore stage), up to the formation of the D-shaped shelled larvae, when it starts its planktotrophic phase (Bayne, 1976). The depth distribution of larvae and juveniles of *M. edulis* is presumed to be the same as in adults (Newell, 1989).

In the present study, the fertilization success and the embryonic and larval development of *Mytilus edulis*, along its entire physiological temperature and pressure tolerance window, is analysed for the first time. The physiological tolerances of the early life stages are examined, as they are of extreme importance in controlling the distribution, colonization pathways and to some extent the speciation within closely related species. The results are discussed in the frame of current theories on the evolution of the macrofauna in chemosynthetic environments.

2.3. Material and methods

2.3.1. Sampling and spawning

Adult specimens of *Mytilus edulis* were collected from intertidal Southampton Water, UK (50°53'N, 1°23'W) and maintained at 15ºC ± 1.5ºC, for at least 7 days, in a running seawater system in the aquarium of the National Oceanography Centre, Southampton, where the experimental work took place in June 2007. Spawning was induced by vigorously shaking all mussels in a bucket (mechanical shock; Costello et al., 1957; Sprung & Bayne, 1984) followed by the

injection of 1 mL of 0.55 M KCl into the mantle cavity of each mussel (salinity shock; Young & Tyler, 1993). To obtain gametes, each mussel was placed in individual glass bowls and left "dry" for 10 minutes, with an ambient temperature of about 20ºC. Mussels were submerged with 5ºC (temperature shock; Costello et al., 1957; Sprung & Bayne, 1984) filtered seawater (1.6 µm retention) and spawning would start after temperature inside the bowls exceeded 15ºC (Fig 2.1.).

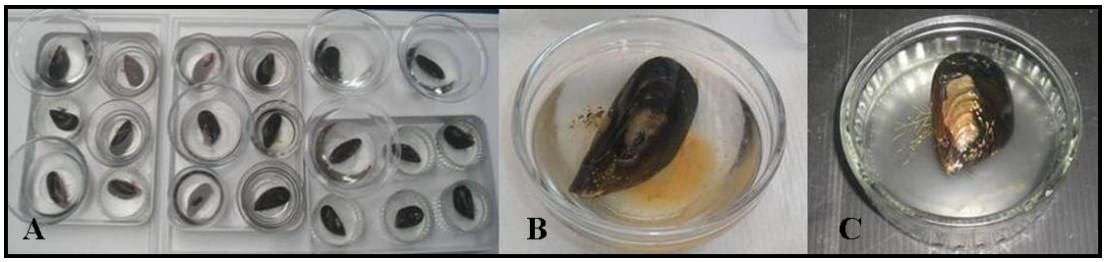

Fig. 2.1. Photograph of *Mytilus edulis* inside individuals bowls (A); female spawning with yellow-orange eggs that deposit in the bottom of the bowl (B); male spawning with water becoming cloudy and whitish colour (C).

2.3.2. Temperature effect on embryonic and larval development

Three cultures of fertilized eggs were prepared by mixing freshly spawned male and female gametes in a 1 L beaker in filtered seawater (1.6 µm retention filter) at 15ºC. Gametes from each male/female pair were used in each culture. Fertilized eggs were transferred into 20 mL glass vials and incubated at 5, 10, 15, 20 and 25ºC, at atmospheric pressure (Fig. 2.2.). Cultures were sampled regularly (every 10 minutes in the first 4 hours after fertilization, and once per hour thereafter) and the time from fertilization and stage of development were noted, until the D-larvae stage was reached for all temperatures. The chosen stages of development were defined according to distinguishable morphological features between stages (Table 2.1.), observable under a compound microscope.

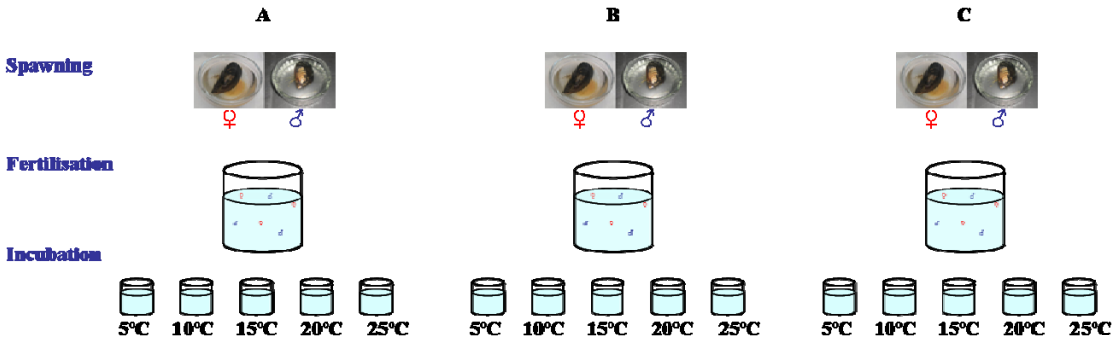

Fig. 2.2. Schematic of the methodology from spawning, fertilization and incubation of the different cultures (A, B, C) at the different temperatures (5, 10, 15, 20 and 25ºC).

Table 2.1. Classification of early ontogenetic stages in *Mytilus edulis*, with reference to the main characteristics observed in each stage (changed after Zardus & Martel, 2002).

stage	main characteristics
A	abnormal development
0	unfertilized egg; no sign of polar body
I	fertilized, uncleaved egg; showing polar body (and polar lobe formation)
II	2-cell stage; first cleavage and extrusion of polar body
III	4-cell stage; 1 large D-cell and 3 smaller cells
IV	8-cell stage; showing spiral unequal cleavage of blastomeres
V	multi-cell; unequal cleavage producing micromeres in the animal pole and macromeres in the vegetal pole; within the fertilization membrane
VI	early blastula; newly released from the fertilization membrane; developing cilia
VII	gastrula; with blastopore (developing gut); invagination of the shell field (developing shell); quadrants with cilia
VIII	early trochophore; developing apical sense organ (apical plate + apical tuft)
IX	late trochophore; with vellum and organic pellicule of first shell
X	D-larva; free-swimming strait hinge veliger with fully formed vellum and prodissoconch I

2.3.3. Pressure effect on embryonic and larval development with fertilization under pressure

The embryonic developmental stage, after 4 and 24 hour treatments, was assessed for pressures of 1, 100, 200 and 300 atm (plus 400 and 500 atm for 4 hour treatments only, as the 24 hours treatment performed first indicated that successful fertilization was also likely at greater pressures) and at temperatures of 10, 15 and

20ºC, with fertilization occurring under pressure. Gametes from three different males and three different females were used, so that three replicates (each from one male/female pair) were assigned to each pressure/temperature combination.

The following method was designed to prevent fertilization before pressurizing eggs and sperm, and at the same time allowing them to mix under pressure: eggs from each female were collected from the glass bowls and re-suspended in ambient seawater, in a 1 L beaker, and then transferred into 6 mL plastic vials; 0.5 mL of diluted sperm suspension was pipetted into a 1 mL microcentrifuge tube (leaving 0.5 mL of air space); one microcentrifuge tube containing the sperm suspension was inserted into each plastic vial containing the egg-suspension (Fig. 2.3a.); the plastic vial was refilled with the egg suspension until it overflowed and the cap closed, avoiding any air being trapped inside; plastic vials were placed inside the pressure vessel (Fig. 2.3b.) and filled with tap water at the test temperature; the pressurization to the desired pressure was continuous. When pressurizing the vessels it was possible to hear the microcentrifuge tubes imploding inside the plastic vials at about 50 atm, due to air deliberately left inside. In all treatments, pressurization was continuous and it took at most 10 seconds until the desired experimental pressure level was reached.

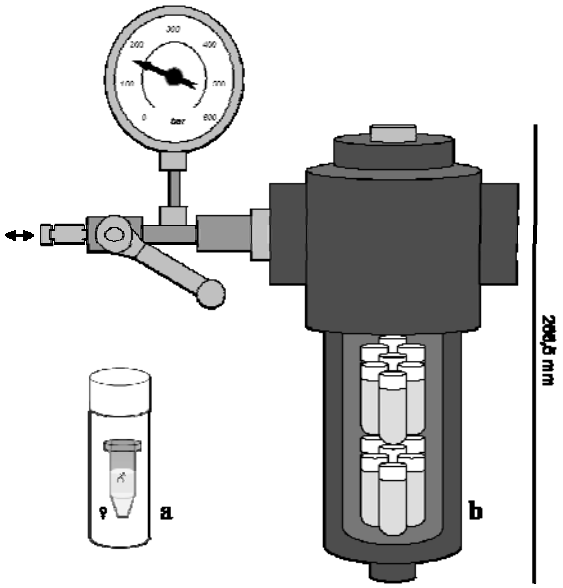

Fig. 2.3. Schematic overview of the experimental pressure vessels: (a) plastic vial filled with the egg suspension and the microcentrifuge tube half-filled with sperm suspension; (b) pressure vessel showing the plastic vials inside (arrow indicates the connection valve to the hydraulic hand-pump).

For the atmospheric pressure cultures, fertilization occurred by mixing eggs and sperm in a 1 L beaker and transferring the solution into 6 mL plastic vials. Pressure chambers and 1 atm cultures were incubated at 10, 15 and 20ºC for 4 and 24 hours. At the end of each trial, pressure vessels were depressurized and samples for the analysis of the developmental stage were quickly preserved in 4% formalin. Fifty embryos from each replicate were randomly selected and staged according to the embryonic development.

After depressurization, sub-samples from the vials containing embryos were immediately preserved in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, and kept refrigerated at 4ºC until used for scanning electron microscopy (SEM). Samples were subsequently rinsed with buffer, dehydrated and critical point dried. Specimens were then mounted on stubs and sputter coated in gold. Scanning electron micrographs were taken using a Hitachi S800 SEM.

2.3.4. Pressure effect on embryonic and larval development with fertilization at atmospheric pressure

For the treatments with fertilization occurring at atmospheric pressure, and embryonic and larval development examined after a 50 hour period, three cultures of fertilized eggs were prepared by mixing freshly spawned male and female gametes in a 1 L beaker in filtered seawater (1.6 µm retention) at 15ºC (ambient temperature). Gametes from each male/female pair were used in each culture. Fertilized eggs were transferred into 6 mL plastic vials, filled to overflowing and cap closed to avoid any air to be trapped inside. Plastic vials were placed inside the pressure chambers, filled with tap water at the test temperature, and the pressurization to the desired pressure was continuous. The fertilized eggs were incubated at 1, 100, 200 and 300 atm, and 5, 10, 15, 20 and 25ºC. After 50 hours, pressure vessels were depressurized and all samples were quickly preserved in 4% formalin. Fifty embryos from each replicate were randomly selected and staged according to the embryonic development.

2.3.5. Statistical analyses

The results are presented as histograms with mean and standard deviation for each stage of development. Data on the proportion of abnormally developing embryos present in each culture failed the assumption of normality, even after data were arcsine transformed. The raw data were used in the nonparametric Kruskal-Wallis *H* test single factor analysis of variance by ranks to test for temperature effects and pressure effects on the proportion of abnormally developing embryos (Sokal & Rohlf, 1995). Temperature and pressure effects were tested separately, for each treatment and each incubation period (e.g. results of pressure effects are for each temperature tested in each incubation period).

2.4. Results

2.4.1. Temperature effects on embryonic and larval development

Mytilus edulis embryos develop faster at higher temperatures. For example, at 1 atm most embryos incubated at 5ºC require 50 hours to reach stage V (multi-cell; Fig. 2.4.; Table 2.1.), but reached stage X (D-larva) at 20ºC. At 10ºC and after 4 hours of incubation, embryos developed to stage III (4-cell stage; Fig. 2.5.), after 24 hours they reached stage VI (early blastula; Fig. 2.6.) and past 50 hours they achieved stage VIII (early trochophore). After 4 hours incubation at 15 and 20ºC, embryos developed to stage V, past 24 hours embryos reared at 15ºC were in stage VII (gastrula) and those reared at 20ºC reached stage X. Embryos incubated at 15ºC needed 50 hours to get to D-larvae stage. At 25ºC, embryos that survived 50 hours incubation, reached stages IX and X (late trochophore and D-larvae, respectively), although 93% of the embryos were developing abnormally or dying, and the culture was quickly degrading.

When testing for effects of temperature on the proportion of abnormally developing embryos reared at atmospheric pressure, it is possible to detect a significant effect in the 50 hours incubation period when all temperatures are analysed together (5-25ºC; Kruskal-Wallis H=6.058, p=0.011; Table 2.2.). There is no significant effect of temperature (p>0.05; tested temperatures: 10, 15 and 20ºC)

on the proportion of embryos developing abnormally for incubation periods of 4 hours and 24 hours for any pressure treatment (Table 2.2.).

Table 2.2. Kruskal-Wallis analysis of variance testing the effects of temperature (10, 15 and 20ºC) on the proportion of abnormally developing embryos of *Mytilus edulis* reared at the different pressures for different incubation periods [*H* statistic (degrees of freedom, *N*=Number of replicates);* for temperatures: 5, 10, 15, 20 and 25ºC].

pressure (atm)	incubation period							
	4 hours		24 hours		50 hours		50 hours *	
	H (2, *N*=9)	*p*-value	*H* (2, *N*=9)	*p*-value	*H* (2, *N*=9)	*p*-value	*H* (4, *N*=15)	*p*-value
1	0.073	0.964	0.615	0.735	6.058	0.048	13.099	0.011
100	0.610	0.737	2.443	0.295	5.695	0.058	12.603	0.013
200	5.728	0.057	5.600	0.061	4.545	0.103	11.070	0.026
300	1.689	0.430	1.107	0.575	7.624	0.022	13.086	0.011

2.4.2. Pressure effects on embryonic and larval development

Fertilization under pressure succeeded in all pressure treatments (Figs. 2.5. and 2.6.). After 4 hours incubation, in both 10 and 15ºC treatments for 100-500 atm, eggs were fertilized but did not develop (Fig. 2.5.). A small proportion of embryos reached stage V (multi-cell), when incubated for 4 hours at 20ºC/100 atm, but from 200 atm up to 500 atm embryos were in less advanced stages, indicating a retarded development. For the 20ºC cultures, with increasing pressure the number of abnormal individuals increased (Fig. 2.5.).

In embryos exposed for 24 hours, the maximum developmental stage reached was for the cultures reared at atmospheric pressure (Fig. 2.6.). A similar pattern occurred for the cultures at 100 atm with maximum development for the 20ºC treatment: stage VIII (early trochophore). At 20ºC/200 atm more than 92% of the embryos did undergo abnormal development, while at 15ºC/200 atm embryos reached stage VI, and at 10ºC/200 atm a small portion of embryos developed to stage V (Fig. 2.6.). At 300 atm and for all temperatures tested embryos did not develop. In all temperatures an increase of abnormal development with increasing pressure was observed.

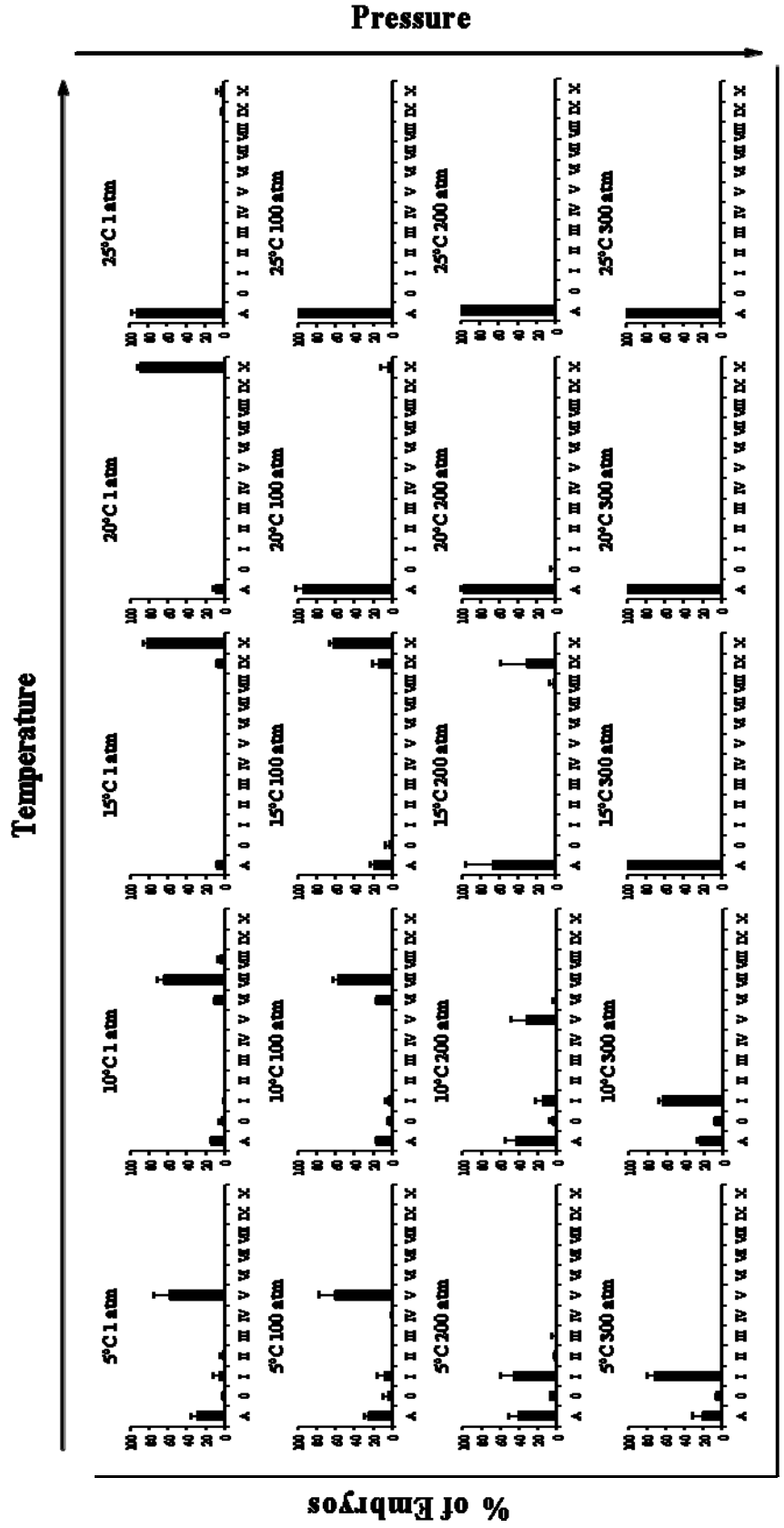

Fig. 2.4. *Mytilus edulis* embryonic and early larval development incubated at different pressure/temperature regimes for 50 hours, with fertilization occurring at atmospheric pressure. Histograms are of percentage mean and standard deviation. Stages of development: A=abnormal; 0=unfertilized; I=fertilized, uncleaved egg; II=2-cell; III=4-cell; IV=8-cell; V=multi-cell; VI=early blastula; VII=gastrula; VIII=early trochophore; IX=late trochophore; X=D-larva (see Table 2.1. for a detailed description of each developmental stage).

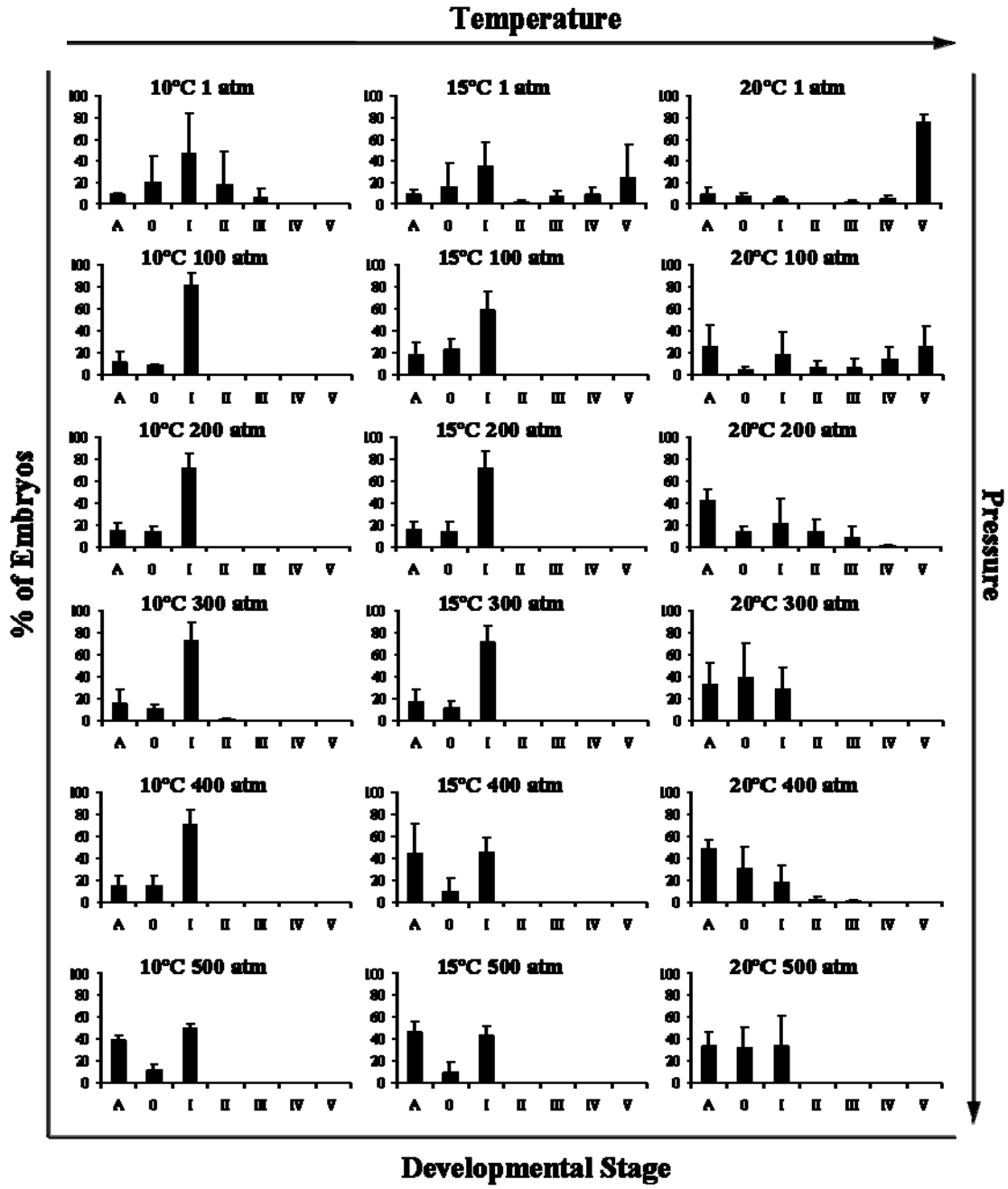

Fig. 2.5. *Mytilus edulis* embryonic development incubated at different pressure/temperature regimes for 4 hours, with fertilization occurring under pressure. Histograms are of percentage mean and standard deviation. Stages of development: A=abnormal; 0=unfertilized; I=fertilized, uncleaved egg; II=2-cell; III=4-cell; IV=8-cell; V=multi-cell (see Table 2.1. for a detailed description of each developmental stage).

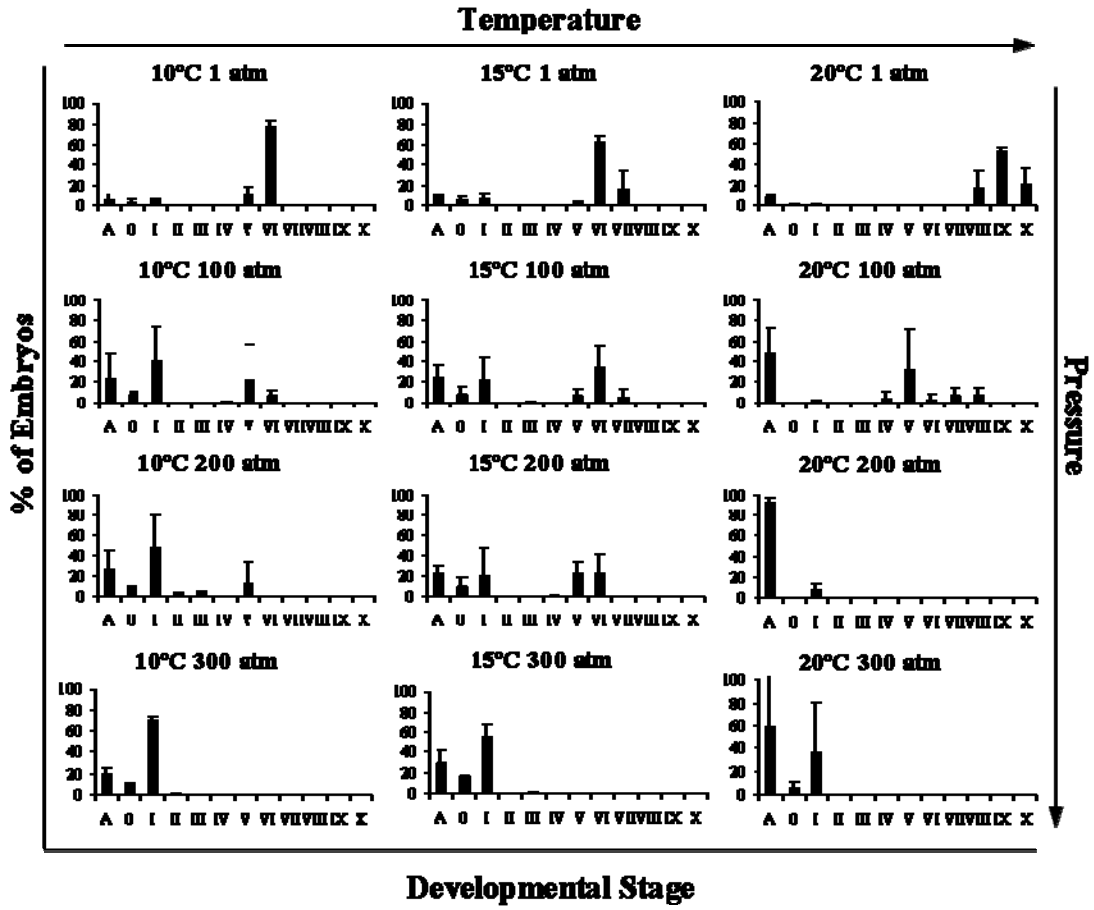

Fig. 2.6. *Mytilus edulis* embryonic and early larval development incubated at different pressure/temperature regimes for 24 hours, with fertilization occurring under pressure. Histograms are of percentage mean and standard deviation. Stages of development: A=abnormal; 0=unfertilized; I=fertilized, uncleaved egg; II=2-cell; III=4-cell; IV=8-cell; V=multi-cell; VI=early blastula; VII=gastrula; VIII=early trochophore; IX=late trochophore; X=D-larva (see Table 2.1. for a detailed description of each developmental stage).

The cultures exposed to 100 atm for 50 hours and incubated at 5, 10 and 15ºC behaved very similarly to those reared at atmospheric pressure, while at higher temperatures most of embryos were abnormal (Fig. 2.4.). In both 20 and 25ºC cultures exposed to 200 and 300 atm the embryos were abnormal or died. In the cultures reared at 300 atm at 5 and 10ºC embryos did not develop, and most of them remained in stage I – uncleaved showing a polar body – after fertilization (Fig. 2.4.). On the other hand, at 10ºC/200 atm more than 30% of embryos reached stage V – multi-cell stage (Fig. 2.4.). At 15ºC/200 atm, the few embryos developing normally

reached stage IX (Fig. 2.4.). In general, the degree of abnormal development observed in embryos increases with both increased pressure and temperature.

Abnormal cleavages, the lack of cell membrane, and extrusions of the cytoplasm originating knob-like structures on the exterior of the embryo are the common abnormalities observed in pressurized cultures (Fig. 2.7. and Fig. 2.8.). The effect of pressure on the proportion of abnormally developing embryos was tested for each temperature and each incubation period (Table 2.3.). Results show no significant effect of pressure for all temperatures analysed (10, 15 and 20ºC) for both incubation periods of 4 and 24 hours, except for treatment 20ºC/24 hours (Kruskal-Wallis H=8.128, p=0.043; Table 2.3.). With a longer incubation period of 50 hours, the effect of pressure on the proportion of abnormally developing embryos is significant for all temperatures tested (Table 2.3.), except for the 5ºC cultures (Kruskal-Wallis H=6.407, p=0.093; Table 2.3.).

Table 2.3. Kruskal-Wallis analysis of variance testing the effects of pressure (1, 100, 200 and 300 atm) on the proportion of abnormally developing embryos of *Mytilus edulis* reared at different temperatures for different incubation periods [H statistic (degrees of freedom, N=Number of replicates)].

temperature (ºC)	incubation period					
	4 hours		24 hours		50 hours	
	H (3, N=12)	p-value	H (3, N=12)	p-value	H (3, N=12)	p-value
5	-	-	-	-	6.407	0.093
10	1.568	0.667	4.351	0.226	10.157	0.017
15	1.977	0.577	6.590	0.086	10.237	0.017
20	5.100	0.165	8.128	0.043	8.194	0.042
25	-	-	-	-	10.735	0.013

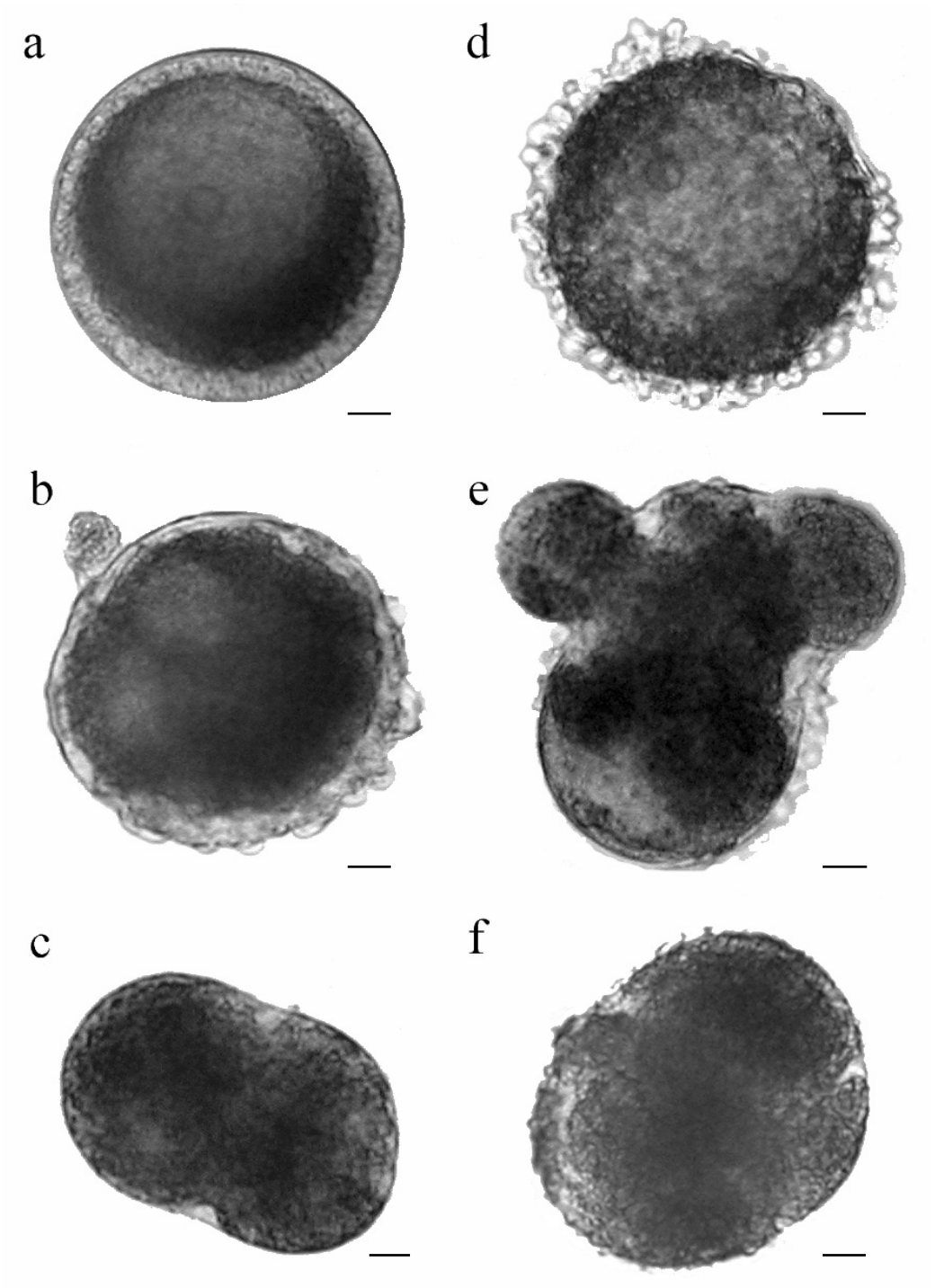

Fig. 2.7. Examples of pressure effects on the morphology of *Mytilus edulis* embryos incubated for 4 hours, with fertilization occurring at incubation pressure (light microscope images). a) unfertilized egg (20ºC/300 atm); b) fertilized egg showing abnormal development with an irregular cell membrane (20ºC/300 atm); c) visible cleavage furrow of the 2-cell stage (20ºC/400 atm); d) unfertilized egg with cytoplasmic extrusions over the surface (10ºC/500 atm); e) abnormally developing embryo (10ºC/500 atm); f) abnormal cleavage furrow of the 2-cell stage with cytoplasmic extrusions over the surface (10ºC/500 atm). Scale bars: 10 µm.

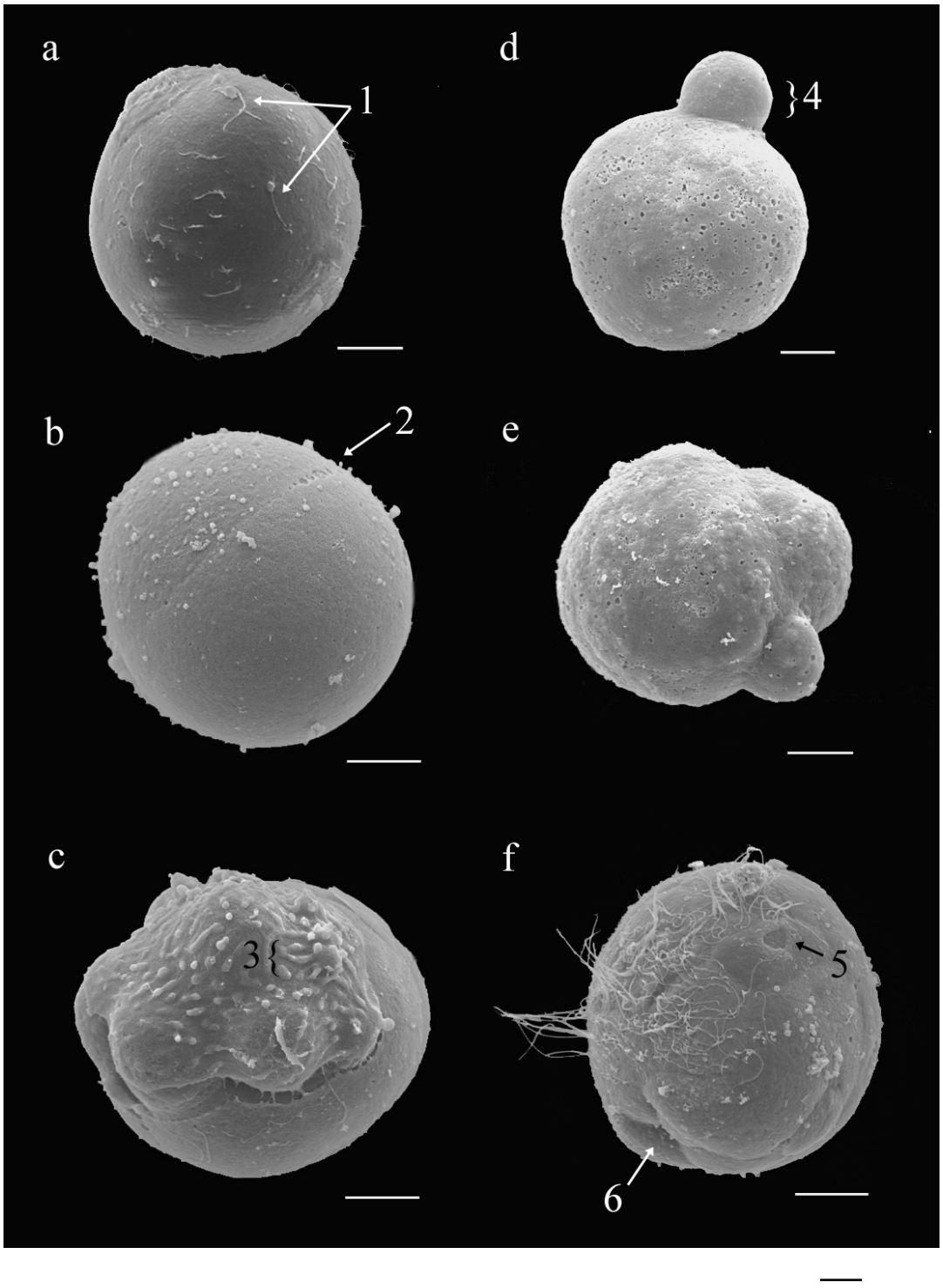

Fig. 2.8. Examples of pressure effects on the morphology of *Mytilus edulis* embryos incubated for 24 hours at 10ºC, with fertilization occurring at incubation pressure (scanning electron micrographs-SEM). a) uncleaved egg, with sperm cells (1) over its surface (200 atm); b) cleavage furrow (2) of the 2-cell stage (200 atm); c) abnormal multi-celled embryo, with membrane breakage and cytoplasmic extrusions (3) over the surface (200 atm); d) fertilized egg with rough surface and polar body (4) (300 atm); e) 2-cell stage embryo with rough surface and polar body (300 atm); f) gastrula stage embryo with cilia, blastopore (5) and shell field invagination (6) (100 atm). Scale bars: 10 μm.

2.5. Discussion

2.5.1. Methodological considerations

The method to induce spawning used in this work results from a combination of different approaches suggested in the literature (e.g. Costello et al., 1957; Sprung & Bayne, 1984; Young & Tyler, 1993). The mechanical, temperature, and salinity shocks proved to be efficient, with at least 15% of mussels spawning per trial.

Fertilization under pressure was 100% effective as all the microcentrifuge tubes imploded under pressure allowing the fertilization to take place. This new and simple method may be used in future pressure studies as a way to mix small volumes of solutions under pressure. As fertilization rates at atmospheric pressure and at tolerated pressures (i.e. 100 atm at 10 and 15ºC, Figs. 2.4.-2.6.) are comparable, I assume that imploding microcentrifuge tube vials do not bias significantly the fertilization process, i.e. by lysing cells. The "popping microcentrifuge" tube approach of fertilization has the advantage of not affecting the pH or seawater density. When developing the experimental design, dissolvable capsules yielded little satisfying results as these drastically affected the viscosity of small volumes of seawater. Fertilization of eggs following rupture of microcentrifuge tubes at about 50 atm and prior to reaching the desired experimental pressure is unlikely. Pressurization was continuous and only lasted 5-7 seconds following rupture of microcentrifuge tubes. Successful fertilization in *Mytilus edulis* eggs took between 40 and approximately 60 seconds when studied at 1 atm under a compound microscope and within the temperature range tested.

The present work focuses on the study of the early embryonic phase of *M. edulis* life cycle that corresponds to the full embryogenic period, from fertilization to the early D-larvae stage. During this period, embryos do not feed and rely on nutritional reserves of maternal origin present in the oocytes. In this study, gametogenesis in *M. edulis* took place at approximately 15ºC, the maintenance temperature of adult blue mussels. Pre-spawning temperatures may affect physiological tolerances in the offspring and thus future studies should focus on the effect of temperature on offspring quality in *M. edulis*. Whereas survival rates in embryos and larvae might be enhanced if acclimation to the experimental

temperature would include gametogenesis, I do not believe that the overall physiological tolerance pattern found in this study would have changed.

2.5.2. Temperature and pressure effect on embryonic and larval development

The data presented here suggest that at atmospheric pressure the temperature tolerance window for successful early development of *Mytilus edulis* ranges from around 10 to 20ºC. The embryo development scales with temperature (for review see Anger, 2001), i.e. embryos take more time at lower temperatures to reach the same embryonic stage than those reared at higher temperatures. At higher temperatures, close to thermal limits, a higher proportion of abnormally developing embryos is observed. This is probably related to the metabolic rates increasing with increasing temperatures; although temperatures might not yet reach lethal state, more energy has to be allocated to the metabolism, which negatively affects the embryo development.

Fertilization is possible from 1 atm up to at least 500 atm, which was the maximum pressure tested. Active sperm cells were observed under compound microscope straight after depressurization when exposed at 500 atm for 4 hours. Based on these data, I hypothesize that pressure presents no barrier to the occurrence of fertilization. It is possible to observe a slower development with increasing pressure, as well as an increase of abnormal development of the embryos. Standard deviations potentially reflect the intraspecific variability found in the early ontogeny of *M. edulis* (Fig. 2.5.), which is higher in the amount of unfertilized eggs and abnormal embryos. Most of the abnormalities observed are due to rupture of the egg membrane, while cleavages apparently continue occurring. The egg fertilization membrane is only supposed to break after the blastula stage (Zardus & Martel, 2002), but was observed at earlier stages of development for pressures ≥200 atm (Fig. 2.8c.).

The cytoplasmic extrusions observed in pressurized embryos (Fig. 2.7b., d.; Fig. 2.8c.) have been described in pressurized protozoans (Landau et al., 1954; Kitching, 1957). According to these authors, elevated pressures cause 1) a reduction

in the protoplasmic gel strength, and 2) a weakness of the more rigid cortical portions of the cell. These effects apparently lead to a separation of the cell membrane from the cytoplasm, and to the formation of knob-like structures in the exterior of the cell membrane – ultimately leading to cell-membrane breakage. Zimmerman and Marsland (1964) found that high pressure affects the mitotic apparatus of dividing cells. In the cleaving eggs of the sea urchin *Arbacia punctulata*, chromosomal movements become retarded at 140 atm and are completely arrested at 280 atm. This effect is in general reversible, but when subject to a pressure in excess of 544 atm for more than 5 minutes leads to a drastic and irreversible disorganization of the mitotic apparatus (Zimmerman & Marsland, 1964). These experiments were for periods of 1 to 15 minutes with fertilization at atmospheric pressure. The authors argue that recovery might be possible but at the same time, the disorganization of the mitotic apparatus observed after a maximum pressurization time of 15 minutes could mean that it was permanently compromised.

The embryonic and larval development in some shallow-water marine invertebrates is inhibited when subjected to high pressures (Marsland, 1950; Salmon, 1975), and this may limit the vertical distributions of species (Young & Tyler, 1993). Pressure can even act differently for each early life-history stage, as found in the sea-urchin *Psammechinus miliaris*, in which the pelagic larva is more resilient to high pressure than the earlier embryonic stages (Aquino-Souza et al., 2008). Based on the present results, it would be possible to estimate that the theoretical maximum distribution depth for the *M. edulis* embryos is, at least 2000 m for the temperatures tested. Marsland (1950, 1970) found that in dividing marine eggs the higher the temperatures, within the temperature tolerance range of a species, the stronger the cortical gel structures and their resistance to high pressures. Although, the results presented here are contrary to this observation, as for example in the 50 hours treatment, when comparing the cultures pressurized at 100 atm at 15 and 20ºC, at 15ºC embryos are more tolerant to pressure and develop to D-larvae, with no increment on the proportion of abnormally developing embryos.

Metabolic rates increase with increasing temperature (for review see Clarke, 2003). In the case of *M. edulis*, the lower the pressure the faster the embryonic or larval development. In the present study, a prolonged time of exposure to the

different temperature/pressure combinations gives a further perspective on this developmental process: after 4 hours of exposure, at 20ºC it is seen higher tolerance to high pressure, as cleavages occur and the number of abnormalities is low (Fig. 2.5.). After 24 hours of exposure, those embryos undergo irregular cleavages and this may indicate that further successful development is unlikely under high pressure (Fig. 2.6.). After 50 hours this is a fact, as embryos do not tolerate pressures greater or equal to 100 atm.

The results show embryos developing normally at high pressures, and low temperatures. Questions remain if embryos developing normally under pressure, but with a slower growth rate due to low temperature, would become abnormal as complexity of developing embryos increases. I assume that there are particular stages in the early ontogeny development that are crucial to tolerate pressure and it is when an embryo reaches these stages that it will define a normal development or not. In similar pressure/temperature studies evidence suggests that low temperature retards the embryonic development (e.g. Young et al., 1997; Tyler & Young, 1998), and in the case of *M. edulis* pressure combined with temperature favours lower temperatures where fewer abnormalities are observed. Thus, it is reasonable to hypothesize that the invasion of the deep sea by *M. edulis* is possible in terms of pressure tolerances in embryos and larvae.

2.5.3. Seeding the deep sea

Wood-, bone-, vent- and seep-associated mussels are phylogenetically closely related and are indicative of a recent common ancestry for vent and non-vent species (Distel et al., 2000). A long isolation of this group of chemosynthetic mussels is proposed to be the cause of divergence from shallow-water mytilids (Distel et al., 2000). It has been hypothesized that wood- and bone-associated mussels worked as an evolutionary step for shallow-water mussels to colonize hydrothermal vents and cold seeps (Distel et al., 2000). Deep-sea colonization by shallow-water species over evolutionary periods of time needs cold-stenothermy adapted species finding a rather stable, low temperature environment, which resulted in the concept of faunistic exchange through low temperature, isothermic water bodies (Kussakin, 1973;

Menzies et al., 1973; Hessler & Thistle, 1975; Hessler & Wilson, 1983; Thatje et al., 2005). On the other hand, the colonization of deep-water chemosynthetic environments would require physiological properties in invertebrates that tolerate greater fluctuations in temperature (= cold-eurythermy). The present study clearly demonstrated that blue mussels are consistent with this scenario, which is furthermore supported by their close phylogenetic relationship with the Bathymodilinae (Distel et al., 2000) and similarities in their reproductive and larval developmental cycle (Lutz et al., 1980; Berg, 1985; Kenk & Wilson, 1985). Supporting this view of similar physiological prerequisites in temperate shallow-water and hydrothermal vent species, bivalves from deep-sea hydrothermal vents have growth rates similar to those of shallow-water mytilids and much higher (several orders of magnitude) than those of bivalve species living in non-chemosynthetic deep-sea habitat (Lutz et al., 1985).

It might be challenging to suggest that from an evolutionary perspective, hydrothermal vent environments have been the only places where shallow-water temperate to sub-tropical invertebrates were able to establish because of similar temperature tolerance regimes. Similar establishment in low-temperature deep-sea environments can only occur if the species possess a cold-stenothermy to a constant, low-temperature environment; a physiological pattern I hypothesize not to be favoured in shallow-water species from sub-tropical to temperate regions. I propose that the development of a thermocline is not necessarily a physiological barrier to pelagic larval stages and drifting stages of any kind to penetrate the deep sea, although its physical properties may influence the direct settlement by invertebrate larvae into deeper waters.

The results presented here challenge the idea by Distel et al. (2000) that mytilids of the genus *Bathymodiolus* colonized eurythermal vent sites via cold-stenothermal chemosynthetic wood and cold-seep environments. This evolutionary pathway would imply that *Bathymodiolus* regained cold eurythermy to colonize hydrothermal vents following the colonization of cold-stenothermal chemosynthetic environments; an evolutionary step that is generally assumed highly unlikely. The results presented herein support the idea that for species of the Mytilinae a direct colonization from shallow-water to deep-water sites, that both of which demand the

same temperature tolerance, is a more likely scenario. In addition, the physiological basis of organismal biology should be increasingly taken into consideration when studying the evolutionary history of deep-sea faunas. It is possible that only advanced larvae have been involved in seeding deep-sea habitats over long evolutionary periods of time owing to lack of food at greater depth. Here, it must be reiterated, however, that invertebrate larvae can arrest development for substantial periods of time when encountering unsuitable habitat conditions, such as lower temperatures in the colder water column, until encountering more suitable habitat for development (Anger, 2001; Thatje et al., 2004), which in addition should facilitate a direct shallow-water – deep-water exchange of floating early life-history stages, provided a hyperbaric tolerance is expressed.

Chapter 3. Temperature and pressure tolerance in the early ontogeny of the shrimp *Palaemonetes varians*

3.1. Summary

The caridean shrimp *Palaemonetes varians* Leach, 1814 inhabits salt marsh ponds, which are rarely deeper than one meter. Nevertheless, this species has a monophyletic relationship with deep-sea hydrothermal vent shrimps of the family Alvinocarididae. In order to understand the evolution of deep-sea fauna and its adaptation to high-pressure, it is important to know the effects of pressure on their shallow-water relatives. In this study I analyse the temperature and pressure effects on the survival, development and energetic contents of the first larval stage of the shallow-water shrimp *Palaemonetes varians*.

Newly hatched larvae from field-collected ovigerous females were reared in the laboratory under controlled conditions of temperature, salinity and food. *P. varians* larval development is planktotrophic with facultative lecithotrophy in the first larval stage. Larval development of *P. varians* at 20ºC in the presence of food (*Artemia salina* nauplii) revealed two distinguishable developmental pathways in larvae from the same female, representing a high degree of phenotypic plasticity. Intraspecific variability in dry weight, carbon, and nitrogen contents was found among the newly hatched larvae from different females collected in different years. The temperature tolerance window of the first larval stage is between 10 and 30ºC. A slower larval development is observed with decreasing temperature. Tolerance to high hydrostatic pressure is greater at 20ºC. With increasing pressure mortality increases and a slower development is observed. Larvae are able to survive and develop at pressures equivalent to at least 1000 m water depth.

The tolerance to high pressure found in the early ontogeny of *P. varians* may be a trace of an adaptation expressed by an ancestor that lived at greater depth. It is likely that any such ancestor would have exhibited similar high variability in larval development and great adaptability to changes in the environment. This ancestor may have lived when an isothermal water column was present (~65 Mya), and given rise

to both shallow-water and hydrothermal vent shrimps, by speciation through geographical and reproductive isolation, after deep water cooled with the re-establishment of deep-sea water circulation patterns (~49 Mya).

Keywords: pressure, temperature, larval development, Palaemonidae, Alvinocarididae, shallow water, deep sea, evolution.

3.2. Introduction

The caridean shrimp *Palaemonetes varians* Leach, 1814 (family Palaemonidae; Appendix II, Fig. II.i.), commonly known as the ditch or variable shrimp, is a widespread species of brackish waters, present in salt marsh areas of northen Europe, south to the Mediterranean basin and some parts of North Africa (Neves, 1973; Fincham, 1979; Yúfera & Rodríguez, 1985; Holthuis, 1987; Dolmen, 1997). Because of the variable appearance of adult and larval stages, there has been controversy over the biogeography of *P. varians* and whether or not it represents a species complex (for discussion see Fincham, 1979, and references therein).

Inhabiting highly turbid and stagnant inland coastal ponds, palaemonid shrimps are exposed to seasonal variations in salinity and temperature (Healey, 1997). These shrimps are euryhaline, living and reproducing in habitats where the local and annual salinity variation can range from 3 up to 80 (Heral & Saudray, 1979), and are highly tolerant of hypoxia (Nielsen & Hagerman, 1998). *Palaemonetes varians* is sensitive to small changes in hydrostatic pressure equivalent of just a few centimetres of water depth. It counteracts the effect of increased pressure by swimming upwards, and of decreasing pressure by swimming downwards (Digby, 1961). It plays an important ecological role in coastal ecosystems in the transfer of nutrients and energy at the various trophic levels (Aguzzi et al., 2005). This shrimp species is a detritivore (Escaravage & Castel, 1990) and at the same time predator of small aquatic organisms (Anderson, 1985; Roberts, 1995).

The breeding season and the occurrence of *Palaemonetes varians* larvae in the plankton takes place from late spring to early summer when both atmospheric and seawater temperatures increase (Fincham, 1979) along with longer periods of daylight (Bouchon, 1991). The first larval stage is facultative lecithotrophic, as newly hatched larvae have sufficient nutritional reserves of maternal origin to survive and successfully moult to the second zoeal stage, and at the same time possess mouthparts that appear functional a few hours after hatching (Heral & Saudray, 1979; Yúfera & Rodríguez, 1985). *Palaemonetes varians* has an abbreviated development consisting of up to five planktonic zoeal stages followed by demersal decapodid stages until it reaches the juvenile stage (Fincham, 1979). *Palaemon serratus* has up to eight or nine zoeal stages, one of the longest larval sequences in the Palaemonidae (Fincham, 1983). Depending on the environmental conditions, different developmental pathways, involving changes in the number of instars, can be found in *P. varians* (Fincham & Figueras, 1986).

The family Palaemonidae has a monophyletic relationship with hydrothermal vent shrimp members of the Alvinocarididae (Tokuda et al., 2006). Members of both families occur in great abundances in their own natural habitats and are exposed to great variations in environmental factors, and especially temperature.

Temperature and pressure tolerance studies of the dispersal stages of deep-sea species and their shallow-water relatives may help understanding of the origins of the deep-sea fauna (Tokuda et al., 2006; Pradillon & Gail, 2007; Mestre et al., 2008). It has been suggested that shallow-water species from temperate zones may have migrated to the deep sea during periods characterized by a warm-isothermal water column, notably the late Mesozoic and early Cenozoic (Menzies et al., 1973; Hessler & Wilson, 1983; Young et al., 1997). Furthermore, a recent study has shown that, because it would have experienced similar cold-eurythermal conditions, the ancestor of deep-sea hydrothermal vent mussels is likely to have been a eurythermal shallow-water relative (Mestre et al., 2008).

The hypothesis being tested here is that *Palaemonetes varians* has inherited a relict physiological pressure tolerance from an ancestor, which lived when an isothermal water column was present. It is suggested that this hypothetical ancestor

gave rise to both shallow-water and hydrothermal vents shrimps, by speciation through geographical and reproductive isolation. Within this context, the physiological potential of the food-independent (lecithotrophic) first larval stage of the shallow-water shrimp *Palaemonetes varians* to survive dispersal to bathyal depths, is investigated by analysing its temperature and pressure tolerance. The intraspecific variability of the offspring of different females, collected from different years, is also analysed, as larval fitness at hatching is likely to constrain the tolerance to changes in the environment. In addition, the variability in larval development is investigated as this reflects the adaptability of larvae to different environmental conditions and its potential to survive and disperse.

3.3. Materials and methods

3.3.1. Sampling and maintenance of ovigerous females

Ovigerous females of the caridean shrimp *Palaemonetes varians* were obtained from salt marsh ponds at Lymington, UK (50°45'N, 1°32'W), in July 2006, 2007 and 2008, using a hand net. Individuals were maintained in the National Oceanography Centre, Southampton, under constant temperature (21.5ºC ± 1.3ºC) and salinity (34.33 ± 0.62) conditions with a 12:12 hours light:dark rhythm. Ovigerous females with eggs close to hatching were isolated in aerated individual buckets (800 mL), with 40% of the water changed every day and fed twice a week with TetraMin flakes. Each bucket was inspected every morning for newly hatched larvae. Using a plastic pipette, larvae were transferred into an 800 mL beaker with filtered seawater (1.6 µm retention) at room temperature. Larvae were quickly examined for any deformities and only the normal and actively swimming ones were assigned to the different experiments.

3.3.2. Larvae maintenance and development

Larvae hatched on the same day and from the same egg batch of one female collected from the field in 2007 were used to study the larval development at 20ºC,

during eight days. All larvae were reared in individual cups (80 mL) filled with filtered seawater (1.6 µm retention), pre-incubated at 20ºC. Every morning larvae were inspected for developmental stage. After the day of moult to zoea II, water was then changed every two days, and larvae fed daily with a small amount (~1 mL) of *Artemia salina* nauplii (from Dohse Aquaristik). Fresh cultures of *A. salina* nauplii were obtained daily, by incubating the eggs, which hatch after 48 hours, in aerated seawater in a glass beaker at 20ºC. On the day following moult, for each developmental stage, larvae were sampled and preserved for elemental composition (Carbon: C; Nitrogen: N) analysis. In order to assess the intraspecific variability, freshly hatched larvae from six different females collected in 2007 (A, B, C, D, E, F) and another six different females ollected in 2008 (G, H, I, J, K, L) were randomly selected and preserved for dry mass, carbon and nitrogen contents analysis.

3.3.2.1. *Developmental stages*

The early ontogenetic stages of *Palaemonetes varians* were distinguished based on the morphological characteristics of each stage, as seen under a stereomicroscope (Yúfera & Rodríguez, 1985; Fincham & Figueras, 1986; Kerr, 2006; Smith, 2008), and by the presence of exuvia in the rearing cup, which confirmed the occurrence of moulting. The different developmental stages found in the analysed larval cultures can be morphologically defined as follows:

Zoea I (ZI) - the rostral spine is straight and simple; the rostrum lacks dorsal teeth; the eyes are sessile; maxillipeds 1-3 are graded in size and have simple setae; developing appendages are relatively short (mainly the exopods on developing pereiopods 1 to 5); the tail fan consists of a broad triangular shaped telson (no budding) with spines on the posterior margin.

Zoea II (ZII) - the rostral spine is slightly more developed than in Zoea I; the base of the rostrum shows two conspicuous dorsal teeth directing anteriorly; the eyes are stalked; the appendages are unsegmented and longer than in the zoea I with setae at the tips; telson spines have increased to stretch the full length of the posterior margin and slightly around the corners of the other two sides of the

triangular telson; a pair of internally developing uropods is visible as buds on either side of the telson, beneath the exoskeleton, in premoult stage.

Zoea III (ZII) - the eyes are stalked, more than in zoeal II individuals; the appendages are unsegmented; there are developed pleopods off every segment of the abdomen; the tail fan has developed a pair of lateral buds indicating the development of uropods.

Decapodid I (DI) - the pereiopods and maxillipeds are fully developed (with segmentation); exopods are still present on the pereiopods; the pleopods are still non-functional; the internal and external uropods are more developed, with the external pair of uropods almost reaching the posterolateral margin of the tail fan; the telson is more elongated than previously, with the posterior margin becoming concave.

Decapodid II (DII) - the antennae are more prominent than in previous stages; the exopods are reduced but still functional; the pleopods are more developed, but still not functional; the tail fan is fully developed, split into five sections made up of two pairs of external uropods and the telson (now rectangular in shape).

Juvenile (J) - the antennae are fully developed; the exopods are no longer present; all other appendages (maxillipeds, pereiopods and pleopods) are fully functional; juveniles are benthic and only rarely swimming.

3.3.3. Temperature and pressure experimental design

In the following experiments and in all different treatments only the zoea I larval stage was studied, i.e. from hatching until the day after moult to zoea II. Newly hatched larvae were maintained unfed in filtered seawater (1.6 μm retention) with no water change throughout the experiments. Larvae were either reared in 80 mL cups or inside 6 mL vials, the latter were always filled to overflow and cap closed. Only the small 6 mL vials were used in pressure treatments. Up to fourteen of these vials could be incubated at a time in each pressure vessel (for a drawing of the pressure vessel, see Appendix I, page I-iii, figure 1). Each pressure vessel was then filled with

tap water pre-incubated at test temperature and closed. The vessel was connected to a stainless steel hand-powered hydraulic pump (Enerpac, model 11-100) and pressurized with an increment of 20 atm every 5 minutes until reaching the desired pressure. Once pressurized, the vessel was disconnected from the pump and transferred to an incubator (LMS series 1 250 model Cooled Incubator) previously set at the test temperature. After the end of the experimental period, pressure inside each vessel was checked on the pressure gauge, the valve opened and the vessel immediately depressurized to atmospheric pressure. The pressure vessel could then be opened and the vials removed. In all treatments, larvae were checked for survival, developmental stage, and any abnormal features registered, using a stereomicroscope.

3.3.3.1. *Preliminary temperature and pressure experiments*

Three preliminary experiments were conducted during 2006. To study the effect of pressure, larvae were reared in small volumes of seawater (6 mL) and in complete darkness, conditions imposed by the technical constrains of the pressures vessels. Experiment 1 tested if the absence of light and the small volumes of the vials affected the survival of larvae in comparison to larvae reared in the presence of a light:dark rhythm and in a larger volume of seawater (80 mL). The volume inside each pressure vessel is limited to a maximum of 14 vials. In experiment 2, the survival of rearing five larvae per 6 mL vials in comparison to one larva per 6 mL vial was tested, at different pressures. In experiment 3, the effects of temperature and pressure on the survival of larvae were analysed. Unless otherwise stated, the newly hatched larvae used in the following experiments were randomly selected from different females, due to a low release (<100) of larvae per night per female.

Experiment 1 – Thirty larvae were reared in individual cups (80 mL) with a 12:12 hours light:dark rhythm. Another thirty larvae were reared in individual vials (6 mL) in total darkness. In both treatments, groups of ten individuals were incubated at 10, 20 and 30ºC (LMS series 1 250 model Cooled Incubator) at atmospheric pressure. Only larvae from one female were used. Larvae reared in cups were checked for moulting and survival every day.

Experiment 2 – In a different experiment, forty-five larvae were reared in groups of five per 6 mL vial (5x/vial) and another 28 larvae were reared individually in 6 mL vials, per pressure treatment at 20ºC and for 72 hours. The pressures analysed were 100, 200 and 300 atm.

Experiment 3 – In a third experiment, up to 28 larvae were assigned for each treatment testing the effect of pressure (1, 100, 200 and 300 atm) at different temperatures (5, 10, 20 and 30ºC). Each pressure vessel enclosed fourteen vials with one newly hatched zoea I larva each; hence, data refer to two replicates for each pressure/temperature treatment. The incubation period per temperature treatment follows that of the developmental time from hatching to moult to zoea II, previously assessed for each temperature at atmospheric pressure.

3.3.3.2. *Temperature and pressure effects on elemental composition of larvae*

In this experiment the effects of temperature and pressure on the survival, dry mass, carbon and nitrogen contents of larvae were analysed. Groups of ten larvae were assigned to each pressure (1, 100, 200 and 300 atm) and temperature (20 and 30ºC) treatment. In addition, ten larvae were reared at 10ºC at atmospheric pressure. All larvae were reared in individual 6 mL vials. This experiment used larvae hatched from two females collected in 2007, with larvae from female AA assigned to 10 and 20ºC treatments and female BB assigned to 30ºC treatments. Larvae were incubated for two days in 20 and 30ºC treatments and for six days in 10ºC treatments. These incubation periods correspond to the developmental time from hatching to moult to zoea II at atmospheric pressure, observed for the different temperatures in 2007. At the end of each treatment larvae were sampled and preserved for dry mass and elemental (CN) analysis.

3.3.4. Dry mass (DM) and elemental composition (CN) analysis

Individual dry mass (DM) and elemental composition in carbon (C) and nitrogen (N) were determined for either zoea I sampled immediately after hatching,

or for the different stages of development sampled on the day after moulting (with n = 5 replicates per treatment; one individual per replicate). The procedure used to determine the elemental composition per larvae contained the following steps: short rinsing in distilled water; blotting on fluff-free Kleenex paper for optical use; placement into pre-weighed tin capsules (HEKAtech Germany); freezing at -80ºC; vacuum drying at $<10^{-2}$ mbar; weighting immediately before analysis; combusting at 1020°C in a Fison (Carlo Erba) 1108 Elemental Analyser (after Anger & Harms, 1990). Dry mass was measured to the nearest 0.1 μg on an autobalance (Mettler MT5). The elemental analyser was calibrated using the standard acetanilide.

3.3.5. Statistical analyses

Statistical differences in elemental composition between decapodid II (developmental pathway 1) and juvenile (developmental pathway 2) were tested by means of a Mann-Whitney U-test (Sokal & Rohlf, 1995). Statistical differences in larval elemental composition at hatching between females within different years (2007 and 2008) were tested by means of a nested design ANOVA, with assumptions of normality tested with the Kolmogorov-Smirnov test (Sokal & Rohlf, 1995).

3.4. Results

3.4.1. Larval development

Palaemonetes varians larval development at 20ºC was analysed during eight days, from hatching up to juvenile stage. Larvae moulted to successive stages every two days from zoea I (ZI) up to decapodid II (DII) or juvenile (J). Two larval development pathways were indentified (Fig. 3.1.).

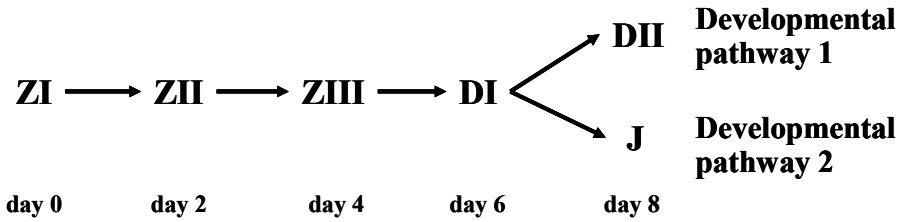

Fig. 3.1. *Palaemonetes varians* larval developmental pathways at 20ºC. Larval stages identified are: ZI – zoea I; ZII – zoea II; ZIII – zoea III; DI – decapodid I; DII – decapodid II; J – juvenile. Larvae hatched on the same day and are from a female collected from the field in 2007.

In both pathways, moult to decapodid II and metamorphosis to juvenile occurred on day eight from hatching. Larvae were first fed on day two, after moulting to zoea II, and it was possible to see an increase in dry weight up to decapodid I accompanied by a decrease in the C:N ratio (Fig. 3.2.; Appendix II, Table II.i.). After decapodid I and in both pathways (i.e. for decapodid II and juvenile), a loss in dry weight was observed but the C:N ratio remained constant. Dry weight and C:N ratio in either pathways were similar (Mann-Whitney U-test, DW: $U = 7.5$, $p = 0.885$; C:N ratio: $U = 5.0$, $p = 0.386$; Appendix II, Table II.ii.).

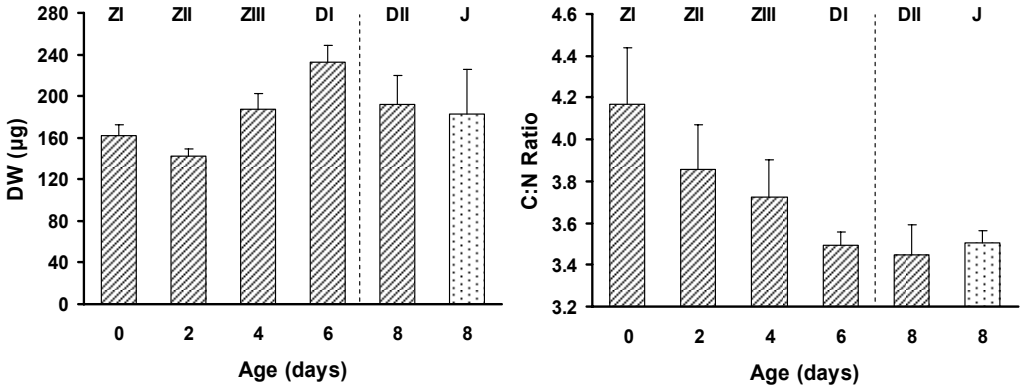

Fig. 3.2. Changes in dry weight (DW µg/individual) and C:N ratio during larval development of *Palaemonetes varians* at 20ºC, in the presence of food from day two ($x \pm$ S.D.). Larvae hatched on the same day and are from a female collected from the field in 2007. Above each error bar is the corresponding larval stage (ZI – zoea I, ZII – zoea II, ZIII – zoea III, DI – decapodid I, DII – decapodid II, J – juvenile). At day eight, two development pathways were identified (Fig. 3.1.): in developmental pathway *1)* larvae moult from DI to DII; in developmental pathway *2)* larvae metamorphosed from DI to juvenile, skipping DII stage (dotted bar). Four larvae were analysed in each stage, except for ZI (5 larvae) and DI (3 larvae).

3.4.2. Intraspecific variability

Dry weight and elemental composition analysis of newly hatched larvae from different females collected from the field in 2007 and 2008, revealed a high degree of variability between females (A-L) within the same year and between years (Fig. 3.3.; Appendix II, Table II.iii.). Statistically significant differences between females within the same year were found for dry weight, carbon and nitrogen contents, with non-significant differences for C:N ratio (nested ANOVA results: F=1.56, p=0.154; Table 3.1.). Newly hatched larvae from 2007 had higher dry weight and C:N ratio than larvae hatched in 2008 (Fig. 3.4.). Between years there were significant differences for all the parameters analysed (nested ANOVA results: DW F=74.610, p<0.001; C F=5.626, p=0.023; N F=14.137, p<0.001; C:N F=11.50, p=0.002; Table 3.1.).

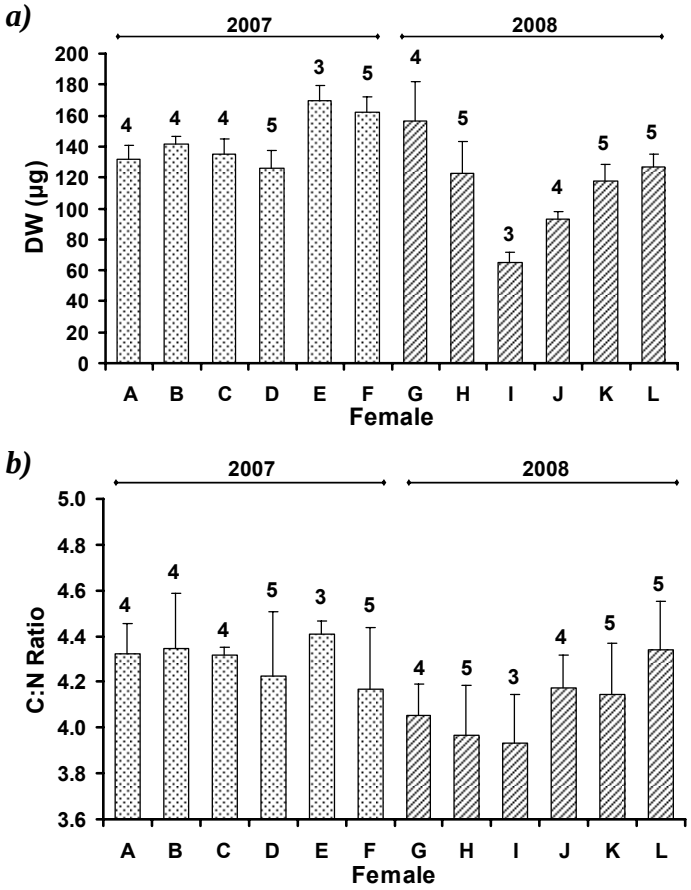

Fig. 3.3. Intraspecific variability in *a)* dry weight (DW µg/individual) and *b)* C:N ratio in newly hatched larvae of *Palaemonetes varians* in 2007 (A-F) and 2008 (G-L; x ± S.D.). Above each bar is the number of larvae analysed per female.

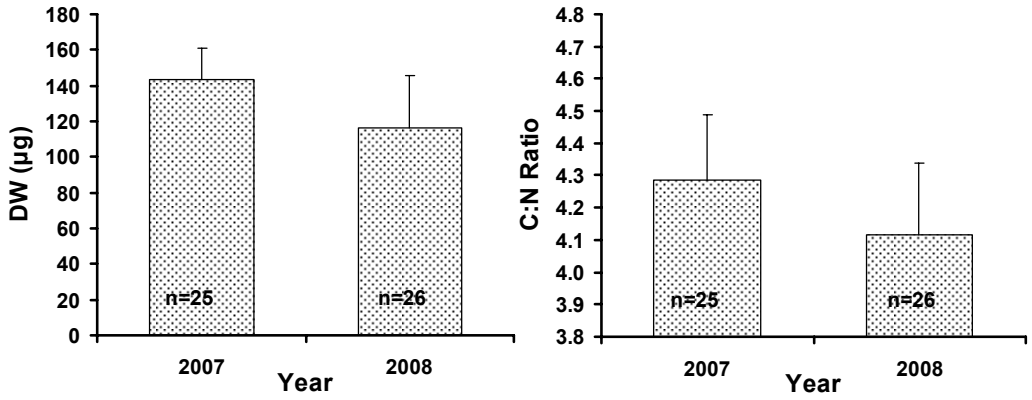

Fig. 3.4. Intra-year variability in dry weight (DW µg/individual) and C:N ratio in newly hatched larvae of *Palaemonetes varians* in 2007 and 2008 (x ± S.D.). On each bar is the number of larvae analysed for six females in each year.

Table 3.1. Nested ANOVA results on individual elemental composition of newly hatched larvae of *Palaemonetes varians* from different females grouped by years (2007 and 2008). DW = dry weight (µg/individual); C = carbon; N = nitrogen; df = degrees of freedom; SS = sum of squares; MS = mean of squares; F = ANOVA statistic. $p<0.05$ significantly different.

dependent variable	source of variation	df	SS	MS	*F*	*p-value*
DW (µg)	**year**	1	11557.40	11557.40	74.61	0.000
	female (year)	10	23347.80	2334.80	15.07	0.000
	error	39	6041.20	154.90		
	total	50	38475.90			
C (%)	**year**	1	71.74	71.74	5.63	0.023
	female (year)	10	2517.25	251.72	19.74	0.000
	error	39	497.38	12.75		
	total	50	3146.11			
N (%)	**year**	1	12.28	12.28	14.14	0.001
	female (year)	10	116.82	11.68	13.45	0.000
	error	39	33.88	0.87		
	total	50	167.35			
C:N Ratio	**year**	1	0.48	0.48	11.50	0.002
	female (year)	10	0.65	0.06	1.56	0.154
	error	39	1.62	0.04		
	total	50	2.65			

3.4.3. Preliminary results on temperature and pressure effects on survival

Experiment 1 – All larvae reared in the absence of light inside the small vials (6 mL), at atmospheric pressure, survived and successfully moulted to the zoea II larval stage. Of the larvae reared in cups (80 mL) with 12:12 hours light:dark rhythm, two died before moulting, one at 10ºC and another at 30ºC (Table 3.2.). The developmental time from hatching to moult to zoea II increased with decreasing temperature (30ºC two days; 20ºC three days; 10ºC seven days; Table 3.2.). The different rearing conditions (cups or vials) had no effect in the developmental time for any temperature tested.

Table 3.2. *Palaemonetes varians.* Duration of development (days) and percentage of survival of zoea I that successfully moulted to zoea II larval stage reared in cups (12:12 hours light:dark) and small vials (24 hours dark) at different temperatures. Larvae hatched on the same day and are from a female collected from the field in 2006. n = initial number of larvae per treatment.

Temperature (ºC)	cups (80 mL)			vials (6 mL)		
	age (days)	n	survival	age (days)	n	survival
10	7	10	90%	7	10	100%
20	3	10	100%	3	10	100%
30	2	10	90%	2	10	100%

Experiment 2 – At 20ºC the percentage of survival in one larva per vial treatment was higher (100% for both 100 and 200 atm, and 36% for 300 atm) when compared with that of rearing five larvae per vial (69% for 100 atm, 24% for 200 atm, and 0% for 300 atm; Fig. 3.5.; Appendix II, Table II.iv.). The larval stage at death was also identified: in the 5 larvae per vial treatment, at 100 atm two larvae were in zoea I stage and twelve in the zoea II stage, at 200 atm all larvae were at moult to zoea II, and at 300 atm thirty seven larvae were in zoea I and eight were in zoea I at pre-moult; in the 1 larva per vial treatment, at 300 atm five larvae were in zoea II, one was at moult and it was not possible to identify the stage of development

of other three. A decline in the percentage of survival with increasing pressure was observed in both treatments (Fig. 3.5.).

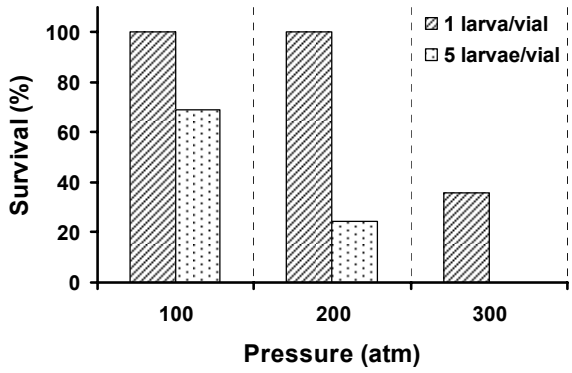

Fig. 3.5. Effect of number of larvae reared per vial at different pressures at 20ºC on the survival of zoea I that successfully moulted to zoea II of *Palaemonetes varians* (in 1 larva per vial treatments initial n=14; in 5 larvae per vial treatments initial n=45). Larvae hatched on the same day and are from a female collected from the field in 2006.

Experiment 3 – All larvae reared at atmospheric pressure (1 atm) at 20 and 30ºC survived and successfully moulted to the second zoeal stage (Fig. 3.6.; Appendix II, Table II.v.). There was a pronounced decrease in survival with an increase of pressure. At 300 atm, no larvae survived. Larvae reared at 5ºC died without moulting to the zoea II, in all the pressure treatments. The highest survivals were observed in larvae reared at 20ºC.

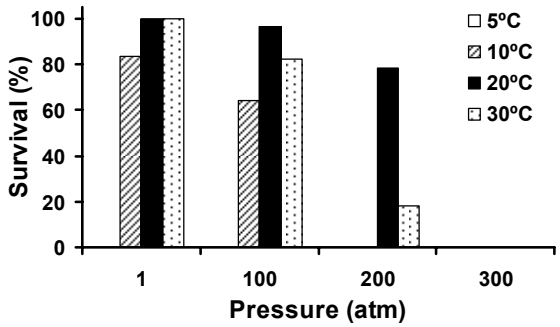

Fig. 3.6. Effect of temperature and pressure on the survival of zoea I that successfully moulted to zoea II of *Palaemonetes varians.* For the pressures 100, 200 and 300 atm the initial number of larvae (n) tested was 28 for each temperature treatment; for 1 atm the initial n was 12 for each temperature of 5, 15 and 20ºC and 18 larvae were tested for the 10ºC treatment. Larvae are from females collected from the field in 2006.

3.4.4. Temperature and pressure effects on survival and elemental composition

In general, there was an increase in mortality with increasing pressure for all temperature treatments, and the optimal temperature for successful development was 20ºC, registering higher survival rates. All larvae reared at atmospheric pressure and 20ºC survived and successfully moulted to the second larval stage, as did 50% of larvae reared at 30ºC (Table 3.3.). Two larvae died without moulting and three died after moulting at 30ºC/1 atm treatment. At 20ºC /100 atm treatment eight larvae survived and two were found dead, all in zoea II stage. All larvae died at 20ºC/200 atm, and it was possible to indentify four larvae in zoea II stage, while the other six larvae were damaged and identification was impossible. Only dead zoea I larvae were found in the 20ºC/300 atm treatment as well as at 30ºC for both 200 and 300 atm treatments. Most larvae incubated at 30ºC/100 atm were dead in zoea I stage, except two larvae that died during moult.

At atmospheric pressure, larvae had the lowest dry weight at 20ºC, and the C:N ratio decreased with increasing temperature (Fig. 3.7.; Appendix II, Table II.vi.). Dry weight was slightly higher in alive zoea II reared 100 atm than at 1 atm, at 20ºC, and there was no difference in the C:N ratio. At higher pressures, dead larvae had lower dry weights and higher C:N ratios. The highest C:N ratio and lower dry weight was for dead zoea I larvae reared at the maximum pressure tested at 20ºC.

Table 3.3. Effect of pressure and temperature on the survival of the zoea I that successfully moulted to zoea II larval stage of *Palaemonetes varians* (survival ZII), with account for the identified stages in dead larvae (dead in ZII stage; dead at moult; dead in zoea I stage – ZI; dead not determined – n.d.). Larvae are from two females collected from the field in 2007; larvae from female AA were assigned to 20ºC treatments, and larvae from female BB were used in 30ºC treatments. n = initial number of larvae per treatment.

temperature (ºC)	pressure (atm)	n	survival ZII	dead ZII	dead at moult	dead ZI	dead n.d.
20	1	10	100%	--	--	--	--
	100	10	80%	20%	--	--	--
	200	10	--	40%	--	--	60%
	300	10	--	--	--	100%	--
30	1	10	50%	30%	--	20%	--
	100	10	--	--	20%	60%	20%
	200	10	--	--	--	100%	--
	300	10	--	--	--	100%	--

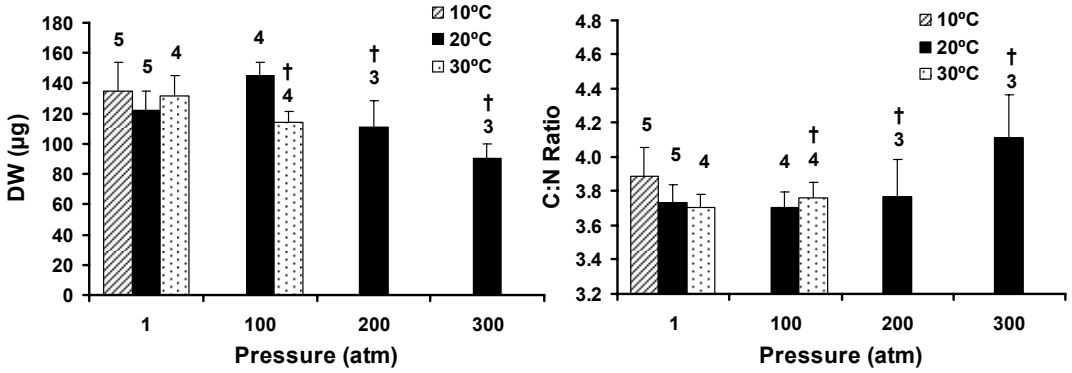

Fig. 3.7. Effect of pressure and temperature on the dry weight (DW µg/individual) and C:N ratio of *Palaemonetes varians* larvae (x ± S.D.). Values are for alive zoea II larval stage (10ºC; 20ºC at 1 and 100 atm; 30ºC at 1 atm), dead (†) zoea II (20ºC at 200 atm) and dead zoea I (20ºC at 300 atm; 30ºC at 100 atm). Above each error bar is the number of larvae analysed. Larvae hatched on the same day and are from two females collected from the field in 2007; larvae from female AA were used in 10 and 20ºC treatments, and larvae from female BB assigned to 30ºC treatments.

3.5. Discussion

3.5.1. Methodological considerations

Only the first larval stage was analysed for its tolerance of different temperatures and pressures, as the zoea I instar of *Palaemonetes varians* is facultative lecithotrophic (Yúfera & Rodríguez, 1985), which allows it to moult to the second zoeal stage in absence of food. This was an essential methodological feature of this study, as the pressure vessels used do not allow for any feeding or water exchange without depressurizing. In some of the preliminary pressure experiments the larvae used were randomly selected from different females, due to low fecundity in this species and low numbers of larvae release per night and female (<100). This may have added variability to the results and is ultimately related to the intraspecific variability found in specimens collected from the field. However, this procedure was considered necessary in order to have a minimum number of larvae per treatment that were starting at the same time.

When designing the experimental setup for the study of the effect of pressure on early larval development it was noticed that larvae would have to be reared in small volumes of seawater and in complete darkness, because of technical constraints

in the use of the pressure vessels. A preliminary control experiment revealed that these factors (absence of light and small volume of the vials) do not affect the first larval stage survival and successful moult to the second zoeal stage, when compared to the so-called normal rearing conditions of the cups, with 12:12 hours light:dark rhythm (Table 3.2.). Based on this first experiment, it was observed that lower temperatures increase the larval developmental time needed to reach the second zoeal stage (30ºC two days; 20ºC three days; 10ºC seven days; Table 3.2.). These timings set the baseline for the preliminary pressure experiments, as it is not possible to control developmental stage of larvae inside the pressure vessel without opening and consequently depressurizing it.

Different pressure vessels were used in the different treatments in order to reduce the variability in time of larval development at pressurization. Similarly, time and equipment constraints did not allow for true independence of data with the analysis of a single vial per pressure vessel. The underlying idea for studying the effect on survival of rearing five larvae per vial in comparison to one larva per vial was in order to assess the possibilities of increasing the sample number per pressure vessel. However, rearing five larvae per vial may have been an extreme value, given the high mortality when compared with that of rearing a single larva per vial (Fig. 3.5.). In the subsequent experiments, each vial contained a single larva and a maximum of fourteen larvae could be reared at a time in each pressure vessel. In the preliminary pressure experiments, two pressure vessels were used per treatment in order to increase the sample number. It was assumed that the use of different pressure vessels would not add variability to the results, although this was not investigated.

3.5.2. Larval development and intraspecific variability

Palaemonetes varians larval development is planktotrophic, but newly hatched larvae have sufficient energetic reserves of maternal origin, which allows them to survive and successfully moult to the second zoeal stage in absence of food (Heral & Saudray, 1979; Yúfera & Rodríguez, 1985). Resistance to starvation in the first larval stage enhances chances of survival in unpredictable environments (Wehrtmann,

1991; Thatje & Bacardit, 2000; Thatje et al., 2004a, b). Both larvae and adult palaeomonid shrimps tolerate a wide range of salinities (e.g. 3 to 80 in *P. varians*, Heral & Saudray, 1979; 1 to 25 in *P. argentinus*, Ituarte et al., 2008). A strong osmoregulation capability in both the larval and adult stages reflects little ecological changes from the larval to the adult habitats (Anger, 2001; Anger et al., 2008; Ituarte et al., 2008).

Females invest a sufficient amount of energy to produce larvae with some degree of lecithotrophy. Possible reasons for this are that: 1) the population habitat is unpredictable in terms of food availability; 2) females may move/migrate to a different location with a low food supply to release their larvae. In both cases it is assumed that larvae may drift away until they find food resources. After moulting to the zoea II stage the larvae probably feed more efficiently than as zoea I larvae. Within a highly unpredictable salt-marsh ecosystem, tidally-induced phytoplankton patchiness is likely to occur, with planktonic predators being found in areas of higher plankton biomass. If the small zoea I is released in a high plankton biomass area it will be more vulnerable to predators. In order to avoid predators, females may release their larvae in a less productive area within the salt marsh ("nursery areas"), possibly with extreme salinity or pH properties that both adults and larvae are likely to tolerate. Newly hatched larvae would then be able to complete development to the second zoeal stage in these locations and move on to food rich areas when they can feed, and swim more efficiently to escape predators, thereby increasing chances of survival.

Development of *P. varians* larvae at 20ºC revealed two developmental pathways (Fig. 3.1.) in larvae from the same female reared in the same constant conditions. Which pathway a particular larva follows may be related to individual differences in the feeding efficiency, with more efficiently feeding larvae having more energy and metamorphosing earlier into juvenile, by skipping a developmental stage (Giménez, 2006). Also, a reduction in larval moults reduces the energetic costs for larval development (Anger, 1998; Thatje 2003; Thatje et al., 2004a, b). From an ecological point of view, different developmental pathways reflect the flexibility of larval instars to adapt to changes in environmental parameters, like temperature, salinity or availability of food. This adaptability (or plasticity) will enhance larval

survival and facilitate the dispersion of larvae over greater distances (Thatje & Bacardit, 2000). It has been demonstrated that Caridea larvae reared in the laboratory may follow different developmental pathways from those developing in the wild. This is mostly due to the difficulty in reproducing the field conditions in aquaria, for example, providing an adequate type of food (Wehrtmann & Albornoz, 2003). Variability in life-cycle complexity through adaptability to the environment is likely to generate different levels of genetic and phenotypic variation (Thatje & Bacardit, 2000; Anger, 2001) in response to potentially disruptive selection pressures being experienced by different life stages.

There is significant intraspecific variability among females within the same year in respect to dry weight and the carbon and nitrogen content of offspring from the same population of *Palaemonetes varians* (Fig. 3.3.). However, the C:N ratio, which is a good indication of lipid:protein ratio, did not significantly differ. Thus, it is assumed that in each year all larvae from this population have a similar energy content, despite differences in dry weight. This is in agreement with the assumption that all individuals within the same population, exposed to the same environmental variables, produce offspring with a similar energetic content, in terms of carbon and nitrogen contents (Anger, 2001).

Newly hatched larvae from 2007 have significantly higher dry weights and C:N ratios than larvae hatched in 2008, reflecting interannual variation. Probably, more energy per female was allocated to reproduction in 2007, increasing the fitness of the first larval stage to develop independent of food (Fig. 3.4.). Facultative lecithotrophy of the first larval stage presents an advantage for less favourable years where adult females are unable to allocate more energy to reproduction. With fully functional mouthparts (Heral & Saudray, 1979) the newly hatched larvae may still thrive by starting to feed at an earlier stage. Differences between years may be explained by a higher water temperature during gametogenesis in 2007, as temperature affects energy allocation into reproduction (Fischer & Thatje, 2008). Variability in energy content between years are comparable to that of populations exposed to different environmental variables, since temperature or food availability/quality are known to affect the number of eggs produced, egg-size and egg energetic composition. These factors will influence the energy content of the

newly hatched larvae (Bernardo, 1996; Fischer & Thatje, 2008). Nevertheless, the presented hypotheses need further investigation and future work should involve larger sample sizes, taken in consecutive years. Future studies should also address the complete reproductive cycle: gametogenesis, fecundity of each individual female and the relation to carapace length, energy contents in embryos and newly hatched larvae, as well as measuring environmental parameters like salinity and temperature.

During larval development, there is a reduction in the C:N ratio, which is related to changes in the mode of nutrition (Fig. 3.2.). In the lecithotrophic stage (zoea I), the elemental composition of larvae is enriched with lipids of maternal origin, which deplete as larvae develop and moult to the second larval stage and start feeding. A decrease in C:N ratio is related to a decrease in lipid:protein ratio. Throughout larval development energy resources shift from a lipid to a more protein based nutrition, gradually relying upon less energy (lipids) of maternal origin (Anger, 2001). There is probably remaining energy from maternal origin up to the third zoeal stage. From the decapodid I instar onwards the C:N ratio remains constant, suggesting that energy gain in larvae is from feeding only. The dry weight increases during larval development up to the decapodid I stage, and decreases after metamorphosis to juvenile in pathway *2)* and after moult to decapodid II in pathway *1)* (Fig. 3.1.; Fig. 3.2.). The loss in weight is related to exuvial loss, which represents an important energy loss at this stage of development. In addition to the energy loss during the complex and energy demanding process of moulting, the larvae stop feeding at ecdysis (Anger, 2001).

3.5.3. Temperature and pressure tolerance

The differences found in larval survival when rearing 5 larvae per vial in comparison to 1 larva per vial points to oxygen limitation affecting both survival and successful development of larvae in this experimental design. Oxygen content measurements were not performed. However, they could improve the interpretation of the effects of pressure and temperature, by adding information on the metabolism of larvae. In this first pressure study performed at 20ºC (experiment 2), all larvae (in 1 larva per vial treatment) survived and successfully moulted to the second zoeal

stage up to a pressure equivalent of 2000 m of water depth, and 36% of larvae survived and develop at 300 atm (Fig. 3.5.). In the 5 larvae per vial treatment many of them were found dead after successful moult, and with increasing pressure less advanced stages were identified, which means that they are able to develop under pressure but then die, which is probably due to oxygen limitation. Hence, low oxygen availability together with increasing pressure causes a retarded development of larvae, similar to the effects of pressure observed in the embryo development of *Mytilus edulis* (Mestre et al., 2008).

The tolerance to high pressure is greater at 20ºC for survival and development of the first larval stage of *Palaemonetes varians* (Fig. 3.6.). The temperature tolerance window for successful development of this larval stage ranges from >10ºC up to at least 30ºC, at atmospheric pressure. The pressure tolerance is reduced at extremes of temperature tolerance and mortality increases with increasing pressure. In this preliminary experiment 3, no larvae survived to 300 atm, in contrast to what was found in experiment 2.

In order to evaluate the effect of pressure and temperature on the elemental composition of larvae, this temperature and pressure experiment was repeated in 2007 with larvae from females collected in the same location. In 2007, the development of zoea I to zoea II, took only two days at both 20 and 30ºC, and six days at 10ºC, in contrast to what was observed in 2006 (Table 3.2.). This may be associated with the previously reported variability in energetic contents at hatching between the 2007 and 2008 larvae (Fig. 3.4.). The larvae hatched in 2006 were probably more robust and able to survive and moult when exposed to higher pressures than those observed in 2007. Although the sample size per treatment was smaller in the 2007 experiment with a total of only 10 larvae, the lack of survival to pressures of 200 and 300 atm was unexpected. Unfortunately, no elemental composition data for newly hatched larvae in 2006 are available to confirm this hypothesis. However, a similar decline in survival with increasing pressure was observed, and a similar temperature tolerance window was detected.

The C:N ratio decreases with increasing temperature (Fig. 3.7.), which may be explained by a decrease in the lipid content of larvae as a result of their development,

as well as a higher metabolic demand at higher temperatures (Anger, 2001). Pressure does not seem to affect the C:N ratio or dry weight in living larvae that successfully moulted to the second zoeal stage (Fig. 3.7.). The high C:N ratios observed in dead larvae reared at 20ºC/300 atm is related to the developmental stage at death – zoea I, which is rich in lipids of maternal origin. In contrast, high C:N ratios can also be explained by a low content of proteins, which may degrade faster than lipids upon death. This could help to explain the low values in dry weight observed in dead larvae. Larvae were unfed in all treatments, and a decline in dry weight is thus expected until they start feeding. The decrease in weight is accompanied by a decrease in C:N ratio due to the consumption of lipids of maternal origin (Anger, 2001).

The natural habitat of *P. varians* is typically that of salt marsh ponds, and larvae are known to hatch in late spring to early summer when both atmospheric and seawater temperatures increase (Fincham, 1979). This explains the temperature tolerance window expressed by early larvae in this study. The salt marsh ponds are typically very shallow, rarely deeper than one meter, and it is thus highly surprising that larvae of *P. varians* are able to survive and develop to the second zoeal stage up to pressures of 300 atm. Despite some ambiguity in the pressure tolerance results between the different sets of experiments, all larvae could tolerate pressures equivalent to at least 1000 meters depth, within the depth range of hydrothermal vent sites.

3.5.4. Variability, adaptability and tolerance to high pressure

Palaemonetes varians is known as the variable shrimp because it exhibits a great variability in its morphology and developmental pathways (Fincham & Figueras, 1986). The physiological plasticity appears to be a characteristic of many palaemonid shrimp, which tolerate wide ranges of temperature, salinity, and oxygen concentrations, probably reflecting the natural variability in habitat conditions (e.g. Heral & Saudray, 1979; Nielsen & Hagerman, 1998; Anger, 2001; Brouwer et al., 2007; Ituarte et al., 2008). Thriving in such variable environments they need to be highly tolerant and able to adapt to environmental changes, a capability that is

believed to trigger phenotypic and genetic changes (Thatje & Bacardit, 2000; Anger, 2001).

While it is possible to understand the physiological plasticity as an adaptation to variable habitat conditions in temperature, salinity or hypoxia, the observed tolerance to high pressure is rather difficult to explain, as these shrimps are not exposed to great gradients of pressure. However, the cellular mechanisms involved in osmoregulation of salinity-induced changes observed in the congener species *P. argentinus* (Ituarte et al., 2008) may be the same as those used to counteract the effects of pressure. The activity of the enzyme Na^+/K^+-ATPase is involved in the osmoregulation of salinity-induced changes in *Palaemonetes argentinus* (Ituarte et al., 2008). The effects of pressure and temperature on Na^+/K^+-ATPase activity were studied in fish gill membrane preparations from shallow and deep-living marine teleosts (Gibbs & Somero, 1989). The enzyme activity in all species studied is conserved at physiological pressures, which may reflect a similar membrane physical state at the actual pressure the animal experiences. Deep-sea and cold-water species are less pressure-sensitive than shallow- and warm-adapted species, whose enzyme activity scales with increasing pressure (Gibbs & Somero, 1989). The authors argue that pressure-adapted enzymes exist in species whose habitat is deeper than 2000 m (Gibbs & Somero, 1989). Could this enzyme activity explain the observed high-pressure tolerance in *P. varians*? Could other euryhaline species also tolerate high-pressure? It has been suggested that osmoregulatory adaptations in decapod crustaceans appeared as consequence of evolutionary transitions from the sea to freshwater and land (e.g. Anger et al., 2008). Although rather speculative, this enzyme may have first evolved for osmoregulation due to pressure changes and then adapted to osmoregulate for salinity changes.

From an evolutionary perspective, the tolerance to high pressure shown in *P. varians* early ontogeny may be a trace adaptation expressed by an ancestor that occupied a greater bathymetric range. Moreover, the Palaemonidae express a monophyletic relationship with the alvinocarid hydrothermal vent shrimps that live at high hydrostatic pressures (Tokuda et al., 2006). Members of both families, live in highly variable habitats characterized by wide temperature gradients. Furthermore, in *P. varians*, lecithotrophy of the first larval stage accompanied with low temperatures

potentially increases the dispersal distance, as larvae can drift for at least seven days in the absence of food. A tolerance to pressure equivalent to 1000 m observed in *P. varians* brings this species within reach of many hydrothermal vent sites. These observations suggest that *P. varians* may have retained a relict high-pressure tolerance, probably from a common ancestor with the deep-sea hydrothermal vent shrimps.

High variability in larval developmental pathways is commonly found in low latitude carideans (Anger, 2001). This variability in life-cycle complexity through adaptation to environmental changes is likely to generate different levels of genetic and phenotypic variation (Thatje & Bacardit, 2000; Anger, 2001). Each life stage is subject to different selection pressures by a changing environment, which is responsible for a genetic drift in populations and ultimately to speciation by isolation or competition. In the presence of a warm isothermal water column (~65 Mya; e.g. Thomas et al., 2006) a species with a high variability in larval development and great adaptability to changes in the environment could extend its depth distribution, while at the same time keeping the high variability in larval developmental pathways associated with warmer temperatures. An adaptation to high-pressure at different stages of the life cycle could lead to a relatively rapid diversification in the genetic and phenotypic traits. Thus, shallow-water and hydrothermal vent shrimps may have originated by speciation through isolation after deep water masses cooled with the re-establishment of deep-sea water circulation patterns (~49 Mya; Thomas et al., 2006).

Chapter 4. Reproductive biology of the hydrothermal vent shrimps *Mirocaris fortunata* and *Rimicaris exoculata* from the Mid-Atlantic Ridge

4.1. Summary

Evolutionary adaptations to changes in the environment may be reflected in the reproductive traits of extant species. Likewise, differences in some of the reproductive traits between populations within the same biogeographic province may be associated to differences in habitat conditions. In this context, I analysed the reproductive biology of the hydrothermal vent shrimps *Mirocaris fortunata* Martin & Christiansen, 1995 and *Rimicaris exoculata* Williams & Rona, 1986 from two distinct hydrothermal vent sites on the mid-Atlantic ridge (MAR), Lucky Strike (37°17'N, 32°16'W) and Rainbow (36°13'N, 33°54'W).

The variety in individual maturity stages found in male gonads (histology) of *M. fortunata* from Lucky Strike, as well as the wide range of individual oocyte size-frequency distributions in females from the same population (direct measurement of dissected oocytes), is consistent with asynchronous gametogenesis, as previously proposed for this species. A similar pattern was found in *R. exoculata* from Rainbow. The population size distribution analysis of *M. fortunata* revealed significant differences in the carapace length (CL) between females and males from Lucky Strike. *M. fortunata* females from the Lucky Strike population have significantly higher total lengths and CL than females collected from Rainbow. This may reflect different habitat conditions between sites in terms of food availability. It is suggested that adaptive maternal effects in response to changes in environmental conditions are likely to induce changes in offspring phenotype. In this sense, it would be possible to find differences in fecundity, size and quality of eggs and consequently differences in larval fitness among populations between sites. Larval dispersal potential, recruitment and resilience of hydrothermal vent populations are also discussed.

Keywords: *Mirocaris fortunata*, *Rimicaris exoculata*, hydrothermal vents, mid-Atlantic ridge, reproduction, maternal effects.

4.2. Introduction

The study of the temporal aspects of reproduction of hydrothermal vent species and the physico-chemical parameters that affect their reproductive traits are important for understanding their population dynamics, and the principals underlying the establishment and maintenance of vent populations. Sampling of populations all year around is not feasible at present, and the physico-chemical conditions at hydrothermal vents are very difficult to reproduce under artificial conditions. As a result, the study of the life history biology of hydrothermal vent species is a very difficult task (Tyler & Young, 1999; Van Dover, 2000). Some hydrothermal vent species reproduce seasonally, but there is no apparent need for a seasonally enhanced food supply to promote reproduction, as these ecosystems are highly productive. On the other hand, the lack of knowledge of the whole life cycle may hide an early life stage dependent on seasonality (Tyler & Young, 1999; Van Dover, 2000; Young, 2003). There is gathering evidence that the life histories of vent organisms are very similar to those of intertidal analogues. Growth is rapid and reproductive maturity is quickly attained, even in small individuals of large macrofauna species. Those traits reflect metabolic and life-history adaptations that favour life in transient and insular habitats (Grassle, 1985; Tunniclife, 1991).

More than 125 species representing 33 families of decapods have been reported from the vicinity of hydrothermal vents and cold seeps (Martin & Haney, 2005; Desbruyères et al., 2006). Of these, the shrimps of the family Alvinocarididae Christoffersen, 1986 and the crabs of the family Bythograeidae Williams, 1980 are obvious endemics, known only from vent or seep sites and presumably restricted to them (Martin & Haney, 2005). The caridean shrimps are among the most common organisms found at hydrothermal vents especially in the Atlantic. All vent-endemic shrimps, most of which were originally treated as members of the family Bresiliidae, are now treated as members of the family Alvinocarididae (Martin & Haney, 2005). The currently recognized vent-associated species of shrimp belong to six genera: *Alvinocaris*, *Chorocaris*, *Mirocaris*, *Nautilocaris*, *Opaepele* and *Rimicaris* (Martin & Haney, 2005). Two species of the genus *Mirocaris* Vereshchaka, 1996 have been described to date, one from the mid-Atlantic ridge (MAR), *Mirocaris fortunata* Martin & Christiansen, 1995, and another recently described species from the central

Indian ocean, *Mirocaris indica* Komai, Martin, Zala, Tsuchida & Hashimoto, 2006. *Mirocaris fortunata* has been found at Menez Gwen, Lucky Strike, Rainbow, Broken Spur, TAG (Trans Atlantic Geotraverse), Snake Pit, and Logatchev vent fields, along the mid-Atlantic ridge between 38ºN and 14ºN and 840-3650 m of depth range. The distribution pattern of the genus *Rimicaris* Williams & Rona, 1986 is nearly identical to that of *Mirocaris*. *Rimicaris exoculata* Williams & Rona, 1986 is known from hydrothermal vents in the mid-Atlantic ridge between 37ºN and 4ºS, present at Lucky Strike (although it was only reported once; Van Dover et al., 1996), Rainbow, TAG, Snake Pit, Broken Spur, Logatchev, Ashadze, and Mephisto sites, within a depth range of 1700-4088 m (Martin & Haney, 2005; Komai & Segonzac, 2008). The other species of the same genus, *Rimicaris karei* Watabe & Hashimoto, 2002, has been found at Karei and Edmond vent fields in the central Indian Ocean (2415-3320 m depth; Martin & Haney, 2005; Komai & Segonzac, 2008). Komai and Segonzac (2008) argue that this distributional pattern would suggest that these taxa are relicts, but these authors are aware of the work by Shank and colleagues (1999) that indicates that vent shrimps comprise the youngest vent- and seep-associated diversification observed to date.

The hydrothermal vent habitats found among the mid-Atlantic ridge differ mainly in their fluid chemistry and mineral deposit pattern, both of which are influenced by water depth (hydrostatic pressure) and the geology of source rocks. The Lucky Strike hydrothermal vent field, located at an average depth of 1690 m, was discovered in 1993 (Langmuir et al., 1993) and has basalt as source rock. In this vent field the temperatures of vent fluid at point sources range between 152 and 333ºC, and the fluid characteristics vary from site to site within the field (Charlou et al., 2000; Desbruyères et al., 2000). At Lucky Strike, the fauna is dominated by dense mussel beds of *Bathymodiolus azoricus* that cover most of the active sulphide structures. The commensal polychaete worm *Branchipolynoe seepensis* is also very abundant, and among the shrimp species *Mirocaris fortunata* is the most common (Van Dover et al., 1996; Desbruyères et al., 2000, 2001). Rainbow was discovered in 1997 (Fouquet et al., 1997) and it is located at about 2300 m deep in ultramafic rocks, with vent fluids reaching temperatures up to 360°C. The fauna is dominated by swarms of *Rimicaris exoculata* over the walls of active chimneys, where *Mirocaris fortunata* is also present. *Bathymodiolus azoricus* are found in the

surrounding blocks within the active area (Desbruyères et al., 2000, 2001). When compared with Lucky Strike, the vent fluids at Rainbow are depleted in hydrogen sulphide and enriched in dissolved methane, hydrogen, and metals (e.g. Fe, Cu, Zn, Mn), what is attributed to differences in the source rocks and bathymetric position between fields (Charlou et al., 1997, 2002; Donval et al., 1997; Douville et al., 1997, 2002; Langmuir et al., 1997; Von Damm et al., 1998; Desbruyères et al., 2000, 2001; Schmidt et al., 2008a). It is important to recognize that both sites are located in oligotrophic zones of the mid-Atlantic ridge (Khripounoff et al., 2008).

Mirocaris fortunata is known to have a rather opportunistic distribution across the whole gradient of vent chimney walls, forming aggregations on mussel free areas in the immediate vicinity of hydrothermal fluid exits (Desbruyères et al., 2001). Studies on stomach contents, lipids and stable isotopes indicate that *M. fortunata* is mixotroph, feeding not only on other animals but also on sulphur-oxidizing bacteria (Van Dover et al., 1988, 1996; Segonzac et al., 1993; Pond et al., 1997c, 2000; Polz et al., 1998; Gebruk et al., 2000; Colaço et al., 2002a, 2007). In addition, there is evidence that juveniles may also rely on photosynthetic-derived material for nutrition (Pond et al., 1997c, 2000; Gebruk et al., 2000; Colaço et al., 2002a). Among the known vent shrimps, *Rimicaris exoculata* is the most specialized for the life in these chemosynthetic environments (e.g. Segonzac et al., 1993; Van Dover, 1995). It exhibits morphological and behavioural adaptations to the vent habitat, and adults' nutrition is based on the epibiotic bacteria they harvest on the branchial cavity (Gebruk et al., 1993; Segonzac et al., 1993). *R. exoculata* may harbour highly diversified epibiotic bacteria at different vent sites, depending on the hydrothermal fluid chemistry. High density swarms are found in a thermal range of 3-18ºC on the vent chimney walls (Schmidt et al., 2008b).

The gametogenic process of deep-sea crustaceans appears identical to that of their shallow-water relatives (Young, 2003). Spermatozoa of all crustaceans are aflagellate, modified for internal fertilization and are often packaged into sperm packets, which are deposited in the oviducts of the female and sperm plugs (Young, 2003). The gonads of decapod crustaceans lie above the stomach within the cephalothorax and extrude mature gametes through gonopores on or near the bases of the pereiopods. Most crustaceans brood their embryos attached to the pleopods on

the underside of the abdomen, either to the juvenile stage or to a larval stage (Young, 2003). Among the various caridean shrimps and other Crustacea common at hydrothermal vents there is little evidence for periodic reproduction (Tyler & Young, 1999).

Studies on reproduction of hydrothermal vent shrimps suggest that the process of gametogenesis is very similar to that of closely-related non-vent species, while the fecundity is higher, probably as a consequence of living in a rich environment with a continuous supply of energy (Tyler & Young, 1999; Ramirez-Llodra, 2000; Ramirez-Llodra et al., 2000). Variability in maturity stages between individuals in samples taken at the same time indicates asynchronous gametogenesis for both *Mirocaris fortunata* and *Rimicaris exoculata* (Ramirez-Llodra et al., 2000). However, the presence of multiple cohorts of oocytes was noted in the gonads of individuals of *Rimicaris exoculata*, suggesting periodicity at the individual level (Copley, 1998; Ramirez-Llodra et al., 2000). In addition, the paucity of ovigerous *R. exoculata* in single-time samples could be explained by seasonal reproduction, but this was found in both summer and autumn samples (Gebruk et al., 1997; Copley, 1998; Ramirez-Llodra et al., 2000; Copley et al., 2007). It is thought that the scarcity of ovigerous specimens may result from their spatial distributional pattern, with ovigerous females of *R. exoculata* moving from the swarms around high temperature vents to the periphery, which is rarely sampled (Ramirez-Llodra et al., 2000; Copley et al., 2007). *Mirocaris fortunata* has highly variable fecundity (25-503 eggs per female) which is much lower than the fecundity found in the single ovigerous female of *Rimicaris exoculata* analysed to date (988 eggs; Ramirez-Llodra et al., 2000).

The scarce data available, however, already indicate that the patterns of development of early larvae are very similar to those of shallow-water relatives (Young, 2003). Studies on the lipid composition of adults and juveniles of *R. exoculata* revealed the presence of photosynthetically derived energetic reserves in the latter. This suggests that larvae feed on phythodetritus that is produced in the euphotic zone (Pond et al., 1997a, b, c; Allen Copley et al., 1998; Allen et al., 2001). Postlarvae ascribed to vent shrimps offspring have been captured more than 100 km away from the hydrothermal vent areas, suggesting that these larvae have a large dispersal potential and a long planktonic phase (Herring & Dixon, 1998). The

presence of embryos of *R. exoculata* was also reported from plankton samples obtained from the hydrothermal vent plume of Broken Spur (Vereschaka, personal communication *in* Tyler & Young, 1999). This raised some controversy regarding the accurate identification of the embryos, because all carideans are known to brood their embryos and this would be a novelty in the Caridea. On the other hand, this observation would help to explain the absence of ovigerous females from the vents (Young, 2003).

Tyler and Dixon (2000) studied the pressure and temperature tolerances of zoea I larvae of *Mirocaris fortunata* that hatched at atmospheric pressure, obtained from sampled berried females, in order to determine the vertical potential for dispersal. The results suggested that larvae of *M. fortunata* could ascend the water column up to the permanent thermocline (~200 m) but are not able to survive at surface waters conditions (1 atm at 20ºC). High levels of gene flow in *Rimicaris exoculata* were found between TAG, Broken Spur and Snake Pit (Creasey et al., 1996; Shank et al., 1998b). These authors postulated the existence of a large single pool of larvae to which all the populations from these hydrothermal vents are contributing and at the same time recruiting individuals (Tyler & Young, 2003).

Evolutionary adaptations to changes in the environment may be illustrated in the reproductive traits of extant species. Likewise, reproductive differences between populations within the same biogeographic province may be associated with differences in habitat conditions, in this case between hydrothermal vent sites. In this context I analysed the reproductive biology of the hydrothermal vent shrimps, *Mirocaris fortunata* and *Rimicaris exoculata*, from Lucky Strike (37°17'N, 32°16'W) and Rainbow (36°13'N, 33°54'W), mid-Atlantic ridge.

4.3. Material and Methods

A sample of 39 individuals of *Mirocaris fortunata* was collected from the Eiffel Tower site (37°17.20'N, 32°16.20'W), in the Lucky Strike hydrothermal vent field, during the EXOMAR cruise in August 2005. In August 2006, during the MOMARETO cruise, a sample of 39 individuals of *M. fortunata* and a sample of 20

individuals of *Rimicaris exoculata* were collected from the Rainbow hydrothermal vent field (36°13.44'N, 33°54.10'W). In both cruises, samples were retrieved using the slurp gun attached to the robot arm of the ROV (Remotely Operated Vehicle) Victor 6000 (BIOCEAN - Ifremer, 2005, 2006).

After collection, all specimens were preserved in 4% buffered seawater formaldehyde and later transferred to 75% propan-2-ol. Male shrimp were distinguished from females by the presence of an asymmetrical mesial extension on the endopod of pleopod 1 and the presence of appendix masculina on pleopod 2 (Williams & Rona, 1986; Williams, 1988). The carapace length (CL) and total length (TL) of *Mirocaris fortunata* specimens were measured from the base of the rostrum between the eyes to the posterior dorsal margin of the carapace, and to the posterior margin of the telson, respectively, to the nearest 1mm using a Vernier calliper.

Direct measurement of oocytes individually dissected from ovaries was used, as it is suggested to be a more accurate method to determine oocyte sizes (Copley et al., 2007) compared to the measurement of cross-sectional areas of oocytes in histological sections (Ramirez-Llodra et al., 2000). When well developed, oocytes occupy a great part of the carapace cavity and often are visible from the exterior through the slightly transparent carapace. In order to remove and measure the oocytes, ovaries were carefully dissected from females under a Leica MZ8 stereomicroscope. The oocytes were measured by placing the cells flat on a Petri dish, under a stereomicroscope, and images were captured using a JVC TK-1280E video camera connected to a PC via a Matrox Rainbow Runner video card. Images were analysed using the image analysis software Jandel SigmaScan Pro to calculate the feret diameter of oocytes, which represents the diameter of a hypothetical circle of equal area to the object measured. Spatial calibration was provided by capturing images of a graticule slide at identical magnification to the oocyte images (Copley et al., 2007). Whenever possible at least 80 oocytes per female were measured.

Twelve male specimens of *Mirocaris fortunata* were dissected and the gonads kept in 75 % propan-2-ol until processed for histology. Gonad tissues were dehydrated in graded alcohols; cleared in Histoclear; impregnated and embedded in paraffin wax; sections cut at 5 μm and mounted on slides; slides were dried and

stained with Mayer's haemalum and Eosin (Ramirez-Llodra et al., 2000). The maturity state of the developing gametes was assessed by examining the microscopic thin-sections under an Olympus BH-2 compound microscope.

The oocyte size measurements were grouped into 25 μm classes in order to determine the oocyte-size frequency distribution for each individual. Statistical differences in total length and carapace length sizes between sexes and between sampling sites were tested by means of a Mann-Whitney U-test (Sokal & Rohlf, 1995).

4.4. Results

4.4.1. Reproductive biology of *Mirocaris fortunata* from Lucky Strike and Rainbow

The samples size available was small, constraining the interpretation of the results, but still gives an idea of the population size distribution at both sites. The sample of 39 individuals of *Mirocaris fortunata* from Lucky Strike (2005) comprised 27 females and 12 males. Total length (TL) ranged from 15 to 31 mm in females and 15 to 25 mm in males (Fig. 4.1.; Table 4.1.; Appendix III, Table III.i.). The median total length was 23 mm in females and 19.5 mm in males, with no significant differences between sexes in this sample from Lucky Strike (Mann-Whitney U-test, U = 102.5, p = 0.070; Appendix III, Table III.ii.). In contrast, females have significantly larger carapace lengths (CL) than males (Fig. 4.2.; Mann-Whitney U-test, U = 79.0, p = 0.012; Appendix III, Table III.ii.). The three ovigerous females present in this sample had total lengths ranging from 19 to 24 mm and a carapace length range of 6-7 mm.

Twenty three females and sixteen males where found in the sample of *M. fortunata* collected from Rainbow in 2006. Males have higher median total length (20.5 mm) than females (18 mm), with equal maximum sizes (24 mm) and no significant difference between sexes (Mann-Whitney U-test, U = 183.5, p = 0.989; Fig. 4.1.; Appendix III, Table III.iii and Table III.iv.). Similarly, no differences in carapace lengths between sexes were found (Mann-Whitney U-test, U = 183.0, p =

0.977; Appendix III, Table III.iv.), with very similar size frequency distributions for both males and females (Fig. 4.2.).

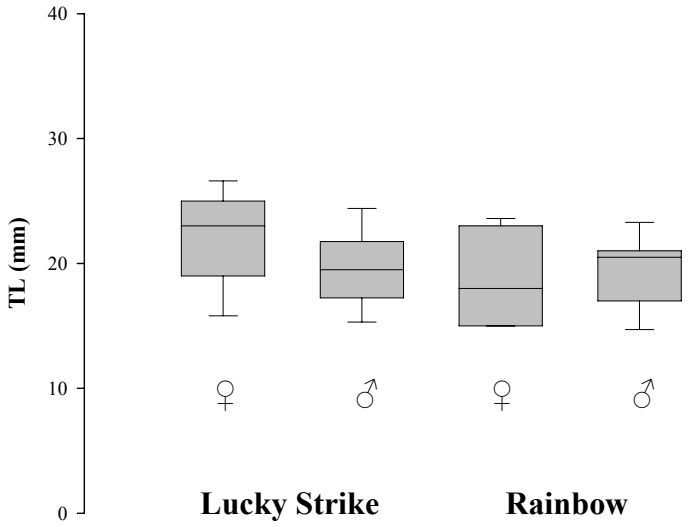

Fig. 4.1. Total length (TL) frequency distributions of *Mirocaris fortunata* from Lucky Strike (females N=27; males N=12) and Rainbow (females N=23; males N=16). Boxes show median, lower and upper quartiles; whiskers denote 5- and 95- percentiles.

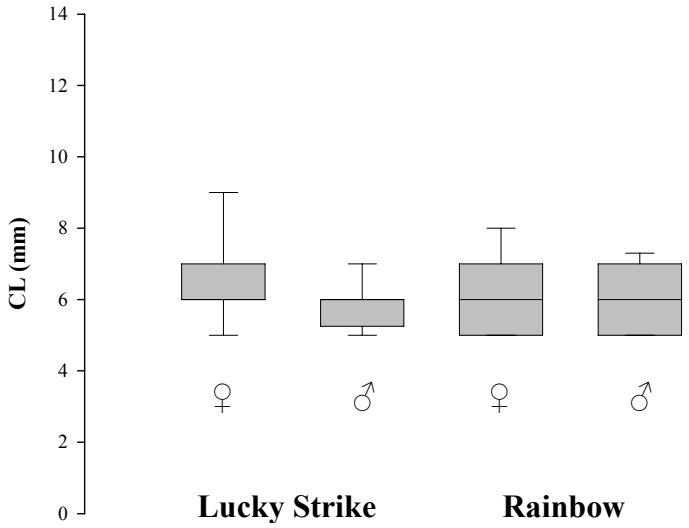

Fig. 4.2. Carapace length (CL) frequency distributions of *Mirocaris fortunata* from Lucky Strike (females N=27; males N=12) and Rainbow (females N=23; males N=16). Boxes show median, lower and upper quartiles; whiskers denote 5- and 95- percentiles.

When comparing total lengths and carapace lengths in males from both samples, none of these parameters show significant differences between sites (Mann-Whitney U-test, TL: $U = 95.5$, $p = 0.981$; CL: $U = 80.5$, $p = 0.472$; Appendix III, Table III.v.). But females from Lucky Strike (2005) have significantly higher total lengths and carapace lengths than females collected from Rainbow in 2006 (Mann-Whitney U-test, TL: $U = 172.5$, $p = 0.007$; CL: $U = 207.5$, $p = 0.045$; Appendix III, Table III.v.).

Three ovigerous females occurred in a sample of 39 specimens collected from Lucky Strike in August 2005, but the egg sizes or fecundity per female were not analysed as only part of the eggs were still attached to the pleopods. Non-ovigerous *Mirocaris fortunata* show a wide range of oocyte size-frequency distributions for individual shrimp in this single sample (Fig. 4.3.; Appendix III, Fig. III.i.). Oocyte size-frequency distributions in this sample varied from 86-252 µm to 420-841 µm (female 1 and female 12 respectively, Fig. 4.3.; Appendix III, Fig. III.i. *A*). There is no linear relation between oocyte size and carapace length.

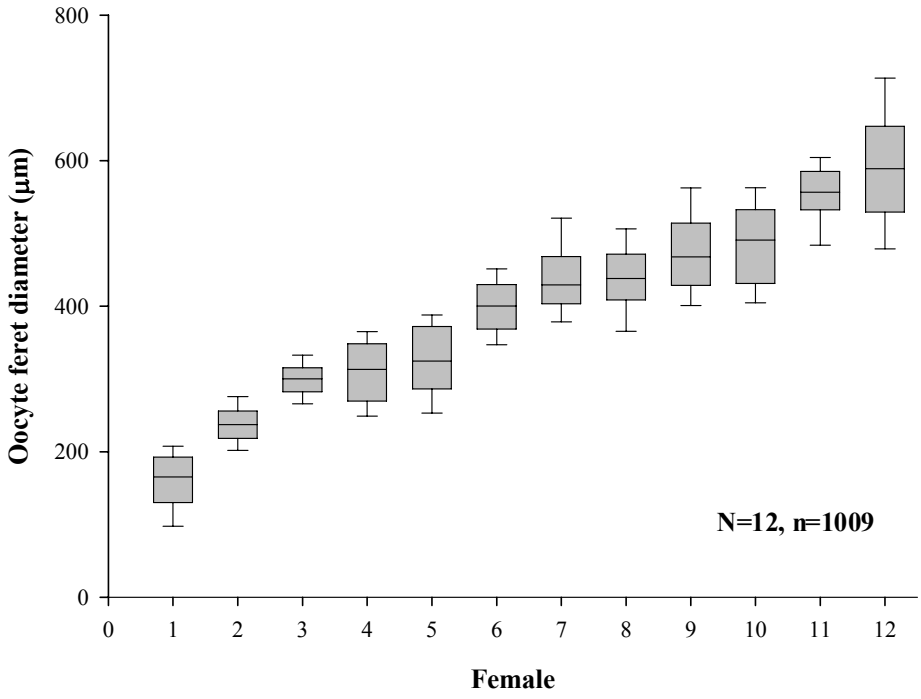

Fig. 4.3. Oocyte size-frequency distributions of individual *Mirocaris fortunata* collected from the Lucky Strike in August 2005. Boxes show median, lower and upper quartiles; whiskers denote 5- and 95-percentiles. Oocyte size-frequency distributions of individual shrimp are presented in order of increasing median values. N=number of females; n=total number of oocytes.

In male *Mirocaris fortunata* it was possible to distinguish different stages of the gonad development in different individuals. The criteria used to distinguish between stages were: morphology of the developing male gametes, round to elongated/flattened shape (less to more developed); position of gametes in the features identified as *vas deferens* (less the distance to the lumen, more developed); and also the presence/abundance of gametes in the spermatoducts (higher the abundance, higher the developmental stage). Using the criteria outlined above, it was possible to classify male individuals, from less to more developed, as: immature (n=2); intermediate (+ and ++) (n=6); intermediate to mature (n=2); mature (n=2; Table 4.1.). There is no linear relation between maturity stage and carapace length.

Table 4.1. Stage of gonad development in individual males of *Mirocaris fortunata* (Mf) collected from Lucky Strike (LS) in 2005. (TL = total length; CL = carapace length; intermediate ++ stands for intermediate to mature; intermediate+ stands between intermediate and intermediate to mature).

Sample ID ♂	TL (mm)	CL (mm)	Maturity stage
Mf LS A	25	7	Mature
Mf LS B	20	6	Intermediate
Mf LS C	22	7	Intermediate+
Mf LS D	16	5	Mature
Mf LS E	21	6	Intermediate+
Mf LS F	18	6	Intermediate
Mf LS G	17	5	Intermediate++
Mf LS H	20	6	Intermediate++
Mf LS I	19	6	Intermediate
Mf LS J	19	6	Immature
Mf LS K	15	5	Immature
Mf LS L	23	6	Intermediate

4.4.2. Reproductive biology of *Rimicaris exoculata* from Rainbow

No males or ovigerous females occurred in this small sample of 20 specimens collected from Rainbow in August 2006. *Rimicaris exoculata* showed a relatively wide range of oocyte size-frequency distributions of individual shrimp ranging from 129-199 μm and 261-470 μm with one individual with larger oocyte sizes and distinct from the other females (376-525 μm, female 20; Fig. 4.4.; Appendix III, Fig.

III.ii.). These twenty females have a mean oocyte feret diameter size of 273.69 ± 71.9 µm.

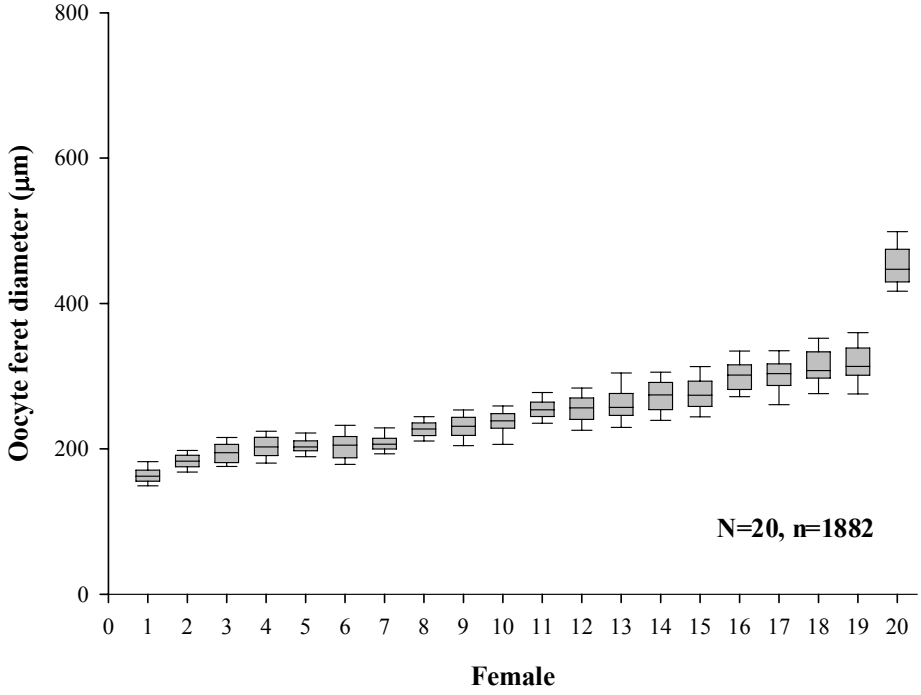

Fig. 4.4. Oocyte size-frequency distributions of individual *Rimicaris exoculata* collected from Rainbow in August 2006. Boxes show median, lower and upper quartiles; whiskers denote 5- and 95-percentiles. Oocyte size-frequency distributions of individual shrimp are presented in order of increasing median values. N=number of females; n=total number of oocytes.

4.5. Discussion

4.5.1. Contrasting vent habitats and population size structure in *Mirocaris fortunata*

To date, there is no study comparing the population size structure of *Mirocaris fortunata* from different vent sites. This is most probably related to sampling restrictions inherent in the study of deep-sea hydrothermal vent fauna. The few data available on total and carapace lengths in the literature appear as accompanying observations to taxonomic descriptions (Komai & Segonzac, 2003; Desbruyères et al, 2006), reproductive biology studies (Ramirez-Llodra, 2000; Ramirez-Llodra et

al., 2000) or biochemical markers studies (Pond et al., 1997c; Gebruk et al., 2000). In the present study, the sample size of *M. fortunata* from both sites is too small for an adequate comparison between sites, but it reflects previously reported size ranges for this species in populations from Lucky Strike and Rainbow. For instance, in a sample collected from Lucky Strike in June 1994 the total length ranged from 17 to 26 mm (Pond et al., 1997c), and in July 1997 it was 17-31 mm (n = 5; Gebruk et al., 2000); in both works there is no distinction between sexes. The carapace length is a parameter usually mentioned in the description of shrimp species, for *Mirocaris fortunata* females are characterized with CL ranging 3.3-8.7 mm and males 3.8-7.2 mm (Desbruyères et al, 2006). Although, females with larger carapace length (ranging from 6.0 to 10.7 mm) have been previously reported from Lucky Strike, in July 2001 (Komai & Segonzac, 2003).

The present results show that females in the sample from Lucky Strike are significantly larger than females in the sample from Rainbow. This could reflect differences in the microenvironment and community composition between the vent fields, or different chemical and temperature gradients between sampling locations, within the vent structure. *Mirocaris fortunata* is known to have a rather opportunistic distribution across the whole gradient of the vent chimney walls, and to form aggregations on mussel free areas in the immediacy of hydrothermal fluid exits (Desbruyères et al., 2001). This gradient also may select for different size classes of shrimps, as it is known to influence the spatial distribution of different size classes of *Bathymodiolus azoricus* from Lucky Strike (Desbruyères et al., 2001). However, this would not apply to males of *M. fortunata* given the similarity between size distributions in both vent fields. Although hydrothermal vents are seen as unlimited source of energy, there could be some degree of energetic limitation in Rainbow when compared to Lucky Strike. Lipid biomarkers and stable isotopes studies suggest that the main food source of *M. fortunata* is the chemoautotrophic bacteria, obtained either by ingestion of a mix of mussel/bacterial detritus or by grazing directly on bacterial mats (Van Dover et al., 1988, 1996; Segonzac et al., 1993; Pond et al., 1997c, 2000; Polz et al., 1998; Gebruk et al., 2000; Colaço et al., 2002a). Furthermore, it is suggested that differences in isotope signatures found between hydrothermal vent sites are associated with the end member fluid concentration in hydrogen sulphide and methane (Gebruk et al., 2000; Colaço et al., 2002a, 2002b).

This may have a direct influence on the relative abundance of different types of chemosynthetic bacteria. In contrast to Rainbow, in Lucky Strike there are extensive bacterial mats covering the vent structures and the dense mussel beds (personal observation). A higher biomass of mussels or bacterial mats may provided an enriched food source at Lucky Strike when compared to Rainbow, which could explain the presence of larger females of *M. fortunata* in the former. Having enhanced quality or quantity of food available, these females may allocate more energy for growth without limiting the energy necessary for reproduction. The present study suggests that the differences in population size distribution of *M. fortunata* may reflect different habitat conditions between Lucky Strike and Rainbow, promoted either by physico-chemical factors (pressure, temperature, or chemical composition of fluids) or biotic factors, such as interspecific competition or quality of food. Nevertheless, samples from Lucky Strike and Rainbow were taken in different years compromising any further interpretations.

4.5.2. Reproductive biology of *Mirocaris fortunata*

There is no evidence of linear relation between total length and maturity stage in *Mirocaris fortunata* collected from Lucky Strike. Only twelve individuals were analysed from this sample; maturity in males is achieved at least at a total length of 16 mm and carapace length of 5 mm. Despite the small sample size, the variety in individual maturity stages found in males (Table 4.1.) and the wide range of individual oocyte size-frequency distributions in females (Fig. 4.3.; Appendix III, Fig. III.i) suggests that reproduction in this population is continuous. These results are consistent with asynchronous gametogenesis, as previously proposed for this species (Ramirez-Llodra et al., 2000). The present results do not show periodic production of oocytes in individual females, in contrast to what has been previously observed (Ramirez-Llodra et al., 2000). However, this is most likely related to the different methods used to determine oocyte sizes. The oocytes measured in the present study are probably only vitellogenic oocytes, given their large size (>100 μm), and in consequence no information is available on the presence or not of previtellogenic oocytes. Hence, it is not possible to confirm the presence of multiple

cohorts of developing oocytes, neither the periodicity in the production of oocytes at the individual level. It is assumed that the population of *M. fortunata* from Rainbow follows a similar reproductive pattern, but unfortunately no data are available at present to proof for any differences.

4.5.3. Reproductive biology of *Rimicaris exoculata*

In the single sample of *Rimicaris exoculata* from Rainbow, obtained in August 2006, the relatively wide range (129-199 μm to 376-525 μm) of individual oocyte size-frequency distributions (Fig. 4.4.; Appendix III, Fig. III.ii.) suggests that different females are in different maturity stages at time of collection. This is a characteristic of asynchronous gametogenesis, as proposed for this species (Ramirez-Llodra et al., 2000), suggesting that reproduction does not reflect any seasonal pattern, i.e., the population generates offspring continuously throughout the year.

There is one individual that has a higher mean value of oocyte size (female 20, Fig. 4.4.), and is thus in a more advanced maturity stage than the rest of the individuals in this sample. This pattern may be a sign of: 1) a higher mortality in mature females and they are less frequent; 2) female 20 belongs to a distinct group of individuals probably situated in a different location within the vent structure than that of where sampling took place.

The first situation, would not justify the highly abundant population of *Rimicaris exoculata*, but is in agreement with previously reported paucity in highly mature females or ovigerous females in previous samples. The second case scenario, assumes that all samples of *R. exoculata* reporting a reduced number of mature females were due to an artefact in the sampling method. Samples are obtained using the slurp gun attached to the arm of the ROV, and thus selective. This collection method usually targets the densest agglomerations of shrimp, and may therefore exclude part of the population. Shrimps at different maturity stages may be distributed along a temperature and chemical gradient over the hydrothermal vent structure, which is believed to influence the distribution of mussels of different size classes (Desbruyères et al., 2001). In addition, juveniles of *R. exoculata* from

Rainbow have been reported from colder zones, away from high temperature vent flow and black smoke emission (Gebruk et al., 2000), and rarely occur in samples of the largest swarms. In these shrimp swarms competition for space is visible in video footages, with shrimps constantly moving trying to get closer to the vent walls. The chemicals released in the vent fluid are used by the epibiotic bacteria present in the carapace of *R. exoculata* and constitute the base of their nutrition (e.g. Segonzac et al., 1993; Zbinden et al., 2008). This competition for space may as well select for different distributions in size classes of shrimps over the sulphide walls and possibly for different maturity stages. Although sample size is very small (only one mature individual), it could be hypothesized that with increasing maturity stage females move to a less populated area of the vent structure, probably under less influence of direct fluid venting. Furthermore, it has been suggested that ovigerous females may move away from direct influence of vent fluids in order to protect their embryos from high temperatures and toxic chemicals (Tyler & Young, 1999; Ramirez-Llodra et al., 2000). Therefore, this single female may have been a "by catch" among the others. The oocytes present in this mature female may have been close to being extruded, which could indicate that females start to migrate away from the vents to brood their embryos at this maturity stage. This could explain the lack of more females with a similarly large oocyte size-frequency distribution. Of the few ovigerous females reported to date, one had eggs of about 0.6 mm in diameter and another collected from TAG in August 1985, the single egg measured was 0.62×0.72 mm in size (Copley, 1998; Ramirez-Llodra et al., 2000). Therefore, the female with oocyte sizes ranging from 376 to 525 µm could be classified as mature which is also in agreement with observations by Copley (1998).

In a previous study by Ramirez-Llodra (2000) analysing oocyte size-frequency distributions of *Rimicaris exoculata*, the author noted that females from TAG (September 1994) had significantly larger oocytes than females from Rainbow (July 1998). The maximum oocyte sizes of 455 µm and 206 µm were observed from TAG and Rainbow samples, respectively (Ramirez-Llodra, 2000). In another study of *R. exoculata*, also collected from TAG in September 1994, the maximum oocyte size was 601 µm (Copley et al., 2007). The difference of about 150 µm between studies can be attributed to differences in the methods used to determined oocyte size-frequency distributions: histological sections of the gonad (Ramirez-Llodra, 2000);

dissection and direct measurement of oocytes (Copley et al., 2007). The sample of *R. exoculata* collected from Rainbow in August 2006 (present study) shows larger oocyte size-frequency distributions, with a maximum oocyte size of 525 μm, than those reported from the same site in July 1998, where the maximum oocyte size was 206 μm (Ramirez-Llodra, 2000). Here again, the differences are likely to be related to the method.

4.5.4. Contrasting vent habitats and reproductive variability

The differences found in sizes of females of *Mirocaris fortunata* from both sites raises the question of whether the maternal effects play a significant role. Maternal effects are "non-genetic effects of the maternal phenotype or environment on the phenotype of offspring" (Marshall et al., 2008). These effects can increase offspring fitness or increase maternal fitness at the expense of offspring fitness, and it has been hypothesized that changes in offspring phenotype are often adaptive maternal effects in response to environmental change (Marshall et al., 2008). In this sense, the presence of smaller females at Rainbow could lead to lower fecundity, and the production of smaller or of less quality eggs in females from Rainbow.

The present results confirm the previously suggested asynchrony in reproduction in *Mirocaris fortunata* and *Rimicaris exoculata*. It is considered that the temperature and chemical gradient found at hydrothermal vents may provide answers for some of the unexplained features of the reproductive biology of these shrimp species. The presence of *R. exoculata* juveniles in colder areas, under less influence of the vent fluid chemicals, may reflect a period of acclimatization to the vent environment, after the long larval phase which, at least part, is believed to be spent in bathypelagic waters (Pond et al., 1997a; Herring, 1998; Herring & Dixon, 1998). It has been suggested that *R. exoculata* may harbour highly diversified episymbionts at different sites, depending on the hydrothermal fluid chemistry (Schmidt et al., 2008b; Zbinden et al., 2008). If there are differences in nutrition between sites, these reflect the adaptability of hydrothermal vent shrimps to different environments, and as a consequence this may have an impact on the offspring. Most studies of different aspects of the biology of hydrothermal vent fauna highlight the need to understand

the complete life cycle of species. Furthermore, it is recognized that differences in biotic and abiotic factors between vent sites may have an impact at different levels of the biology of a single species (e.g. Kadar et al., 2006; Gonzalez-Rey et al., 2007, 2008; Schmidt et al., 2008a, b).

4.6. Other perspectives on larval dispersal potential and recruitment

The patterns of production of energetic reserves in an organism are conservative across their taxonomic group and will affect the rate of egg production, the fecundity and the larval development mode (Van Dover & Williams, 1991; Eckelbarger, 1994; Eckelbarger & Watling, 1995). Yet, habitat variability can affect the final reproductive output by slowing down vitellogenesis or by changing the amount of energy allocated to reproduction (Qian & Chia, 1991; Bridges et al., 1994; Eckelbarger, 1994; Levin et al., 1994). Planktonic larvae may follow two general modes of development: lecithotrophic or planktotrophic. In the former, larvae are non-feeding and rely on maternal reserves for nutrition; in the latter larvae are able to feed on small particles available in the plankton. Accordingly, the energetic investment per female to produce lecithotrophic larvae will be much larger than that involved in planktotrophic development. In contrast to anomuran crabs, there is no account of entirely lecithotrophic development (from hatching up to juvenile) in marine shrimp larvae. However, intermediate types of larval development have been noted. In general, these are characterized by an abbreviated development, and/or the first larval stages being lecithotrophic and the later stages planktotrophic. This pattern is known from many caridean shrimps and can be related to the larval developmental plasticity, allowing for energy saving traits as an adaptation to variable natural conditions (Anger, 2001; Thatje et al., 2001, 2005; Thatje, 2004).

All of the above will affect the larvae dispersal potential, which is a complex function of temperature, abundance of food, hydrographic regimes, larval behaviour and time spent in the water column (e.g. Thorson, 1950; Strathmann, 1974; Lutz et al., 1984; Scheltema, 1986; Gage & Tyler, 1991). Recruitment will rely on the ability of larvae to locate and settle, e.g. a vent site, without being able to actively swim in any direction (Tyler & Young, 1999, 2003).

Not surprisingly, it has been suggested that warmer waters are necessary for larval development and may provide a cue for larval recruitment (Lutz et al., 1980; Marsh et al., 2001; Pradillon et al., 2001). Experimental work carried out on the embryo development of the hydrothermal vent polychaete worm *Alvinella pompejana* (Desbruyères & Laubier, 1980) showed that embryos do not develop at 2ºC, but once in the presence of warmer waters (>10ºC) embryonic development is rapid (Pradillon et al., 2001). Hence, larvae may arrest development under very low temperatures, but once they are exposed to warmer waters, such as a new vent site, it is likely that larval development rates increase. Arresting development is likely to increase larvae dispersal potential.

Tyler and Dixon (2000) studied the pressure and temperature tolerances of zoea I larvae, obtained from berried females of *Mirocaris fortunata*, with hatching occurring at atmospheric pressure, in order to determine the vertical potential for dispersal. The results suggested that larvae of *M. fortunata* could ascend up to the permanent thermocline (~200 m) but would not be able to survive at surface waters conditions (1 atm at 20ºC). The estimated position of larvae in the water column is consistent with evidence that *M. fortunata* juveniles have biomarkers of photosynthetic derived material used for nutrition (Pond et al., 1997c, 2000; Gebruk et al., 2000; Colaço et al., 2002a). Nevertheless, larvae may develop faster at higher temperatures but it has to be within their thermal tolerance range, which in this case probably established the upper vertical limit of distribution of *M. fortunata* larvae.

Studies based on lipid biomarkers and plankton samples suggest that *R. exoculata* larvae have a large dispersal potential and a long planktotrophic larval phase, feeding on phytodetritus (Pond et al., 1997a; Allen Copley et al., 1998; Herring, 1998; Herring & Dixon, 1998). In addition, molecular analysis studies support the theory of large dispersal potential of these larvae, as there is evidence of high gene flow between Broken Spur, TAG and Snake Pit vent fields, and estimations of about 250 migrants per generation exchanged among sites (Creasey et al., 1996; Shank et al., 1998b).

However, no information is available on the developmental mode of either *M. fortunata* or *R. exoculata*. It is therefore rather speculative to assume that the full

larval development is planktotrophic, and an intermediate mode of development should not be rejected. Moreover, two ovigerous females of the shrimp *Alvinocaris* sp. collected from 1157 m at cold-seep sites in Sagami Bay, Japan, were successfully maintained in the laboratory until the larvae hatched (Koyama et al., 2005). Larvae were reared at atmospheric pressure and 4.5ºC in the presence of food. According to the authors the larvae hatched to prezoea and after 15 days developed compound eyes, dying after 74 days without metamorphosing. The presence of massive yolk reserves in the documented larval stages suggests that these could be lecithotrophic, or at least facultative lecithotrophic. Lecithotrophy and a prolonged larvae life are likely to enhance the potential for larval dispersal.

Other studies suggest that larvae are entrained in the hydrothermal vent plumes, to get to the surface waters to feed and develop (Kim et al., 1994; Mullineaux et al., 1995; Mullineaux & France, 1995). Although, this is unlikely to apply to *Rimicaris exoculata*, as it is suggested that ovigerous females move away from the vents to protect embryos from high-temperatures and potentially toxic chemicals (Tyler & Young, 1999; Ramirez-Llodra et al., 2000).

In a recent study analysing phenotypic traits dependent on gene expression, an extremely high rate of microevolution within a single geographic vent field was found in populations of the vent shrimp *Alvinocaris markensis* (Lunina & Vereshchaka, 2008). The authors argue that such high rates of change in the populations inhabiting the same vent field cannot be caused only by high rates of microevolution within the same hydrothermal community, but that a directional gene flow between hydrothermal fields, caused by larval migration, is more probable (Lunina & Vereshchaka, 2008). They suggest that a single genetic wave moving northwards passed along the mid-Atlantic ridge between 1998 and 2002 (Lunina & Vereshchaka, 2008). This adds to other evidence that most of the shrimp species found at hydrothermal vents, at least from the mid-Atlantic ridge, have a high dispersal ability.

Dispersing larvae of hydrothermal vent organisms could exit the vent environment either directly into the cold deep-sea environment, or through the buoyant plume. If early larvae are released into the cold deep sea they will start their

dispersal at very low temperatures, well below temperatures encountered during gametogenesis. On the other hand, larvae dispersed by the vent plume may avoid, to some extent, the extreme cold deep-sea temperatures and even feed on particles transported in the plume, but they would be exposed to the toxic chemicals. Either way, larvae apparently need warm temperatures to develop but where this takes place is still not clear; it could be either in the warmer surface waters or near the vent sites. Larvae dispersing in the cold deep sea will depend on hydrographic regimes for transport to the upper surface, or will stay drifting in bathypelagic waters until favourable currents take them to a warmer hydrothermal vent. It is unlikely that they can derive enough energy from the small amount of phytodetritus arriving to bathypelagic depths to survive for an indeterminate time before finding warmer waters. Hence, it is likely that these larvae have at least some degree of lecithotrophy. Planktotrophic larvae are probably dispersed by the vent plume and possibly feed within the plume and while in the upper ocean; lecithotrophs could also be entrained in the vent plume as a faster way to escape the vents, aiding dispersal. In addition, larvae may also be transported by their mothers, in species where adults are able of active swimming, either to the upper water column or to an adjacent vent site where spawning/hatching could take place.

The metapopulation structure typical of hydrothermal vent biogeographic provinces results in heterogeneous mosaics of local populations (vent sites or fields), with limited population sizes, making them vulnerable to potential near-extinction events (particularly on fast spreading ridges). Maintenance of this metapopulation structure will rely mostly on drift and gene flow (e.g. Jollivet, 1996; Vrijenhoek, 1997; Tyler & Young, 2003). High levels of gene flow between different populations are considered to indicate the presence of a large single pool of larvae. All populations within a metapopulation would contribute with individuals and at the same time would recruit individuals from this single pool of larvae (e.g. Tyler & Young, 2003). The existence of a well-mixed single pool of larvae would reflect a high dispersal potential, a prolonged planktonic life and favourable hydrographic conditions for dispersion. Nevertheless, a high gene flow could also be explained by the ability of adults to commute between vent sites. This would depend on the ability of a species to actively swim, survive starvation for a potentially long period, and detect a vent site. Sensory adaptations have been recorded in some vent species.

These include the highly sensitive photoreceptors of the modified eyes of adult *Rimicaris exoculata* and chemosensory organs, the use of which is believed to help vent organisms detect the presence of hydrothermal vents (e.g. Pelli & Chamberlain, 1989; Van Dover et al., 1989; Renninger et al., 1995; Charmantier-Daures & Segonzac, 1998). Thus, either the larval dispersal or the ability adults to migrate would contribute to the maintainance of a high gene flow between hydrothermal vent populations and foster a rapid colonization of new vent sites.

Species with a complex life-cycle, including a high dispersal ability, are likely to generate higher levels of genetic variation, as different environmental factors will have different impacts in each life stage. Most vent species seem to express a wide physiological tolerance to the different environmental variables at play in hydrothermal vent ecosystems. The environmental variables are also responsible for promoting phenotypic diversity in life history stages, which is believed to be a characteristic of most vent metazoans. Nevertheless, more comprehensive studies on the reproductive traits of vent species are needed, as these may illustrate past evolutionary adaptations to changes in the environment and potentially help to elucidate the origin and evolution of chemosynthetic biogeographic provinces.

Chapter 5. Synthesis

This study provides important clues on the common evolutionary history of both deep-sea hydrothermal vent species and their shallow-water relatives, based on the species' physiological scope. A species habitat boundary is determined by its physiological limitations, which may be related to their temperature or pressure tolerance and/or availability of food necessary to fulfil energetic requirements. Adult populations of *Mytilus edulis* and *Palaemonetes varians* are only found in shallow-water environments but it was shown here that their early life history stages have the physiological ability to tolerate a wide range of temperatures and pressures, far beyond conditions encountered in their natural habitat. This is likely to relate to past environmental changes during the evolutionary history of both species. The fact that these species only occur in shallow water and their early life-history stages tolerate deep-sea conditions, in terms of high-pressures, presumes that they had ancestors which had to adapt to high pressures during their evolutionary history, and that were probably occurring over a wide depth gradient. Because *M. edulis* and *P. varians* have a close phylogenetic relationship with deep-sea hydrothermal vent species, belonging to Bathymodiolinae and Alvinocarididae, respectively (Distel et al., 2000; Tokuda et al., 2006), it is likely that common ancestors existed and gave rise to both extant deep-sea species and their shallow-water relatives.

5.1. On the origins of hydrothermal vent fauna and their shallow-water relatives

The role of pressure in driving species evolution and speciation is still unclear. This study, however, elucidated some physiological connections between phylogenetically closely-related species, through the pressure tolerances of their early ontogenetic stages. Theoretically, the drifting stages of *M. edulis* and *P. varians* are able to disperse into deep-water depths, within their physiological limits to high-pressure. The study of the effects of pressure combined with those of temperature reveals that the former narrows the tolerance to the later. A common observation in

both species studied is that lower temperatures and higher pressures cause a slow-down in metabolism, prolonging embryonic or larval development. This would have consequences for dispersal potential of larvae, which is a complex function of temperature, abundance of food, hydrographic regimes, larval behaviour and time spent in the water column (e.g. Thorson, 1950; Strathmann, 1974; Lutz et al., 1984; Scheltema, 1986; Gaje & Tyler, 1991).

Larvae from cold-eurythermal shallow-water organisms dispersing into cold deep-sea waters would possibly face a prolonged larval life increasing the dispersal distance, by arresting development until finding warmer conditions. Dispersing larvae could develop in warmer upper ocean waters until reaching more advanced stages of development, settling into cold deeper waters, which would most likely cause a delay in metamorphosis until finding warmer conditions suitable for settlement. Larvae would probably only be successful if settling into a warm, hydrothermal vent habitat, where they could complete development. It is thus likely to be the thermal tolerance, and not the pressure tolerance alone, that currently keeps these shallow-water species away from the deep-sea.

In order to be able to colonize hydrothermal vents, species would have to tolerate the toxic fluids emitted from the vents, and this would favour species that are highly tolerant to changes in the environment. In addition, these species would probably be scavengers (e.g. shrimps) or able to filter feed (e.g. mytilids; from particulate organic matter put into the water column surrounding the vents by turbulent mixing of the vent fluid flow). Both *M. edulis* and *P. varians* could be good candidates, given their high physiological tolerance, adaptability to variable conditions and, equally important, some similarities in their physiological requirements to hydrothermal vent species.

Besides of their phylogenetic relatedness (Distel et al., 2000; Tokuda et al., 2006), existing evidence suggests that both shallow-water and vent species are similar in their reproductive and larval developmental cycle (Lutz et al., 1980; Berg, 1985; Kenk & Wilson, 1985). Moreover, bivalves from deep-sea hydrothermal vents have also shown growth rates similar to mytilids found in shallow-water environments, and their growth rates are much higher (several orders of magnitude)

than those in bivalve species living in non-chemosynthetic deep-sea habitat (Lutz et al., 1985). In addition, variability in life-cycle complexity through adaptability to the environment is likely to generate different levels of genetic and phenotypic variation (Thatje & Bacardit, 2000; Anger, 2001) in response to potential disruptive selection pressures being experienced by different life stages. Phenotypic plasticity in larval development may be an important feature of both hydrothermal vent and shallow-water species, which eventually could be able to colonize hydrothermal vents. This appears to be a characteristic of *P. varians,* enabling this species to adapt to variable environmental conditions, characteristic of its natural habitat. Similarly, vent shrimps live in a highly variable habitat characterized, for example, by wide temperature gradients. Adult populations of *Mirocaris fortunata* show phenotypic variability between different vent sites (Martin & Christiansen, 1995; Vereshchaka, 1996; Komai & Segonzac, 2003), which is most likely related to phenotypic variability during larval development, as an adaptation to different environmental conditions existent between sites.

Furthermore, the presence of massive yolk reserves in the documented larval stages of the shrimp *Alvinocaris* sp. (Koyama et al., 2005) suggests that these could be lecithotrophic, or at least facultative lecithotrophic. The first larval stage of *P. varians* is facultative lecithotrophic (Heral & Saudray, 1979; Yúfera & Rodríguez, 1985), what enables larvae to develop in the absence of food, and when this is accompanied with low temperatures it potentially increases the dispersal distance. Some degree of lecithotrophy, phenotypic plasticity and a complex life cycle, are all trade-offs likely to enhance survival under unpredictable and variable environmental conditions (Wehrtmann, 1991; Thatje & Bacardit, 2000; Anger, 2001; Thatje et al., 2004a, b). These trade-offs are likely to be characteristic of some hydrothermal vent species.

However, the ability of shallow-water species to survive, and adapt to a combination of all environmental conditions of vents at a time is more unlikely. I do not believe that the shallow-water species *Mytilus edulis* and *Palaemonetes varians* could actually colonize the vents, but their particular characteristics, which are likely to coincide with those of some deep-sea hydrothermal vent species, may be very similar to those of hypothetical ancestors of vent endemic fauna. In this sense,

ancestors of hydrothermal vent-endemic fauna should have the following pre-requisites:

- Cold-eurythermal;
- Wide tolerance range to variable habitat conditions;
- Scavenger, detritivor, or filter-feeding;
- Phenotypic plasticity in larval development;
- Complex life cycle, with a long dispersal stage;
- Some degree of lecithotrophy in the first larval stages.

The existing hypotheses for the origin of the deep-sea fauna assume that deep-sea species have a narrow and conservative thermal tolerance. Hence, shallow-water species could only migrate to the deep-sea *via*:

1) high latitudes cold-isothermal water column (Kussakin, 1973; Menzies et al., 1973; Hessler & Thistle, 1975; Hessler & Wilson, 1983; Tyler & Young, 1998; Thatje et al., 2005);

2) low latitudes warm-isothermal water column, existent during the late Mesozoic and early Cenozoic (~ 65 Mya; Menzies et al., 1973; Benson, 1975; Berger, 1979; Schopf, 1980; Hessler & Wilson, 1983; Young et al., 1997).

5.1.1. Evidence from past oceanic currents and deep-sea conditions

Continents were in a different position at the end of the Cretaceous (about 65 Mya) than they are at present, and there was little latitudinal gradient in seawater temperatures (e.g. Kussakin, 1973; Thomas et al., 2006). The asteroid impact at this time caused a mass-extinction of many groups of the upper ocean fauna, with the deep-sea benthic fauna being less affected. It is suggested that this event caused global darkness, decreasing photosynthesis and a subsequent decline in the flux of organic matter to the deep-seafloor (e.g. D'Hondt, 2005; Thomas et al., 2006). Phytoplankton biomass could have recovered quickly, as the surviving

phytoplankton would bloom as soon as light conditions allowed, but phytoplankton species diversity apparently remained low (D'Hondt, 2005). In the sea, some groups of organisms were apparently more resistant to this impact than others, and the key to success appears to be associated with favourable life-history traits (e.g. formation of cysts in planktonic foraminifera) or the mode of nutrition independent of photosynthesis (e.g. detritus feeding macrofauna; D'Hondt, 2005). Although the world cooled for a few millennia, the ecosystems recovered with progressive global warming during the following ~10 Myrs (Thomas et al., 2006).

Foraminiferal carbon and oxygen isotope changes from an Antarctic sedimentary sequence indicate that at the end of the Palaeocene (~57 Mya) a rapid global warming accompanied by oceanographic changes caused one of the major deep-sea benthic faunal extinctions (Kennett & Stott, 1991). In contrast, at shallower depths (<100 m), the fauna appears to have been less affected by this event, except in Antarctica where temperatures are likely to have increased 5ºC (Kennett & Stott, 1991). These benthic extinctions are likely to have been caused by a rapid (<3 kyr) increase in deep water temperature (>15ºC) and associated reduction of oxygen concentration (Kennett & Stott, 1991).

The opening of the Arctic Ocean basin, containing low salinity seawater, to the north Atlantic at about 49 Mya may have promoted global cooling by changing deep-water circulation patterns (Thomas et al., 2006), initiating the gradual cooling of the deep-sea waters. After the climatic optimum in the early Eocene (~50 Mya) there was a gradual temperature decrease of about 14-15ºC in the deep-sea waters to reach present values (Kussakin, 1973; Thomas et al., 2006). Because this occurred over a long time period marine fauna were probably able to gradually adapt to increasingly cold conditions (Kussakin, 1973).

According to the fossil record of the different extinction events, the taxonomic patterns of macrofaunal extinction varied considerably from region to region, according to the different selection factors operating in different regions and ecosystems (Hansen et al., 1993; McClure & Bohonak, 1995). Geographic distribution may have played some role in selecting the genera that survived among bivalves (Jablonski, 2003) and calcareous dinoflagellates (Wendler & Willems,

2002). In the case of echinoids, survival may have been independent of geographical distribution and correlated instead with the adult feeding strategies, by selecting in favour of genera with omnivory and deposit feeding (Smith & Jeffrey, 1998).

The previously proposed pre-requisites for ancestors of hydrothermal vent fauna (cold-eurythermal; wide tolerance range to variable habitat conditions; scavenger, detritivor, or filter-feeding; phenotypic plasticity in larval development; complex life cycle; some degree of lecithotrophy in the first larval stages) may prove to be congruent with the characteristics of organisms (e.g. formation of cysts in dinoflagellates, a life history trait making it easier to survive starvation; detritus feeding macrofauna; D'Hondt, 2005), that are likely to have survived the mass extinction events. During these critical periods, adaptation to new nutrition modes may have evolved, for example, mussels or shrimp ancestors could have evolved adaptations to derive food from symbioses with chemoautotrophic bacteria and colonize hydrothermal vents.

Nevertheless, adaptation to high pressure by shallow-water ancestors was necessary, and an increasingly eurybathyal distribution was more likely in the presence of an isothermal water column. In addition, shallow-water species migrating to the deep sea through a warm isothermal water column could develop at higher rates than at lower temperatures, probably accompanied by higher phenotypic variability, and potentially a faster turnover of generations leading to a faster evolution. In a subsequent stratified ocean, species diversity would increase as a result of specialization, adaptive radiation and geographic or reproductive isolation leading to speciation. Apparently, the ancestors' phenotypic/genetic adaptation to tolerate a wide range of pressures is still imprinted in extant species, such as *Mytilus edulis* and *Palaemonetes varians*.

Other more recent global and local climatic events, such as glaciations of shallow continental shelves, tectonic events that changed global and local circulation patterns of oceanic water masses, may also have contributed to further species adaptations to changes in the environment, speciation and evolution. However, evidence from the geological record points to a stratified global ocean after about 50

Mya, and a warm isothermal water column was since no longer available to serve as a route for cold-eurythermal shallow-water species to colonize the deep sea.

5.2. Do contrasting vent habitats affect reproductive variability?

Global biogeographic provinces of chemosynthetic driven ecosystems have been proposed based on species diversity, distribution and abundance (Tyler et al., 2003). It has been shown that differences in fluid composition between different vent sites within the same biogeographic province affect the abundance and distribution of species (Desbruyères et al., 2001). Different geological, physical and chemical settings are found in different vent and seep sites worldwide, and similarities in fauna composition may be explained by similarities in these settings. The chemoautotrophic bacteria are the basis of the food chain in these chemosynthetic ecosystems, hence different chemicals available will be exploited by different types of bacteria (e.g. Dubilier et al., 2008). This is likely to have a major impact on the macrofauna diversity and abundance, by affecting the mode of nutrition, and ultimately the reproductive output and dispersal of offspring, possibly having consequences for the evolution and biogeography of species.

The vent shrimp *Mirocaris fortunata* is widespread along the mid-Atlantic ridge hydrothermal vents, and different morphological features are found in populations from different sites; in earlier studies it was even classified as different species (Martin & Christiansen, 1995; Vereshchaka, 1996; Komai & Segonzac, 2003). The differences found in morphological characters between populations from different sites might be related to variations in habitat conditions between sites.

In this study, the population size structure analysis of *Mirocaris fortunata* revealed significant differences in carapace size between females from two contrasting vent habitats in the mid-Atlantic ridge – Lucky Strike and Rainbow. To date, there is no other study comparing the population's size structure of *Mirocaris fortunata* from different vent sites and the patterns of gametogenesis in *M. fortunata* have only been assessed from Lucky Strike samples. However, different environmental conditions between sites have been reported (e.g. Desbruyères et al.,

2001), and may induce different adaptive maternal effects, which are likely to affect the offspring phenotype and/or quality.

It has been hypothesized that changes in offspring phenotype are often adaptive maternal effects in response to environmental change (Marshall et al., 2008). The same species could develop differences in the reproductive traits in contrasting chemosynthetic habitats, as a result of differences in the maternal fitness in response to the environment. More specifically, maternal fitness may affect fecundity, and eggs size or quality, which is likely to have consequences for the larval development and dispersal potential. Environmentally induced phenotypic changes may have consequences for offspring genotype. Hence, any differences in these reproductive traits may reflect past evolutionary adaptations to changes in the environment and potentially help to elucidate the origin and evolution of chemosynthetic biogeographic provinces.

To date reproductive traits of only a few vent species have been studied in detail and most of these works lack detailed temporal and spatial resolution, often as a result of sample size and effort constraints. It is necessary to integrate all data on biotic and abiotic properties available for each deep-sea chemosynthetic site within each biogeographic province. This should include data about the geological background of each site (e.g. source rock, spreading rates, type of sulphide structure); the physical and chemical properties (e.g. depth, end-member fluids composition; habitat temperature and chemical features); the type of symbionts/free-living bacteria present at each site; and the species diversity and ecology (e.g. reproduction, nutrition). Such a dataset could provide a baseline of knowledge and guidance for future research in order to 1) explore relationships between diversity, distribution and abundance of vent and seep species and the physical and chemical properties of chemosynthetic environments, 2) relate these to phylogenetics, or 3) find gaps of knowledge where further sampling and/or study are needed.

The present study also demonstrated the importance of studying the physiological aspects of the early life stages of the shallow-water relatives to hydrothermal vent species, as they may yield new insight regarding the origin of hydrothermal vent faunas. This should be applied to other shallow-water species with

hydrothermal vent relatives (e.g. limpets, crabs, scale worms). The physiological tolerance of shallow-water species to other physical and chemical properties prevailing at hydrothermal vents should also contribute to a better understanding of the adaptations of hydrothermal vent fauna to their environment. Nevertheless, for the study of other properties of the deep-sea hydrothermal vents, the factor pressure should always be considered.

5.3. Future perspectives

Pressure tolerance studies on the early life history stages of species seem to be a very useful tool to identify potential evolutionary links between species. An increased effort should be made in order to screen other species for their pressure and temperature tolerance, as this should not be ignored when analysing phylogenetic relationships. The pioneer experiment demonstrating the fertilization under pressures of up to 500 atm of *Mytilus edulis* eggs raises interesting questions:

1) What are the structural properties of the gametes and zygotes that facilitate the tolerance to high pressure?
2) Is this a common feature of reproductive cells in other animals?
3) Can this be a divide in the evolution of protostomes and deuterostomes?
4) Is this related to the existence of eurybathyal ancestors?

In order to start the quest for screening other species for fertilization success under pressure, I applied the same method for analysing temperature and pressure tolerance effects on fertilization success of eggs in *M. edulis* to eggs of the shallow-water sea-urchin *Psammechinus miliaris* from temperate European waters (for details on method and results see Appendix IV). Briefly, results show that in this species, fertilization is inhibited at pressures greater than 50 atm (Fig. 5.1.), while at pressures equivalent of 500 m depth the percentage of fertilized embryos is very low. As in the case of *M. edulis*, a slower developmental rate with increasing pressure is observed in *P. miliaris*. In contrast, the development rates do not increase with increasing

temperature, and are higher at 15ºC, which corresponds to the temperature of adults' maintenance and probably the optimum temperature for embryonic development.

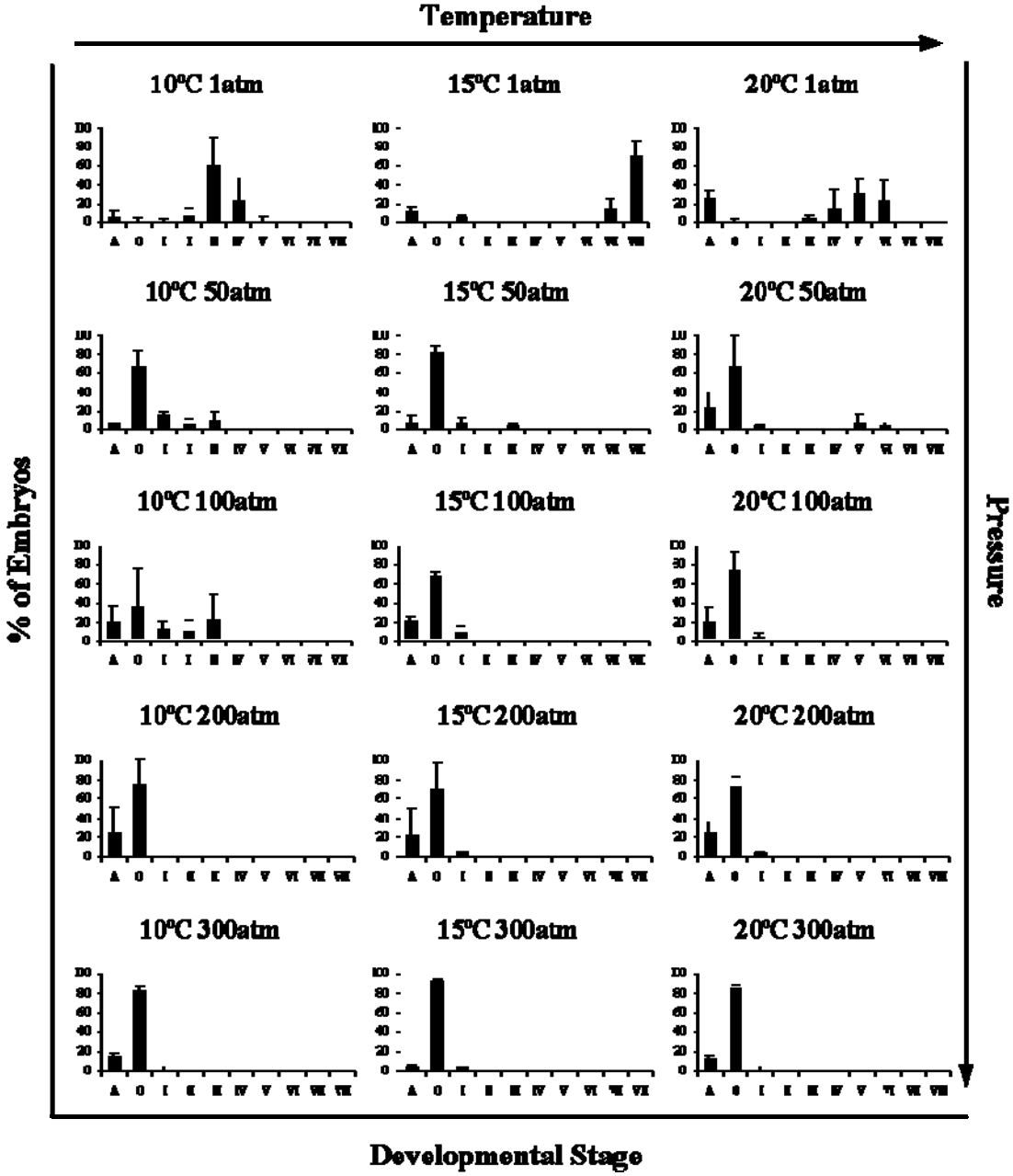

Fig. 5.1. *Psammechinus miliaris* embryonic development incubated at different pressure/temperature regimes for 4h, with fertilization occurring under pressure. Histograms are of percentage mean and standard deviation. Stage of development is A=abnormal; 0=unfertilized; I=fertilized with fertilization membrane; II=2-cell; III=4-cell; IV=8-cell; V=16-cell; VI=32-cell; VII=64-cell up to pre-blastula; VIII=early blastula (see Appendix IV, Table IV.i. for a detailed description of each developmental stage).

A ten-fold higher degree of tolerance to pressure is observed in *Mytilus edulis*. Both species are broadcast spawners, occupying neighbouring habitats in shallow water, so the eggs and sperm will find equal environmental conditions once in the water column. This suggests that there are important differences probably at the cell-structure level regarding tolerance to pressure, which may be phylogenetically determined. Furthermore, the temperature and pressure effect on embryonic and larval development of *P. miliaris* with fertilization occurring at atmospheric pressure (Aquino-Souza et al., 2008) revealed that embryos reared at 15ºC survive and develop normally up to 100 atm, at the same rate as at atmospheric pressure. In addition, when embryos were reared at atmospheric pressure up to the gastrula and prism stage and then incubated for 24 hours, the survival rates were high for all temperature (5, 10, 15, 20ºC) and pressure (1, 50, 100, 150 and 200 atm) treatments (Aquino-Souza et al., 2008). Thus, *P. miliaris* exhibits remarkable ontogenetic changes in terms of physiological tolerance range for pressure and temperature. Fertilization in this species appears to be constrained to shallow water (1 atm, 15ºC), dispersing stages are able to drift to greater depths (200 atm, 5ºC), and the maximum recorded depth distribution of adults is 100 meters (Mortensen, 1927; Kelly et al., 2006).

The observed differences between species, at the fertilization stage, could possibly relate the fact that the sea-urchin belongs to the deuterostomes and the mytilid to the protostomes; groups with distinctive embryonic development (Chapter 1, Table 1.1.). These groups diverged earlier in animal evolutionary history, and it could be interesting to explore the deuterostomes – protostomes dichotomy regarding the pressure effects in other species from different depth habitats.

The few studies on temperature and pressure tolerance in the early ontogeny of marine invertebrates from different depth habitats mostly show earlier stages having a wider tolerance to pressure than the recorded depth distribution of the adults (Fig.5.2.). Most of these studies focused on echinoderms either from shallow or deep populations, and the exceptions are the shallow-water species presented in this study, *Mytilus edulis* and *Palaemonetes varians*; and the vent shrimp *Mirocaris fortunata* (Tyler & Dixon, 2000). In most studies, a wider pressure tolerance range is observed

for the optimum temperature for normal development, i.e. temperature conditions of their natural habitat.

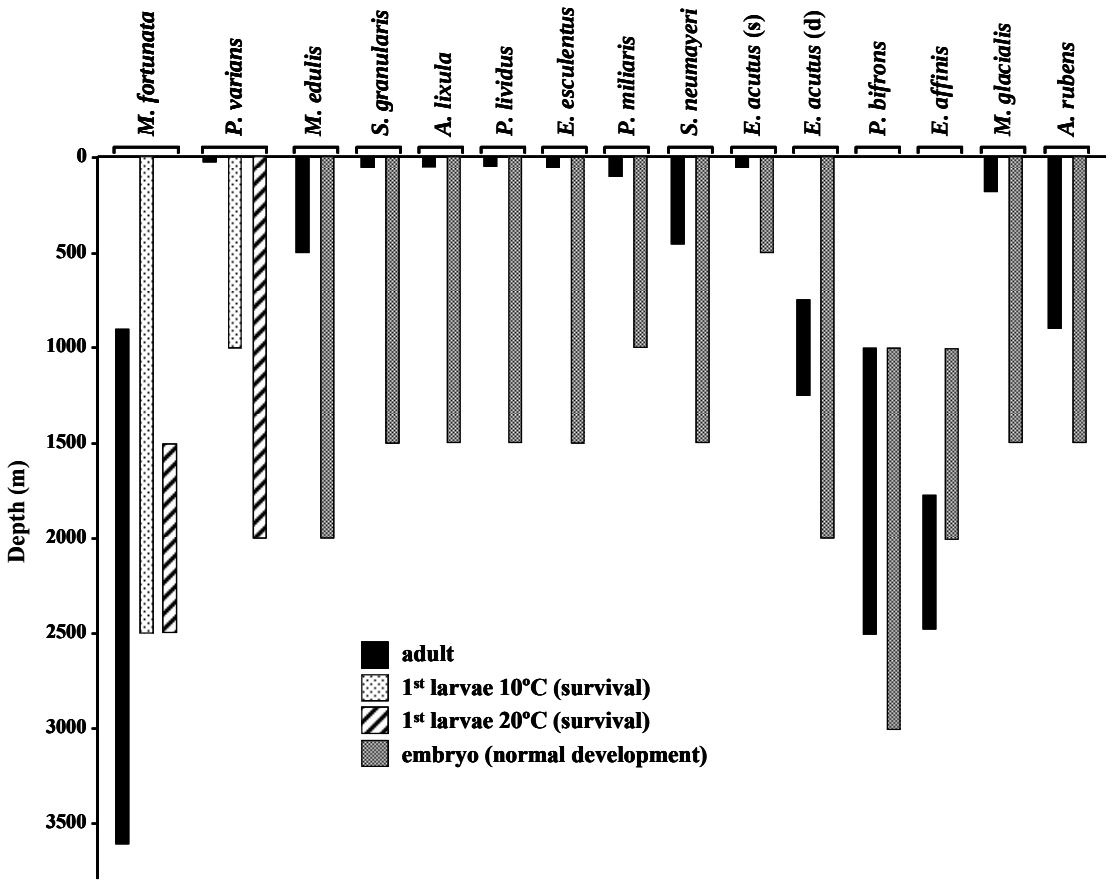

Fig. 5.2. Comparison of depth distribution recorded for adult populations and potential depth distribution of early ontogeny of some invertebrate taxa. The potential depth distribution of early ontogeny (1st larvae or embryos) was estimated according to their pressure tolerance, after *in vitro* studies. Data on the pressure tolerance for normal embryonic development are for the optimum temperature, with incubation starting straight after fertilization. For more information see the respective reference for each species: *Mirocaris fortunata* (Tyler & Dixon, 2000); *Palaemonetes varians* and *Mytilus edulis* (present study); *Sphaerechinus granularis*, *Arbacia lixula* and *Paracentrotus lividus* (Young et al., 1997); *Echinus esculentus* and *E. acutus* (Tyler & Young, 1998); *Psammechinus miliaris* (Aquino-Souza et al., 2008); *Sterechinus neumayeri* (Tyler et al., 2000); *Plutonaster bifrons* (Young et al., 1996); *Echinus affinis* (Young & Tyler, 1993); *Martasterias glacialis* and *Asterias rubens* (Benitez Villalobos et al., 2006). (s) refers to shallow population; (d) refers to deep population.

The particularly wide pressure tolerance range observed in embryos of the deeper population of *Echinus acutus,* plus the known existence of a shallower population, led the authors to hypothesize that this species is in the process of invading deeper waters and in process of speciation (Tyler & Young, 1998; Fig. 5.2.). Furthermore, *Echinus affinis* normal embryonic development is restricted to deeper waters, although the upper limit of vertical distribution is higher than in adults (Young & Tyler, 1993). In this case, the lower depth limit of distribution of embryos corresponds to the maximum pressure tested; hence it is likely that they are able to tolerate higher pressures (Fig. 5.2.). Assuming that the genus *Echinus* has shallow water origins (Tyler & Young, 1998), it appears that with speciation at greater depths embryos loose the ability to tolerate shallower depths. In another deep-sea echinoderm *Plutonaster bifrons*, the upper limit of depth distribution of adults and embryos is the same (Young, et al., 1996). It could be suggested that *P. bifrons* may have colonized the deep-sea before *E. affinis*, given the upper vertical distribution of embryos, which coincides with that of the adults; meaning that there is no trace of shallower existence in the early ontogeny.

In the closely-related shrimp species, *Mirocaris fortunata* and *Palaemonetes varians* from deep-sea hydrothermal vents and shallow water, respectively, there is an overlap of the depth tolerance ranges of the first larval stage for each temperature (Fig. 5.2.). This supports the previously proposed theory for the existence of a common ancestor expressing a wide physiological tolerance range to pressure, giving origins to both species. These observations highlight the importance of pressure tolerance studies, and physiological studies in general when interpreting phylogenetic data, as it may help to understand the evolutionary pathway leading to speciation.

For a better understanding of the effects of pressure on early life-history development of marine invertebrates, future experiments should include other environmental variables such as pH, salinity, or toxic chemicals such as those present in chemosynthetic environments. DNA damage assessment and/or proteomic studies could be used to further investigate the effects of pressure at the molecular level. The DNA damage assessment has been attempted. Logistical constraints did not allow the study to be completed but it will certainly be important for this to be done in future

experiments. For instance, at high pressures and low temperatures it was seen that embryos take more time to develop, with fewer abnormalities occurring. Analysing the DNA damage could be useful to check if these embryos are likely to complete development and survive or if their development has been permanently compromised at an earlier stage. Another technique is the proteomics. This could provide information on what proteins are conferring tolerance to high pressure. It could be applied, for example, to compare closely-related species occurring at different depth habitats, and with knowledge on the protein(s) involved in pressure adaptation it could be possible to understand the cellular mechanism behind this adaptation. However, this approach was only recently developed and presents some major challenges at present. The proteome is only known for a few species (Dowling & Sheehan, 2006), and even fewer marine invertebrates.

As previously mentioned, the study of embryonic and larval development of hydrothermal vent animals is limited to just a few species. In an attempt to study the larval development, specimens of *Bathymodiolus azoricus* collected from Menez Gwen (850 m), mid-Atlantic ridge, in January 2007, were maintained in the laboratory for more than a year, at atmospheric pressure, 8ºC and fed with live microalgae. Spawning was noted, and some embryos at the four-cell stage of development, very similar to those of *M. edulis*, were found in the adults maintenance aquaria. Unfortunately, in all the trials and when spawning was induced, it was not possible to obtain female and male gametes at the same time, and for this reason the embryonic and larval development could not be studied. Despite remarkable survival of these symbiotic mytilids at atmospheric pressure, fed with microalgae, their degree of fitness is uncertain. *B. azoricus* suffered decompression from about 850 m and were deprived of their symbionts, which are unable to survive without the chemical supply to sustain chemosynthesis. It is likely that all these factors may have contributed to the lack of success in obtaining gametes. Efforts should be made to collect *B. azoricus* with a pressurized recovery device (e.g. Periscope; Shillito et al., 2008) and maintain them under pressure, until the first evidence of spawning is noted. This would ensure that most of the specimens were mature and ready to release their gametes. If eventually gametes survive to depressurization, a pressure and temperature tolerance experiment with fertilization

under pressure could be done, in order to study their embryonic and larval development.

Finally, studies aiming to explain the biogeography or evolutionary history of chemosynthetic environments should assess not only the species diversity, distribution and abundance, but also integrate information on the abiotic settings, reproductive patterns and nutrition strategies for each chemosynthetic site. Different environmental conditions between contrasting chemosynthetic sites are proposed to induce different adaptive maternal effects, which are likely to affect the reproductive biology of species. It is of particular importance to elucidate the role of reproductive traits in shaping distribution patterns, depth distribution limits of species and populations, and the resilience potential of vent communities to environmental change.

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The ocean is not deep enough: pressure tolerances during early ontogeny of the blue mussel *Mytilus edulis*

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Early ontogenetic adaptations reflect the evolutionary history of a species. To understand the evolution of the deep-sea fauna and its adaptation to high pressure, it is important to know the effects of pressure on their shallow-water relatives. In this study we analyse the temperature and pressure tolerances of early life-history stages of the shallow-water species *Mytilus edulis*. This species expresses a close phylogenetic relationship with hydrothermal-vent mussels of the subfamily Bathymodiolinae. Tolerances to pressure and temperature are defined in terms of fertilization success and embryo developmental rates in laboratory-based experiments. In *M. edulis*, successful fertilization under pressure is possible up to 500 atm (50.66 MPa), at 10, 15 and 20°C. A slower embryonic development is observed with decreasing temperature and with increasing pressure; principally, pressure narrows the physiological tolerance window in different ontogenetic stages of *M. edulis*, and slows down metabolism. This study provides important clues on possible evolutionary pathways of hydrothermal vent and cold-seep bivalve species and their shallow-water relatives. Evolution and speciation patterns of species derive mostly from their ability to adapt to variable environmental conditions, within environmental constraints, which promote morphological and genetic variability, often differently for each life-history stage. The present results support the view that a direct colonization of deep-water hydrothermal vent environments by a cold eurythermal shallow-water ancestor is indeed a possible scenario for the Mytilinae, challenging previous hypothesis of a wood/bone to seep/vent colonization pathway.

Keywords: pressure; temperature; Mytilidae; evolution; shallow water; deep sea

1. INTRODUCTION

Bacterial and higher animal life has been found in the deepest parts of the ocean, including the Challenger Deep in the Marianas Trough, where pressure is near 1100 atm. Hypotheses on the origins of the deep-sea fauna say that extant deep-sea organisms dispersed for long distances through isothermal water masses in the past glacial periods (Tyler *et al.* 2000; Thatje *et al.* 2005). Therefore, today's faunal exchange between deep-sea and shallow-water animals could occur via cold-adapted species living at high latitudes, where the water column is isothermal (Kussakin 1973; Menzies *et al.* 1973; Hessler & Thistle 1975; Hessler & Wilson 1983; Tyler & Young 1998; Thatje *et al.* 2005). At lower latitudes, during the Mesozoic and early Cenozoic, the deep sea was warmer than at present, and could have allowed shallow-water species to invade deeper ocean habitats (Menzies *et al.* 1973; Benson 1975; Berger 1979; Schopf 1980; Hessler & Wilson 1983; Young *et al.* 1997). To further elucidate this hypothesis, increasing relevance has recently been given to studies on pressure and thermal tolerance of the early life stages of both shallow-water and deep-sea invertebrates, together with studies on past changes in deep-sea hydrography (Tyler *et al.* 2000; Tokuda *et al.* 2006; Pradillon & Gaill 2007).

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In the marine environment, pressure is the single variable that has a continuous relationship with depth, increasing by approximately 1 atm (atmosphere; 10^5 Pascals) per 10 m of water depth. Marine species' habitats are often also defined in relation to upper and lower depth limits and these limits are ultimately related to pressure tolerances of the organisms. Ideally, these habitat boundaries should be assessed for each life stage, as the depth tolerance range in each stage is different in some species (Anger 2001; Aquino-Souza *et al.* 2008). The early life stages of marine benthic invertebrates, for instance, are the most likely opportunities they have to disperse over long distances and to colonize new habitats (Tyler 1995; Young *et al.* 1996; Macdonald 1997; Tyler & Young 1998).

An ecological factor of great importance in defining the distribution of species is the temperature. The temperature tolerances for each life stage of a marine invertebrate can vary in a single species and affect the survival and the ability of a species to colonize new habitats. Temperature accelerates or delays the rates of metabolism and thus affects larval growth, development and survival as well as seasonal variation in the occurrence of larvae in the plankton (Anger 2001; for review see Clarke 2003). A stenothermal response is generally associated with species from the tropics or high latitudes, while those adapted to pronounced seasonal and regional temperature variations are typical of intermediate climatic zones and show a eurythermal response (Anger 2001, and references therein).

In order to understand the evolution/adaptation of the deep-sea fauna in terms of its sensitivity to pressure, it is important to know how their biological structures and processes differ from their shallow-water relatives (Childress & Fisher 1992; Somero 1992). These questions motivated scientists to develop high-pressure equipment to study the effect of pressure on marine organisms (Quetin & Childress 1980; Young & Tyler 1993; Young *et al.* 1996; Shillito *et al.* 2001). Furthermore, pressure is a physical property affecting molecular interactions and in consequence all biological processes on the Earth, and is largely unaffected by other factors. According to Le Chatelier's principle, if a chemical system at equilibrium experiences a change in concentration, temperature, volume or total pressure, the equilibrium will shift in order to counteract the imposed change (Somero 1992; Pradillon & Gaill 2007).

In biological systems the effect of pressure causes a compression of the system, conditioning physiological and biochemical processes involved in increasing or decreasing cell volume. A combination of the pressure effect with other physico-chemical factors affecting biological processes, e.g. temperature, pH and salinity, will intensify or reduce the effect of pressure alone. Pressure sensitivities of enzymes, structural proteins and membrane-based systems differ markedly between shallow-water and deep-sea species (Somero 1992; Pradillon & Gaill 2007).

The subfamily Mytilinae is the shallow-water closest taxonomic group to the hydrothermal vent and seep mussels of the subfamily Bathymodiolinae (Distel *et al.* 2000). Jones *et al.* (2006) revealed that vent species evolved multiple times, with moments of habitat reversals, but evidence is that there was a progressive evolution from shallow to deep habitats. Mid-ocean hydrothermal vent species may represent a monophyletic group with one noticeable reversal, and this is in agreement with previous hypotheses regarding evolution from wood/bone to seeps/vents (Jones *et al.* 2006).

The blue mussel *Mytilus edulis* Linné, 1758, is a widespread semi-sessile epibenthic bivalve found on rocky shores, shallow sublittoral zones and estuaries (Newell 1989). It is very common in northern Europe and parts of the Atlantic coast off Canada, but also colonizes temperate zones in the Southern Hemisphere (Gosling 1992). The upper vertical limit of *M. edulis* populations' distribution on rocky shores is determined by its tolerance to temperature and desiccation (Seed & Suchanek 1992). This species is relatively tolerant to extreme cold and freezing and it can survive occasional short frost events, but may not be resistant to persistent very low temperatures (Bourget 1983). The lower depth limit of distribution of *M. edulis* is presumably mainly influenced by predation (Seed 1969). In addition, the burial and abrasion by shifting sands are also of relevant importance (Daly & Mathieson 1977; Holt *et al.* 1998). The maximum depth distribution registered for this species in the Baltic Sea is 40 m, and its depth limit distribution is associated with the presence/absence of a hard bottom substratum (Bubinas & Vaitonis 2003). Subtidal populations have been reported on seamounts, dock pilings and offshore oil platforms, where they grow to a larger size, probably owing to a lack of predators (Seed & Suchanek 1992). Uncommonly, it has been found in deeper and cooler waters (100–499 m, Theroux & Wigley 1983) owing to shallower lack of habitat and/or presence of hard substratum at greater depths. The genus *Mytilus* is also an important invasive species (Carlton 1999).

Mytilus edulis is dioecious with rare occasions of hermaphroditism (Seed 1976; Micallef & Tyler 1988), and has a large number of small-sized eggs and a planktotrophic larva (Bayne 1976). Gametogenesis is synchronous and spawning occurs when eggs and sperm are fully ripe and are released through the exhalent siphon into the water column where fertilization takes place (Bayne 1976; Newell 1989). The embryonic development takes a few days and comprises cleavages, the formation of cilia, velum and shell gland—trochophore stage, up to the formation of the D-shaped shelled larvae, when it starts its planktotrophic phase (Bayne 1976). The depth distribution of larvae and juveniles of *M. edulis* is presumed to be the same as in adults (Newell 1989).

In the present study, the embryonic and larval development of *M. edulis*, along its entire physiological temperature and pressure tolerance window, is analysed, to our knowledge, for the first time. The physiological tolerances of the early life stages are examined, as they are of extreme importance in controlling the distribution, colonization pathways and to some extent the speciation within closely related species. The results are discussed in the frame of current theories on the evolution of the macrofauna in chemosynthetic environments.

2. MATERIAL AND METHODS

(a) *Sampling and spawning*

Adult specimens of *M. edulis* were collected from Southampton Water (UK) and maintained at 15±1.5°C in a running seawater system in the aquarium of the National Oceanography Centre, Southampton, where the experimental work took place in June 2007. Spawning was induced by heavily shaking all mussels in a bucket (mechanical shock; Costello *et al.* 1957; Sprung & Bayne 1984), followed by the injection of 1 ml of 0.55 M KCl into the mantle cavity of each mussel (salinity shock; Young & Tyler 1993). To obtain gametes, each mussel was placed in individual glass bowls and left 'dry' for 10 min, with an ambient temperature of approximately 20°C. The mussels were submerged with 5°C (temperature shock; Costello *et al.* 1957; Sprung & Bayne 1984) filtered seawater (1.6 µm retention) and spawning started after temperature inside the bowls exceeded 15°C.

(b) *Temperature effect on embryonic and larval development*

Three cultures of fertilized eggs were prepared by mixing freshly spawned male and female gametes in a 1 l beaker in filtered seawater (1.6 µm retention filter) at 15°C. Gametes from each male/female pair were used in each culture. Fertilized eggs were transferred into 20 ml glass vials and incubated at 5, 10, 15, 20 and 25°C, at atmospheric pressure. Cultures were sampled regularly (every 10 min in the first 4 hours after fertilization, and once per hour thereafter) and the time from fertilization and stage of development were noted, until the D-larvae stage was reached for all temperatures. The chosen stages of development were defined according to distinguishable morphological features between stages (table 1), observable under a compound microscope.

Table 1. Classification of early ontogenetic stages in *M. edulis*, with reference to the main characteristics observed in each stage (changed after Zardus & Martel 2002).

stage	main characteristics
A	abnormal development
0	unfertilized egg; no sign of polar body
I	fertilized, uncleaved egg; showing polar body (and polar lobe formation)
II	two-cell stage; first cleavage and extrusion of polar body
III	four-cell stage; one large D-cell and three smaller cells
IV	eight-cell stage; showing spiral unequal cleavage of blastomeres
V	multi-cell; unequal cleavage producing micromeres in the animal pole and macromeres in the vegetal pole; within the fertilization membrane
VI	early blastula; released from the fertilization membrane; developing cilia
VII	gastrula; with blastopore (developing gut); invagination of the shell field (developing shell); quadrants with cilia
VIII	early trochophore; developing apical sense organ (apical plate+apical tuft)
IX	late trochophore; with velum and organic pellicule of first shell
X	D-larva; free-swimming straight hinge veliger with fully formed velum and prodissoconch I

(c) ***Pressure effect on embryonic and larval development with fertilization under pressure***

The embryonic developmental stage, after 4 and 24 hour treatments, was assessed for pressures of 1, 100, 200 and 300 atm (plus 400 and 500 atm for 4 hour treatments only, as the 24 hour treatment performed first indicated that successful fertilization was likely at greater pressures) and at temperatures of 10, 15 and 20°C, with fertilization occurring under pressure. Gametes from three different males and three different females were used, so that three replicates (each from one male/female pair) were assigned to each pressure/temperature combination.

The following method was designed to prevent fertilization before pressurising eggs and sperm, and at the same time allowing them to mix under pressure: eggs from each female were collected from the glass bowls and re-suspended in ambient seawater, in a 1 l beaker, and then transferred into 6 ml plastic vials; 0.5 ml of diluted sperm suspension was pipetted into a 1 ml microcentrifuge tube (leaving 0.5 ml of air space); one microcentrifuge tube containing the sperm suspension was inserted into each plastic vial containing the egg suspension (figure 1*a*); the plastic vial was refilled with the egg suspension until it overflowed and the cap closed, avoiding any air being trapped inside; plastic vials were placed inside the pressure vessel (figure 1*b*) and filled with tap water at the test temperature; the pressurization to the desired pressure was continuous. When pressurising the vessels, it was possible to hear the microcentrifuge tubes imploding inside the plastic vials at approximately 50 atm, due to air left inside on purpose. In all treatments, pressurization was continuous and took at most 10 s until reaching the desired experimental pressure level.

For the atmospheric pressure cultures, fertilization occurred when eggs and sperm were mixed in a 1 l beaker and the solution was transferred into 6 ml plastic vials. Pressure chambers and 1 atm cultures were incubated at 10, 15 and 20°C for 4 and 24 hours. At the end of each trial, pressure vessels were depressurized and samples for the analysis of the developmental stage were quickly preserved in 4 per cent formalin. Fifty embryos from each replicate were randomly selected and staged according to the embryonic development.

After depressurization, sub-samples from the vials containing embryos were immediately preserved in 3 per cent glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, and kept refrigerated at 4°C until used for scanning electron microscopy (SEM). Samples were subsequently rinsed with buffer, dehydrated and critical point dried. Specimens were then mounted on stubs and sputter-coated in gold. SEMs were taken using a Hitachi S800 SEM.

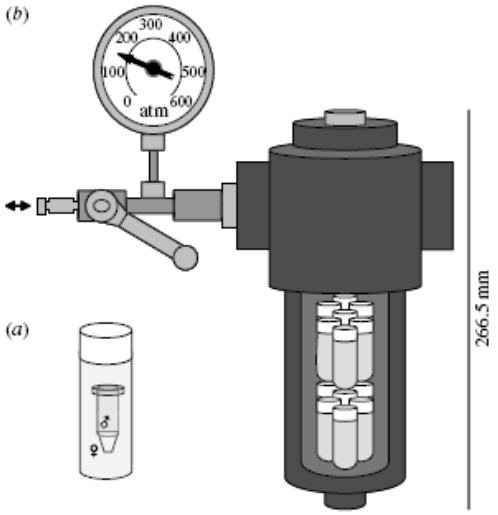

Figure 1. Schematic of the experimental pressure vessels: (*a*) plastic vial filled with the egg suspension and the microcentrifuge tube half-filled with sperm suspension; (*b*) pressure vessel showing the plastic vials inside (arrow indicates the connection valve to the hydraulic pump).

(d) ***Pressure effect on embryonic and larval development with fertilization at atmospheric pressure***

For the treatments with fertilization occurring at atmospheric pressure, analysing at 50 hours of embryonic and larval development, three cultures of fertilized eggs were prepared by mixing freshly spawned male and female gametes in a 1 l beaker in filtered seawater (1.6 µm retention) at 15°C (ambient temperature). Gametes from each male/female pair were used in each culture. Fertilized eggs were transferred into 6 ml plastic vials, filled until overflowing and with the cap closed to avoid any air being trapped inside. Plastic vials were placed inside the pressure chambers, filled with tap water at the test temperature, and the pressurization

to the desired pressure was continuous. The fertilized eggs were incubated at 1, 100, 200 and 300 atm, and 5, 10, 15, 20 and 25°C. After 50 hours, pressure vessels were depressurized and all samples were quickly preserved in 4 per cent formalin. Fifty embryos from each replicate were randomly selected and staged according to the embryonic development.

(e) *Statistical analyses*

Data on the proportion of abnormally developing embryos present in each culture failed the assumption of normality, even after the data were arcsine transformed. The raw data were used in the non-parametric Kruskal–Wallis single factor analysis of variance by ranks to test for temperature effects and pressure effects on the proportion of abnormally developing embryos. Temperature and pressure effects were tested separately, for each treatment and each incubation period (e.g. results of pressure effects are for each temperature tested in each incubation period).

3. RESULTS

(a) *Temperature effects on embryonic and larval development*

Mytilus edulis embryos develop faster at higher temperatures. For example, at 1 atm most embryos incubated at 5°C require 50 hours to reach stage V (multi-cell; figure 2; table 1) but reached stage X (D-larva) at 20°C. At 10°C and after 4 hours of incubation, the embryos developed to stage III (four-cell stage; figure 3), after 24 hours they reached stage VI (early blastula; figure 4) and past 50 hours they reached stage VIII (early trochophore). After 4 hours incubation at 15 and 20°C, the embryos developed to stage V, past 24 hours, the embryos reared at 15°C were in stage VII (gastrula) and those reared at 20°C reached stage X. The embryos incubated at 15°C needed 50 hours to get to D-larvae stage. At 25°C, those that survived 50 hours incubation reached stages IX and X (late trochophore and D-larvae, respectively), although 93 per cent of the embryos were developing abnormally or dying, and the culture was quickly degrading.

When testing for effects of temperature in the proportion of abnormally developing embryos, reared at atmospheric pressure, it is possible to detect a significant effect in the 50 hours incubation period when all temperatures are analysed together (5–25°C; Kruskal–Wallis $H=6.058$, $p=0.011$; table 2). There is no significant effect of temperature (tested temperatures: 10, 15 and 20°C) in the proportion of embryos developing abnormally for incubation periods of 4 hours and 24 hours for any pressure treatment (table 2).

(b) *Pressure effects on embryonic and larval development*

Fertilization under pressure succeeded in all pressure treatments (figures 3 and 4). After 4 hours incubation, in both 10 and 15°C treatments for 100–500 atm, eggs were fertilized but did not develop (figure 3). A small proportion of embryos reached stage V (multi-cell), when incubated for 4 hours at 20°C per 100 atm, but from 200 atm up to 500 atm embryos were in less advanced stages, indicating a retarded development. For the 20°C cultures, with increasing pressure the number of abnormal individuals increased (figure 3).

In the embryos exposed for 24 hours, the maximum developmental stage reached was for the cultures reared at atmospheric pressure (figure 4). A similar pattern occurred for the cultures at 100 atm with a maximum development for the 20°C treatment: stage VIII (early trochophore). At 20°C per 200 atm more than 92 per cent of the embryos did undergo abnormal development, while at 15°C per 200 atm embryos reached stage VI, and at 10°C per 200 atm a small portion of embryos developed to stage V (figure 4). At 300 atm and for all temperatures tested, embryos did not develop. In all temperatures, an increase of abnormal development with increasing pressure was observed.

The cultures exposed to 100 atm for 50 hours and incubated at 5, 10 and 15°C behaved very similarly to those reared at atmospheric pressure, while at higher temperatures most of embryos were abnormal (figure 2). In both 20 and 25°C cultures exposed to 200 and 300 atm, the embryos were abnormal or died. In the cultures reared at 300 atm at 5 and 10°C, embryos did not develop, and most of them remained in stage I—uncleaved showing a polar body—after fertilization (figure 2). On the other hand, at 10°C per 200 atm more than 30 per cent of embryos reached stage V—multi-cell stage (figure 2). At 15°C per 200 atm, the few embryos developing normally reached stage IX (figure 2). In general, the degree of abnormal development observed in embryos increases with both increased pressure and temperature.

Abnormal cleavages, the lack of cell membrane, and extrusions of the cytoplasm originating knob-like structures on the exterior of the embryo are the common abnormalities observed in pressurized cultures (see figures in the electronic supplementary material). The effect of pressure on the proportion of abnormally developing embryos was tested for each temperature and each incubation period (table 3). Results show no significant effect of pressure on all the temperatures analysed (10, 15 and 20°C) for both the incubation periods of 4 and 24 hours, except for treatment of 20°C per 24 hours (Kruskal–Wallis $H=8.128$, $p=0.043$; table 3). With a longer incubation period of 50 hours, the effect of pressure on the proportion of abnormally developing embryos is significant for all temperatures tested (table 3), except for the 5°C cultures (Kruskal–Wallis $H=6.407$, $p=0.093$; table 3).

4. DISCUSSION

(a) *Methodological considerations*

The method to induce spawning used in this work results from a combination of different approaches suggested in the literature (e.g. Costello *et al.* 1957; Sprung & Bayne 1984; Young & Tyler 1993). The mechanical, temperature and salinity shocks proved to be efficient, with at least 15 per cent of mussels spawning per trial.

Fertilization under pressure was 100 per cent effective as all the microcentrifuge tubes imploded under pressure allowing fertilization to take place. This new and simple technique may be used in future pressure studies as a way to mix small volumes of solutions under pressure. As fertilization rates at atmospheric pressure and at tolerated pressures (i.e. 100 atm at 10 and 15°C, figures 2–4) are comparable, we do not assume imploding microtube vials to significantly bias the fertilization process, i.e. by lysing

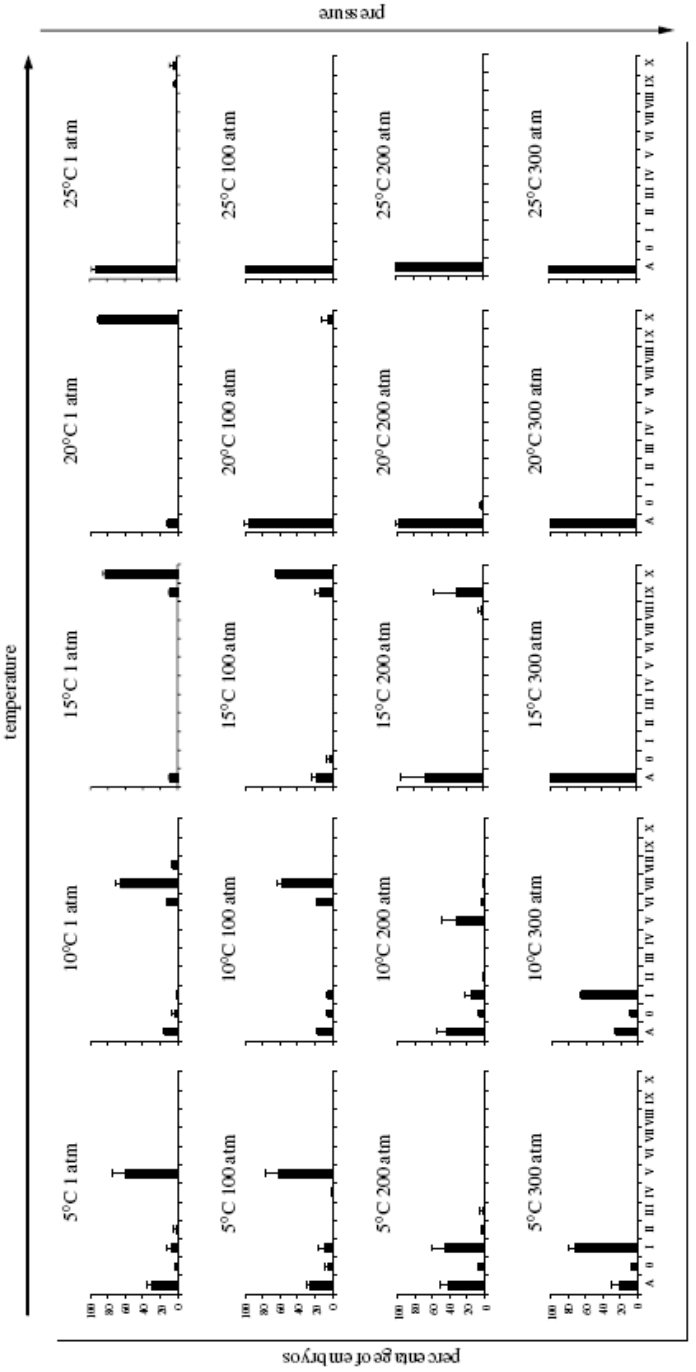

Figure 2. *Mytilus edulis* embryonic and early larval development incubated at different pressure/temperature regimes for 50 hours, with fertilization occurring at atmospheric pressure. Histograms are of percentage mean and standard deviation. Stages of development: A = abnormal; 0 = unfertilized; I = fertilized, uncleaved egg; II = two-cell; III = four-cell; IV = eight-cell; V = multi-cell; VI = early blastula; VII = gastrula; VIII = early trochophore; IX = late trochophore; X = D-larva (see table 1 for a detailed description of each developmental stage).

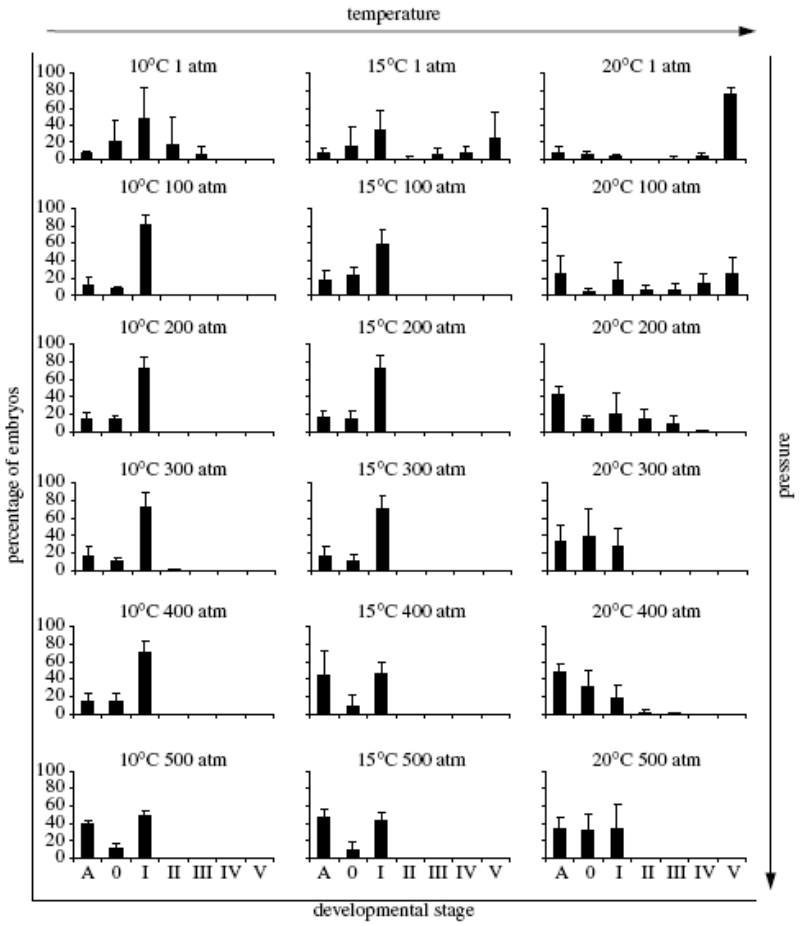

Figure 3. *Mytilus edulis* embryonic development incubated at different pressure/temperature regimes for 4 hours, with fertilization occurring under pressure. Histograms are of percentage mean and standard deviation. Stages of development: A=abnormal; 0=unfertilized; I=fertilized, uncleaved egg; II=two-cell; III=four-cell; IV=eight-cell; V=multi-cell (see table 1 for a detailed description of each developmental stage).

cells. The 'popping microcentrifuge' tube approach of fertilization has the advantage of not affecting the pH or seawater density. When developing the experimental design, dissolvable capsules yielded little satisfying results as the dissolving capsules drastically affected the viscosity of small volumes of seawater. Fertilization of eggs following rupture of microcentrifuge tubes at approximately 50 atm and prior to reaching the desired experimental pressure is unlikely. Pressurization was continuous and only lasted 5–7 s following rupture of microcentrifuge tubes. Successful fertilization in *M. edulis* eggs took between 40 and approximately 60 s when studied at 1 atm under a compound microscope and within the temperature range tested.

The present work focuses on the study of the early embryonic phase of the *M. edulis* life cycle that corresponds to the full embryogenic period, from fertilization to the early D-larvae stage, when embryos do not feed and rely on nutritional reserves of maternal origin present in the oocytes. In our study, gametogenesis in *M. edulis* took place at approximately 15°C, the maintenance temperature of adult blue mussels. Pre-spawning temperatures may affect physiological tolerances in the offspring and thus future study should focus on the effect of temperature on offspring quality in *M. edulis*. Whereas survival rates in embryos and larvae might be enhanced if acclimation to the experimental temperature would include gametogenesis, we do not believe that the overall physiological tolerance pattern found in this study would have changed.

(b) *Temperature and pressure effect on embryogenesis*

Our data suggest that at atmospheric pressure the temperature tolerance window for successful early embryogenesis of *M. edulis* ranges from approximately 10–20°C. The embryo development scales with temperature (for review see Anger 2001), i.e. embryos take more time at lower temperatures to reach the same embryonic stage than those reared at higher temperatures. At higher temperatures, close to thermal limits, a higher proportion of abnormally developing embryos are observed. This

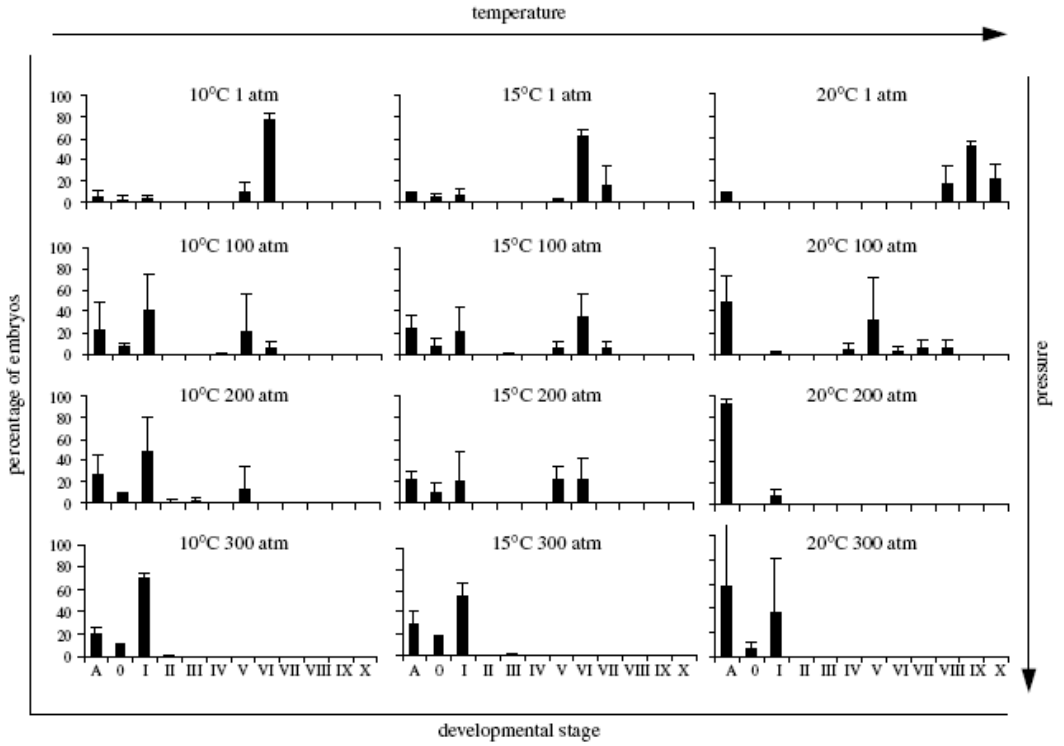

Figure 4. *Mytilus edulis* embryonic and early larval development incubated at different pressure/temperature regimes for 24 hours, with fertilization occurring under pressure. Histograms are of percentage mean and standard deviation. Stages of development: A=abnormal; 0=unfertilized; I=fertilized, uncleaved egg; II=two-cell; III=four-cell; IV=eight-cell; V=multi-cell; VI=early blastula; VII=gastrula; VIII=early trochophore; IX=late trochophore; X=D-larva (see table 1 for a detailed description of each developmental stage).

Table 2. Kruskal–Wallis analysis of variance testing the effects of temperature (10, 15 and 20°C) on the proportion of abnormally developing embryos reared at the different pressures for different incubation periods. (*H* statistic (degrees of freedom, *N*=number of replicates); *for temperatures: 5, 10, 15, 20 and 25°C.)

	incubation period							
	4 hours		24 hours		50 hours		50 hours*	
pressure (atm)	*H* (2, *N*=9)	*p*-value	*H* (2, *N*=9)	*p*-value	*H* (2, *N*=9)	*p*-value	*H* (4, *N*=15)	*p*-value
1	0.073	0.964	0.615	0.735	6.058	0.048	13.099	0.011
100	0.610	0.737	2.443	0.295	5.695	0.058	12.603	0.013
200	5.728	0.057	5.600	0.061	4.545	0.103	11.070	0.026
300	1.689	0.430	1.107	0.575	7.624	0.022	13.086	0.011

Table 3. Kruskal–Wallis analysis of variance testing the effects of pressure (1, 100, 200 and 300 atm) on the proportion of abnormally developing embryos reared at different temperatures for different incubation periods. (*H* statistic (degrees of freedom, *N*=number of replicates).)

	incubation period					
	4 hours		24 hours		50 hours	
temperature (°C)	*H* (3, *N*=12)	*p*-value	*H* (3, *N*=12)	*p*-value	*H* (3, *N*=12)	*p*-value
5	—	—	—	—	6.407	0.093
10	1.568	0.667	4.351	0.226	10.157	0.017
15	1.977	0.577	6.590	0.086	10.237	0.017
20	5.100	0.165	8.128	0.043	8.194	0.042
25	—	—	—	—	10.735	0.013

is probably related to the metabolic rates increasing with increasing temperatures; although temperatures might not yet have reached a lethal state, more energy has to be allocated to the metabolism, which negatively affects the embryo development.

Successful embryo development is possible from 1 atm up to 500 atm, which was the maximum pressure condition tested. Active sperm cells were observed under a compound microscope straight after depressurization when exposed at 500 atm for 4 hours. With the presented pressure range tested, we hypothesize that pressure presents no barrier to fertilization. It is possible to observe a slower development with increasing pressure, as well as an increase of abnormal development of the embryos. Standard deviations potentially reflect the intraspecific variability found in the early ontogeny of *M. edulis* (figure 3), which is higher in the amount of unfertilized eggs and abnormal embryos. Most of the abnormalities observed are due to rupture of the egg membrane, while cleavages apparently continue occurring. The egg fertilization membrane is only supposed to break after the blastula stage (Zardus & Martel 2002), but was observed for earlier stages of development for pressures $\geq$200 atm (figure S2*c* in the electronic supplementary material).

The cytoplasmic extrusions observed in pressurized embryos (figures S1*b*,*d* and S2*c* in the electronic supplementary material) have been described in pressurized protozoans (Landau *et al.* 1954; Kitching 1957). According to these authors, elevated pressures cause (i) a reduction in the protoplasmic gel strength, and (ii) a weakness of the more rigid cortical portions of the cell. These effects apparently lead to a separation of the cell membrane from the cytoplasm, and to the formation of knob-like structures in the exterior of the cell membrane—ultimately leading to cell-membrane breakage. Zimmerman & Marsland (1964) found that high pressure affects the mitotic apparatus of dividing cells. In the cleaving eggs of the sea urchin *Arbacia punctulata*, chromosomal movements become retarded at 140 atm and are completely arrested at 280 atm. This effect is in general reversible, but when subject to a pressure in excess of 544 atm for more than 5 min leads to a drastic and irreversible disorganization of the mitotic apparatus (Zimmerman & Marsland 1964). These experiments were for periods of 1–15 min with fertilization at atmospheric pressure. The authors argue that recovery might be possible, but at the same time the disorganization of the mitotic apparatus observed after a maximum pressurization time of 15 min could be permanently compromised.

The embryonic development in some shallow-water marine invertebrates is inhibited when subjected to high pressures (Marsland 1950; Salmon 1975), and this may limit the vertical distributions of species (Young & Tyler 1993). Pressure can even act differently for each early life-history stage, as found in the sea-urchin *Psammechinus miliaris*, in which the pelagic larva is more resilient to high pressure than the earlier embryonic stages (Aquino-Souza *et al.* 2008). With the results presented here, it would be possible to estimate that the theoretical maximum distribution depth for the *M. edulis* embryos is, at least 2000 m for the temperatures tested. Marsland (1950, 1970) found that in dividing marine eggs, the higher the temperatures, within the temperature tolerance range of a species, the stronger the cortical gel structures and their resistance to high pressures. Although, our results are contrary to this observation, as for example in the 50 hours treatment when comparing the cultures pressurized at 100 atm at 15 and 20°C, at 15°C embryos are more tolerant to pressure and develop to D-larvae, with no increment on the proportion of abnormally developing embryos.

Metabolic rates increase with increasing temperature (for review see Clarke 2003). In the case of *M. edulis*, the lower the pressure the faster the embryonic development. In the present study, a prolonged time of exposure to the different temperature/pressure combinations gives us a further view on this developmental process: after 4 hours of exposure, at 20°C we see higher tolerance to high pressure, as cleavages occur and the number of abnormalities is low. After 24 hours of exposure, it is possible to observe that those embryos undergo irregular cleavages and this may indicate that further successful progression in development is unlikely under high pressure. After 50 hours this is a fact, as embryos do not tolerate pressures greater than or equal to 100 atm.

Our results show embryos developing normally at high pressures, and low temperatures. Questions remain about whether embryos developing normally under pressure, but with a slower growth rate due to low temperature, would become abnormal as the complexity of developing embryos increases. We assume that there are particular stages in the early ontogeny which are crucial to tolerate pressure. When an embryo reaches these stages it will be defined whether there will be normal development or not. In similar pressure/temperature studies, evidence suggests that low temperature retards the embryonic development (e.g. Young *et al.* 1997; Tyler & Young 1998), and in the case of *M. edulis* pressure combined with temperature favours lower temperatures where fewer abnormalities are observed. Thus it is reasonable to hypothesize that the invasion of the deep sea by *M. edulis* is possible in terms of pressure tolerances in embryos and larvae.

(c) *Seeding the deep sea*

Wood-, bone-, vent- and seep-associated mussels are phylogenetically closely related and are indicative of a recent common ancestry for vent and non-vent species (Distel *et al.* 2000). A long isolation of this group of chemosynthetic mussels is proposed to be the cause of divergence from other mytilids from shallow water (Distel *et al.* 2000). It has been hypothesized that wood- and bone-associated mussels worked as an evolutionary step for shallow-water mussels to colonize hydrothermal vents and cold seeps (Distel *et al.* 2000). Deep-sea colonization by shallow-water species over evolutionary periods of time needs cold-stenothermy adapted species finding a rather stable, low temperature environment, which resulted in the concept of faunistic exchange through low temperature, isothermic water bodies (Kussakin 1973; Menzies *et al.* 1973; Hessler & Thistle 1975; Hessler & Wilson 1983; Thatje *et al.* 2005). Contrarily, the colonization of deep-water chemosynthetic environments would indeed require physiological properties in invertebrates that tolerate greater fluctuations in temperature (=cold-eurythermy), and the present study clearly demonstrated that blue mussels are a likely proof for such a scenario, which is furthermore supported by their close

phylogenetic relationship with the Bathymodilinae (Distel *et al.* 2000) and similarities in their reproductive and larval developmental cycle (Lutz *et al.* 1980; Berg 1985; Kenk & Wilson 1985). Supporting this view of similar physiological prerequisites in both temperate shallow-water and hydrothermal vent species, bivalves from deep-sea hydrothermal vents have also shown growth rates similar to mytilids found in shallow-water environments and their growth rates are much higher (several orders of magnitude) than those in bivalve species living in non-chemosynthetic deep-sea habitat (Lutz *et al.* 1985). It might be challenging to suggest that from an evolutionary perspective, hydrothermal vent environments have been the only places where shallow-water temperate to sub-tropical invertebrates were able to establish, owing to similar temperature-tolerance regimes. Similar establishment in low-temperature deep-sea environments can only occur if the species possess a cold stenothermy to a constant, low-temperature environment—a physiological pattern, we hypothesize, not favoured in shallow-water species from sub-tropical to temperate regions. We propose that the development of a thermocline is not necessarily a physiological barrier to pelagic larval stages and drifting stages of any kind to penetrate the deep sea, although its physical properties may influence the direct settlement by invertebrate larvae into deeper waters.

The results presented here challenge the idea by Distel *et al.* (2000) that mytilids of the genus *Bathymodiolus* did colonize eurythermal vent sites via cold-stenothermal chemosynthetic wood and cold-seep environments. This evolutionary pathway would imply that *Bathymodiolus* regained cold eurythermy to colonize hydrothermal vents following the colonization of cold-stenothermal chemosynthetic environments—an evolutionary step that is generally assumed highly unlikely. The herein presented results indeed support the fact that for species of the Mytilinae, a direct colonization from shallow water into deep-water sites that both demand the same temperature tolerance is a more likely scenario and that the physiological basis of organismal biology should be increasingly taken into consideration when studying the evolutionary history of deep-sea faunas. It is possible that only advanced larvae have been involved in seeding deep-sea habitats over long evolutionary periods of time owing to lack of food at greater depth. Here, it must be reiterated, however, that invertebrate larvae can arrest development for substantial periods of time when encountering unsuitable habitat conditions such as lower temperatures in the colder water column, until encountering a more suitable habitat for development (Anger 2001; Thatje *et al.* 2004), which in addition should facilitate direct shallow water—deep water exchange of floating early life-history stages, provided a hyperbaric tolerance is expressed.

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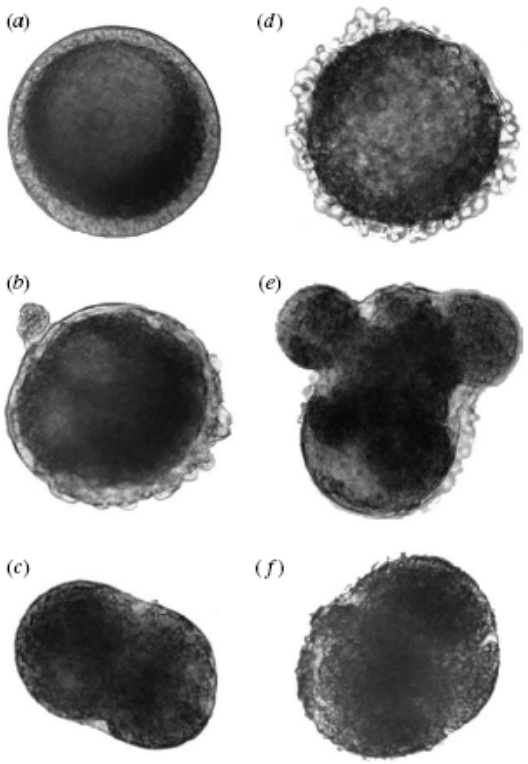

Figure S1. Examples of pressure effects on the morphology of *M. edulis* embryos incubated for 4 hours, with fertilization occurring at incubation pressure (light microscope images). (*a*) unfertilized egg (20°C, 300 atm); (*b*) fertilized egg showing abnormal development with an irregular cell membrane (20°C, 300 atm); (*c*) visible cleavage furrow of the two-cell stage (20°C, 400 atm); (*d*) unfertilized egg with cytoplasmic extrusions over the surface (10°C, 500 atm); (*e*) abnormally developing embryo (10°C, 500 atm); (*f*) abnormal cleavage furrow of the two-cell stage with cytoplasmic extrusions over the surface (10°C, 500 atm).

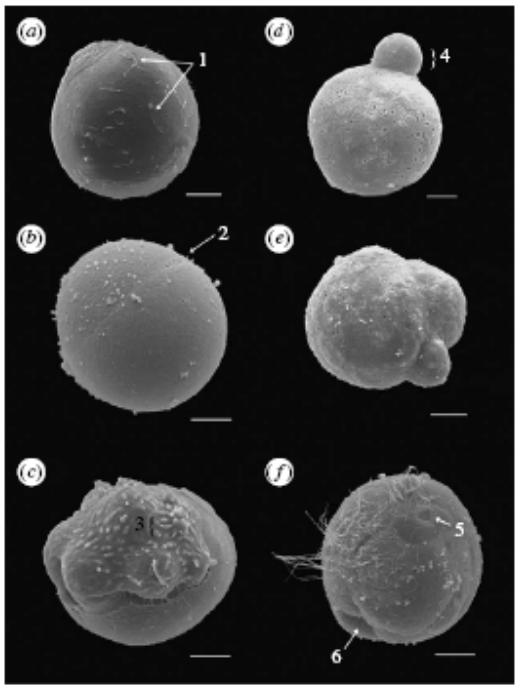

Figure S2. Examples of pressure effects on the morphology of *M. edulis* embryos incubated for 24 hours at 10°C, with fertilization occurring at incubation pressure (scanning electron micrographs, SEM). (*a*) uncleaved egg, with sperm cells (1) over its surface (200 atm); (*b*), cleavage furrow (2) of the two-cell stage (200 atm); (*c*) abnormal multi-celled embryo, with membrane breakage and cytoplasmic extrusions (3) over the surface (200 atm); (*d*) fertilized egg with rough surface and polar body (4) (300 atm); (*e*) two-cell stage embryo with rough surface and polar body (300 atm); (*f*) gastrula stage embryo with cilia, blastopore (5) and shell field invagination (6) (100 atm). Scale bars: 10 µm.

Appendix II. Tables and Figures – Pressure tolerance in *Palaemonetes varians*

Figure II.i. Live adult specimen of *Palaemonetes varians*.

Table II.i. Elemental composition of *Palaemonetes varians* larvae in different stages of development (ZI = zoea I, ZII = zoea II, ZIII = zoea III, DI = decapodid I, DII = decapodid II, J = juvenile; d = days; DW = dry weight, C = carbon, N = nitrogen). All larvae hatched on the same day and were reared at constant 20ºC (±0.5ºC) in the presence of food (*Artemia* nauplii) from day two. Larvae are from one female collected from the field in 2007.

stage, age (d)	DW (µg)	C (%)	N (%)	C:N Ratio
ZI (0)	157.00	37.91	8.74	4.34
ZI (0)	162.00	31.78	8.14	3.91
ZI (0)	172.00	33.13	8.54	3.88
ZI (0)	148.00	37.52	8.37	4.48
ZI (0)	171.00	36.49	8.59	4.25
x	**162.00**	**35.36**	**8.48**	**4.17**
±S.D.	**10.02**	**2.75**	**0.23**	**0.27**
ZII (2)	151.00	32.36	8.55	3.79
ZII (2)	142.00	31.29	7.82	4.00
ZII (2)	135.00	38.15	9.43	4.05
ZII (2)	140.00	33.99	9.46	3.59
x	**142.00**	**33.95**	**8.81**	**3.86**
±S.D.	**6.68**	**3.02**	**0.79**	**0.21**
ZIII (4)	186.00	38.58	10.34	3.73
ZIII (4)	174.00	39.73	11.05	3.60
ZIII (4)	209.00	40.11	10.09	3.98
ZIII (4)	182.00	33.89	9.42	3.60
x	**187.75**	**38.08**	**10.22**	**3.73**
±S.D.	**15.02**	**2.87**	**0.67**	**0.18**

stage, age (d)	DW (µg)	C (%)	N (%)	C:N Ratio
DI (6)	251.00	37.79	10.63	3.56
DI (6)	220.00	34.93	9.98	3.50
DI (6)	225.00	37.86	11.05	3.43
x	**232.00**	**36.86**	**10.55**	**3.49**
±S.D.	**16.64**	**1.68**	**0.54**	**0.06**
DII (8)	223.00	47.76	13.13	3.64
DII (8)	171.00	41.16	12.45	3.31
DII (8)	164.00	37.32	11.06	3.37
DII (8)	208.00	41.15	11.82	3.48
x	**191.50**	**41.85**	**12.12**	**3.45**
±S.D.	**28.52**	**4.34**	**0.88**	**0.14**
J (8)	182.00	40.03	11.67	3.43
J (8)	121.00	38.04	10.67	3.57
J (8)	218.00	35.53	10.10	3.52
J (8)	208.00	37.33	10.64	3.51
x	**182.25**	**37.73**	**10.77**	**3.51**
±S.D.	**43.56**	**1.86**	**0.65**	**0.06**

Table II.ii. *Palaemonetes varians.* Mann-Whitney U-test results table analysing statistical differences in elemental composition (DW = dry weight, C = carbon, N = nitrogen) between decapodid II – DII (developmental pathway 1) and juvenile – J (developmental pathway 2). *U* = Mann-Whitney statistic; *Z* = normal distribution variate value; *p* = level of significance; * = significantly different.

variable	valid n DII	valid n J	Rank Sum DII	Rank Sum J	*U*	*Z*	*p*
DW (µg)	4	4	18.50	17.50	7.50	0.144	0.885
C (%)	4	4	23.00	13.00	3.00	1.443	0.149
N (%)	4	4	25.00	11.00	1.00	2.021	0.043*
C:N Ratio	4	4	15.00	21.00	5.00	-0.866	0.386

Table II.iii. Individual dry weight (DW) and elemental composition in carbon (C) and nitrogen (N) of newly hatched larvae of *Palaemonetes varians*. Female A to F were collected from the field in 2007 and females G to L were obtained from the field in 2008.

female 2007	DW (µg)	C (%)	N (%)	C:N Ratio
A	135	42.14	9.43	4.47
A	141	40.82	9.31	4.39
A	120	44.40	10.66	4.17
A	131	45.36	10.62	4.27
x	**131.75**	**43.18**	**10.01**	**4.32**
±S.D.	**8.85**	**2.07**	**0.74**	**0.13**
B	145	42.31	10.34	4.09
B	134	47.43	11.09	4.28
B	144	46.44	10.70	4.34
B	143	43.63	9.34	4.67
x	**141.50**	**44.95**	**10.37**	**4.35**
±S.D.	**5.07**	**2.38**	**0.75**	**0.24**
C	134	45.83	10.59	4.33
C	143	41.91	9.66	4.34
C	141	42.23	9.74	4.34
C	122	44.73	10.47	4.27
x	**135.00**	**43.68**	**10.11**	**4.32**
±S.D.	**9.49**	**1.91**	**0.48**	**0.03**
D	140	34.53	7.72	4.47
D	118	37.86	8.54	4.43
D	113	42.27	10.07	4.20
D	135	31.90	8.46	3.77
D	124	31.77	7.45	4.26
x	**126.00**	**35.67**	**8.45**	**4.23**
±S.D.	**11.34**	**4.45**	**1.02**	**0.28**
E	175	34.13	7.85	4.35
E	158	37.20	8.43	4.41
E	175	35.89	8.04	4.47
x	**169.33**	**35.74**	**8.11**	**4.41**
±S.D.	**9.81**	**1.54**	**0.30**	**0.06**
F	157	37.91	8.74	4.34
F	162	31.78	8.14	3.91
F	172	33.13	8.54	3.88
F	148	37.52	8.37	4.48
F	171	36.49	8.59	4.25
x	**162.00**	**35.36**	**8.48**	**4.17**
±S.D.	**10.02**	**2.75**	**0.23**	**0.27**
2007				
x	**143.24**	**39.58**	**9.24**	**4.29**
±S.D.	**18.02**	**5.00**	**1.10**	**0.20**

female 2008	DW (µg)	C (%)	N (%)	C:N Ratio
G	163	31.65	7.55	4.19
G	140	38.78	9.47	4.09
G	189	28.98	7.13	4.07
G	134	28.03	7.24	3.87
x	**156.50**	**31.86**	**7.85**	**4.06**
±S.D.	**25.01**	**4.86**	**1.10**	**0.13**
H	123	41.8	11.03	3.79
H	144	31.8	7.77	4.09
H	121	35.85	9.12	3.93
H	91	47.82	12.78	3.74
H	135	34.58	8.09	4.28
x	**122.80**	**38.37**	**9.76**	**3.97**
±S.D.	**20.08**	**6.42**	**2.11**	**0.22**
I	58	34.24	8.58	3.99
I	70	29.12	7.87	3.7
I	68	31.42	7.65	4.11
x	**65.33**	**31.59**	**8.03**	**3.93**
±S.D.	**6.43**	**2.56**	**0.49**	**0.21**
J	89	58.28	13.81	4.22
J	96	54.22	13.38	4.05
J	98	56.2	13.81	4.07
J	88	61.22	14.06	4.36
x	**92.75**	**57.48**	**13.77**	**4.18**
±S.D.	**4.99**	**2.99**	**0.28**	**0.14**
K	120	47.91	10.99	4.36
K	122	41.59	10.23	4.07
K	113	44.13	11.63	3.79
K	103	44.35	10.5	4.22
K	131	41.24	9.63	4.28
x	**117.80**	**43.84**	**10.60**	**4.14**
±S.D.	**10.47**	**2.68**	**0.76**	**0.22**
L	117	54.24	11.85	4.58
L	119	52.06	11.72	4.44
L	131	47.36	11.3	4.19
L	133	48.01	11.84	4.06
L	134	47.84	10.8	4.43
x	**126.80**	**49.90**	**11.50**	**4.34**
±S.D.	**8.14**	**3.07**	**0.45**	**0.21**
2008				
x	**116.54**	**42.80**	**10.38**	**4.11**
±S.D.	**29.39**	**9.83**	**2.21**	**0.23**

Table II.iv. Effect of number of larvae reared per vial (1x / vial = 1 larva per vial; 5x / vial = 5 larvae per vial) on the percentage of survival of the zoea I that successfully moulted to zoea II larval stage of *Palaemonetes varians*. Larvae were incubated at 20ºC and different pressures. Larvae hatched on the same day and are from females collected from the field in 2006; n = initial number of larvae per treatment.

pressure (atm)	1x / vial		5x /vial	
	n	survival	n	survival
100	14	100%	45	68.9%
200	14	100%	45	24.4%
300	14	35.7%	45	0.0%

Table II.v. Effect of pressure and temperature on the survival of the zoea I that successfully moulted to zoea II larval stage of *Palaemonetes varians* (survival ZII), with account for the identified stages in dead larvae (dead in ZII stage; dead at moult; dead in zoea I stage – ZI; dead not determined – n.d.). Larvae are from females collected from the field in 2006. n = initial number of larvae per treatment.

temperature (ºC) age (days)	pressure (atm)	n	survival ZII	dead ZII	dead at moult	dead ZI	dead n.d.
5 (10)	1	24	--	--	--	100%	--
	100	14	--	--	--	100%	--
	200	14	--	--	--	100%	--
	300	14	--	--	--	100%	--
10 (7)	1	18	83.3%	--	--	16.7%	--
	100	28	64.3%	--	21.4%	14.3%	--
	200	28	0%	--	--	100%	--
	300	28	0%	--	--	100%	--
20 (3)	1	12	100%	--	--	--	--
	100	28	96.4%	3.6%	--	--	--
	200	28	78.6%	7.1%	3.6%	--	10.7%
	300	28	0%	3.6%	--	42.9%	53.6%
30 (2)	1	12	100%	--	--	--	--
	100	28	82.1%	14.3%	--	--	3.6%
	200	28	17.9%	--	67.9%	--	14.3%
	300	28	0%	--	--	100%	--

Table II.vi. Individual elemental composition of *Palaemonetes varians* larvae after each pressure and temperature treatment. (DW = dry weight; C = carbon; N= nitrogen). Data are for live zoea II larval stage, dead zoea II (††) and dead zoea I (†). Larvae hatched on the same day and are from females AA (10 and 20ºC treatments) and BB (30ºC treatments), collected from the field in 2007.

pressure (atm)	temperature (ºC)	DW (µg)	C (%)	N (%)	C:N Ratio
1	**10**	148.00	34.13	8.81	3.87
1	**10**	112.00	39.48	10.98	3.60
1	**10**	116.00	42.66	10.82	3.94
1	**10**	149.00	34.26	8.55	4.01
1	**10**	148.00	35.10	8.75	4.01
	x	**134.60**	**37.13**	**9.58**	**3.89**
	±S.D.	**18.86**	**3.79**	**1.21**	**0.17**
1	**20**	128.00	38.72	10.49	3.69
1	**20**	124.00	38.66	10.40	3.72
1	**20**	124.00	37.91	10.07	3.76
1	**20**	135.00	38.10	10.59	3.60
1	**20**	101.00	39.72	10.22	3.89
	x	**122.40**	**38.62**	**10.35**	**3.73**
	±S.D.	**12.78**	**0.71**	**0.21**	**0.11**
1	**30**	140.00	37.88	9.97	3.80
1	**30**	144.00	37.70	10.42	3.62
1	**30**	129.00	38.63	10.48	3.69
1	**30**	113.00	40.31	10.82	3.72
	x	**131.50**	**38.63**	**10.42**	**3.71**
	±S.D.	**13.87**	**1.19**	**0.35**	**0.08**
100	**20**	157.00	34.54	9.49	3.64
100	**20**	134.00	38.66	10.52	3.68
100	**20**	143.00	34.43	8.97	3.84
100	**20**	145.00	34.47	9.41	3.66
	x	**144.75**	**35.53**	**9.60**	**3.70**
	±S.D.	**9.46**	**2.09**	**0.66**	**0.09**
100 (†)	**30**	122.00	37.52	10.03	3.74
100 (†)	**30**	118.00	39.82	10.32	3.86
100 (†)	**30**	110.00	46.71	12.28	3.80
100 (†)	**30**	107.00	36.63	10.04	3.65
	x	**114.25**	**40.17**	**10.67**	**3.76**
	±S.D.	**6.95**	**4.56**	**1.08**	**0.09**
200 (††)	**20**	111.00	42.38	11.87	3.57
200 (††)	**20**	94.00	53.75	14.38	3.74
200 (††)	**20**	129.00	37.62	9.40	4.00
	x	**111.33**	**44.58**	**11.88**	**3.77**
	±S.D.	**17.50**	**8.29**	**2.49**	**0.22**
300 (†)	**20**	85.00	38.52	10.08	3.82
300 (†)	**20**	101.00	46.26	10.91	4.24
300 (†)	**20**	86.00	39.33	9.20	4.27
	x	**90.67**	**41.37**	**10.07**	**4.11**
	±S.D.	**8.96**	**4.25**	**0.86**	**0.25**

Appendix III. Tables and Figures – Reproductive biology of vent shrimps

Table III.i. Total length (TL) and carapace length (CL) female (♀) specimens of *Mirocaris fortunata* (Mf) collected from Lucky Strike (LS) in August 2005.

Sample ID ♀	TL (mm)	CL (mm)	Sample ID ♀	TL (mm)	CL (mm)
Mf LS 1	24	7	**Mf LS 15**	24	7
Mf LS 2	31	10	**Mf LS 16**	25	8
Mf LS 3	20	6	**Mf LS 17**	19	6
Mf LS 4	20	7	**Mf LS 18**	19	6
Mf LS 5	23	7	**Mf LS 19**	24	7
Mf LS 6	24	7	**Mf LS 20**	23	7
Mf LS 7	29	10	**Mf LS 21**	23	7
Mf LS 8	24	8	**Mf LS 22**	20	6
Mf LS 9	25	8	**Mf LS 23**	18	6
Mf LS 10	26	9	**Mf LS 24**	17	6
Mf LS 11	26	9	**Mf LS 25**	22	7
Mf LS 12	25	8	**Mf LS 26**	15	5
Mf LS 13	16	5	**Mf LS 27**	15	5
Mf LS 14	18	6			

Table III.ii. Mann-Whitney U-test results table analysing statistical differences in total length (TL) and carapace length (CL) between males (♂) and females (♀) of *Mirocaris fortunata* collected from Lucky Strike in 2005. *U* = Mann-Whitney statistic; *Z* = normal distribution variate value; *p* = level of significance; * = significantly different.

variable	valid n ♀	valid n ♂	Rank Sum ♀	Rank Sum ♂	*U*	*Z*	*p*
TL (mm)	27	12	599.50	180.50	102.50	1.811	0.070
CL (mm)	27	12	623.00	157.00	79.00	2.526	0.012*

Table III.iii. Total length (TL) and carapace length (CL) for male (♂) and female (♀) specimens of *Mirocaris fortunata* (Mf) collected from Rainbow (Rb) in August 2006.

Sample ID ♀	TL (mm)	CL (mm)	Sample ID ♂	TL (mm)	CL (mm)
Mf Rb 1	24	7	**Mf Rb A**	17	5
Mf Rb 2	22	7	**Mf Rb B**	17	5
Mf Rb 3	21	6	**Mf Rb C**	16	5
Mf Rb 4	18	6	**Mf Rb D**	21	6
Mf Rb 5	23	7	**Mf Rb E**	19	6
Mf Rb 6	17	6	**Mf Rb F**	21	7
Mf Rb 7	15	5	**Mf Rb G**	21	7
Mf Rb 8	16	5	**Mf Rb H**	19	6
Mf Rb 9	17	6	**Mf Rb I**	23	7
Mf Rb 10	18	6	**Mf Rb J**	24	8
Mf Rb 11	15	5	**Mf Rb K**	21	6
Mf Rb 12	23	7	**Mf Rb L**	20	7
Mf Rb 13	16	5	**Mf Rb M**	21	7
Mf Rb 14	22	8	**Mf Rb N**	14	5
Mf Rb 15	15	5	**Mf Rb O**	15	5
Mf Rb 16	12	4	**Mf Rb P**	21	7
Mf Rb 17	15	5			
Mf Rb 18	15	5			
Mf Rb 19	24	8			
Mf Rb 20	23	8			
Mf Rb 21	22	7			
Mf Rb 22	21	7			
Mf Rb 23	23	7			

Table III.iv. Mann-Whitney U-test results table analysing statistical differences in total length (TL) and carapace length (CL) between males (♂) and females (♀) of *Mirocaris fortunata* collected from Rainbow in 2006. *U* = Mann-Whitney statistic; *Z* = normal distribution variate value; *p* = level of significance.

variable	valid n ♀	valid n ♂	Rank Sum ♀	Rank Sum ♂	*U*	*Z*	*p*
TL (mm)	23	16	459.50	320.50	183.50	-0.014	0.989
CL (mm)	23	16	459.00	321.00	183.00	-0.029	0.977

Table III.v. Mann-Whitney U-test results table analysing statistical differences in total length (TL) and carapace length (CL) from males (♂) and females (♀) of *Mirocaris fortunata* between the two sites Lucky Strike (LS) 2005, and Rainbow (Rb) 2006. *U* = Mann-Whitney statistic; *Z* = normal distribution variate value; *p* = level of significance; * = significantly different.

variable	valid n LS	valid n Rb	Rank Sum LS	Rank Sum Rb	*U*	*Z*	*p*
TL ♀	27	23	826.50	448.50	172.50	2.686	0.007*
CL ♀	27	23	791.50	483.50	207.50	2.005	0.045*
TL ♂	12	16	173.50	232.50	95.50	-0.023	0.981
CL ♂	12	16	158.50	247.50	80.50	-0.720	0.472

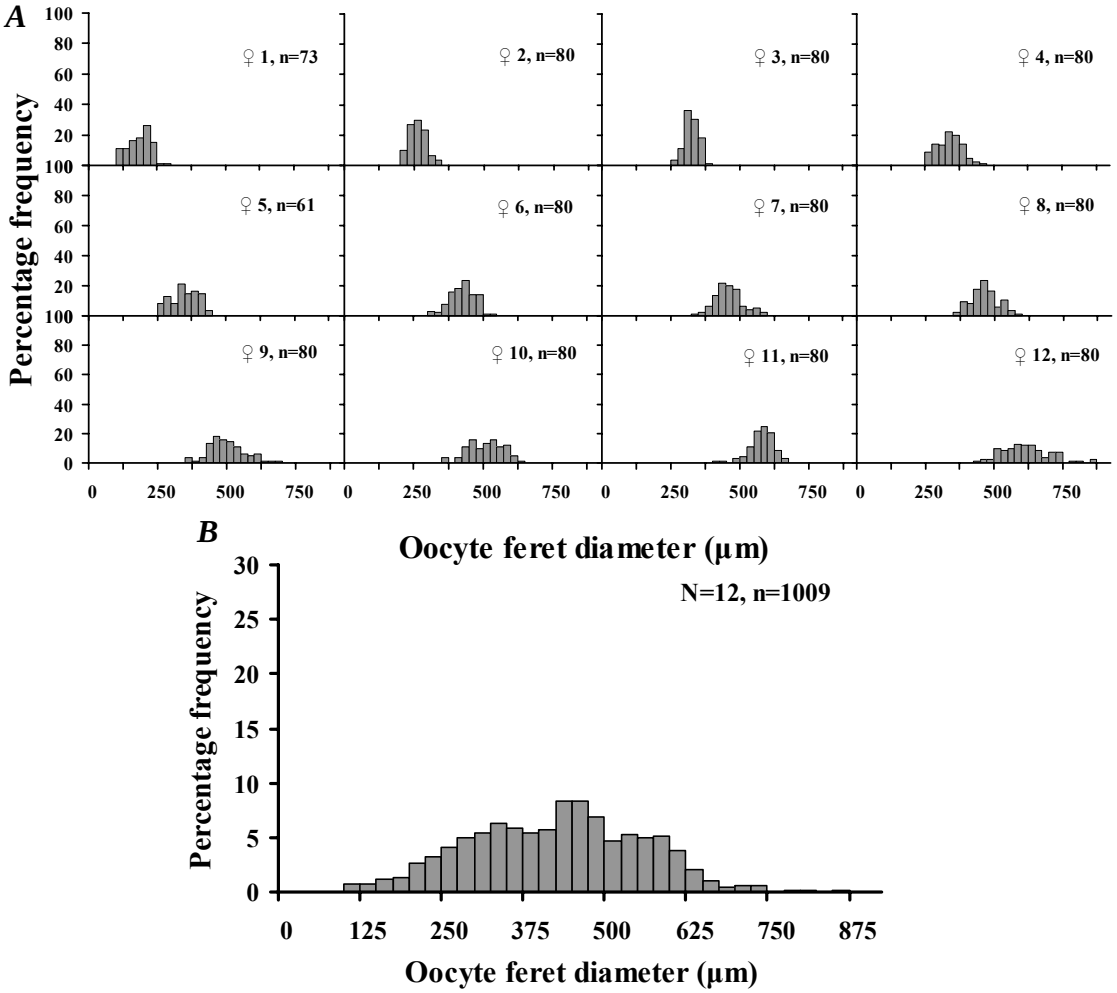

Fig. III.i. Oocyte size-frequency distributions of *Mirocaris fortunata* (Lucky Strike, August 2005). *A*-Oocyte size-frequency distributions of individuals. *B*- Summated oocyte size-frequency distribution for twelve individuals. N=number of females; n=number of oocytes.

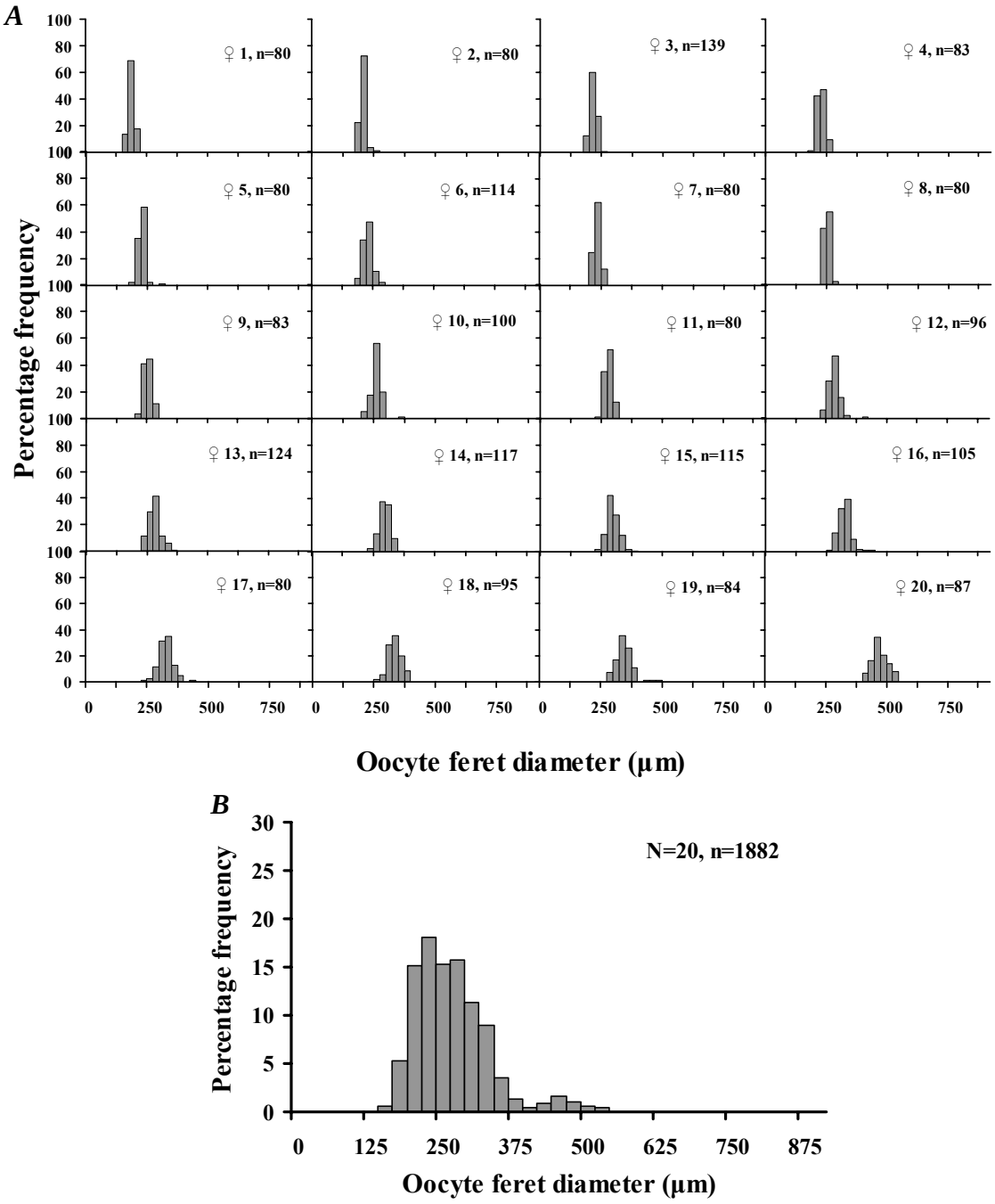

Fig. III.ii. Oocyte size-frequency distributions of *Rimicaris exoculata* (Rainbow, August 2006). *A*-Oocyte size-frequency distributions of individuals. *B*- Summated oocyte size-frequency distribution for twenty individuals. N=number of females; n=number of oocytes.

Appendix IV. Methods and Results – Temperature and pressure tolerance in the early ontogeny of *Psammechinus miliaris*

Material and Methods

Sampling and spawning

Adult specimens of *Psammechinus miliaris* were collected from Torquay, Devon, UK (50°28'N, 3°31'W) and maintained at 15ºC ± 1.5ºC in a running seawater system in the aquarium of the National Oceanography Centre, Southampton, where the experimental work took place during April 2008. Spawning was induced by injecting of 0.3 mL of 0.55 M KCl through the mouth of each sea urchin (salinity shock; Young & Tyler, 1993). To obtain gametes, each sea urchin was placed in individual glass bowls with filtered seawater at ambient temperature of about 20ºC and spawning would start almost immediately in males and after a maximum of 30 minutes in females.

Temperature and pressure effect on embryonic development with fertilization under pressure

The embryonic development stage, after 4 hours incubation was accessed for pressures of 1, 50, 100, 200 and 300 atm and at temperatures of 10, 15 and 20ºC, with fertilization occurring under pressure for test pressures 50-300 atm. For the atmospheric pressure treatments, fertilization occurred by mixing eggs and sperm in a 1 L beaker and transferring the solution into 6 mL plastic vials. Gametes from three different males and three different females were used, so that three replicates were assigned to each pressure/temperature combination (Fig. IV.i.).

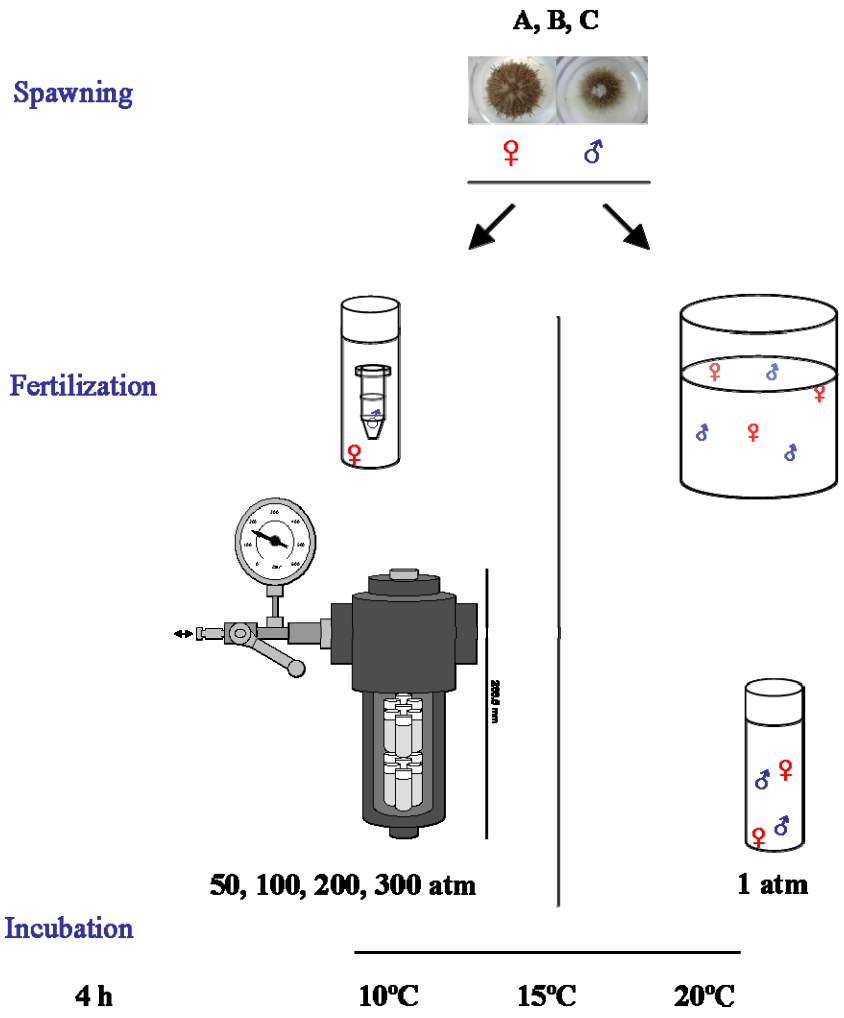

Fig. IV.i. Schematic of the experimental set up: spawning; fertilization under pressure (left) and at atmospheric pressure (right); incubation for 4 hours of the different pressure and temperature treatments (replicates A, B and C).

The method for fertilization under pressure (after Mestre et al., 2008) is described in Chapter 2: eggs from each female were collected from the glass bowls and re-suspended in ambient seawater, in a 1 L beaker, and then transferred into 6 mL plastic vials; 0.5 mL of diluted sperm suspension was pipetted into a 1 mL microcentrifuge tube; one microcentrifuge tube containing the sperm suspension was inserted into each plastic vial containing the egg-suspension; the plastic vial was refilled with the egg suspension until it overflowed and the cap closed, avoiding any air being trapped inside; plastic vials were placed inside the pressure vessel and filled

with tap water at the test temperature; the pressurization was continuous and did take at most 10 seconds until reaching the desired pressure level.

At the end of each trial, pressure vessels were depressurized and all samples were quickly preserved in 4% formalin. Fifty embryos from each replicate were randomly selected and staged according to the embryonic development (Table IV.i.).

Table IV.i. Classification of early ontogenetic stages in *Psammechinus miliaris*, with reference to the main characteristics in each stage (changed after Conn, 1991).

stage	main characteristics
A	abnormal development
0	unfertilized egg
I	uncleaved egg; with fertilization membrane
II	2-cell stage
III	4-cell stage
IV	8-cell stage
V	16-cell stage
VI	32-cell stage; showing cell elongation, with cells positioning on the edge of the membrane
VII	64-cell up to pre-blastula stage
VIII	early blastula, 128-256 cells, with cells arranged on the edge of the membrane recently released from the fertilization membrane

Statistical analyses

Data on the proportion of abnormal developing embryos from each culture failed the assumption of normality, even after data were arcsine transformed. The raw data were used in the nonparametric Kruskal-Wallis single factor analysis of variance by ranks to test for temperature effects and pressure effects on the proportion of abnormal developing embryos (Sokal & Rohlf, 1995). Temperature and pressure effects were tested separately, i.e. for each treatment (e.g.: results of pressure effects are for each temperature tested).

Results

Temperature and pressure effect on embryonic development

Psammechinus miliaris embryonic development at 1 atm was faster at 15 ºC and slower at 10ºC (Chapter 5, Fig. 5.1.). After 4 hours of incubation at 1 atm, the most advanced stage of development attained at 10ºC was stage V (16-cell stage); at 15ºC was stage VIII (early blastula); and at 20ºC was stage VI (32-cell stage; Table IV.i.; Chapter 5, Fig. 5.1.). At atmospheric pressure, higher temperatures significantly increase the proportion of abnormally developing embryos (Kruskal-Wallis H=6.269, p=0.044; Table IV.ii; Chapter 5, Fig. 5.1.).There is no significant effect of temperature on the proportion of embryos developing abnormally for pressure treatments greater than 50 atm, although at 300 atm the p-value is very close to significant (Table IV.ii).

Table IV.ii. Kruskal-Wallis analysis of variance testing the effects of temperature (10, 15 and 20ºC) on the proportion of abnormally developing embryos of *Psammechinus miliaris* reared at the different pressures for 4 hours [H statistic (degrees of freedom, N=Number of replicates)].

Pressure (atm)	H (2, N=9)	p-value
1	6.269	0.044
50	3.525	0.172
100	0.022	0.989
200	0.371	0.831
300	5.915	0.052

In *Psammechinus miliaris*, successful fertilization under pressure was compromised in all high-pressure treatments (50-300 atm; Chapter 5, Fig.5.1.). The percentage of unfertilized eggs cultured at high-pressures was greater than 60% in most treatments. There was no significant effect of pressure in the proportion of unfertilized eggs for temperatures of 10 and 20ºC (Table IV.iii.). At 15ºC, there is a significant effect of pressure on the proportion of unfertilized eggs, as at 1 atm there

were no unfertilized eggs for all three cultures, and this proportion significantly increased with pressures ≥50 atm (Table IV.iii.; Chapter 5, Fig.5.1.)

After 4 hours of incubation at 50 atm, a few embryos were able to be fertilized and develop normally (28% at 10ºC, 11% at 15ºC and 12% at 20ºC). Despite the low percentage of fertilized eggs at 50 atm, it was possible to observe that some embryos reach stage III (4-cell) at 10 and 15ºC, and stage VI (32-cell) at 20ºC (Chapter 5, Fig.5.1.). At 10ºC/100 atm treatment, 45% of embryos were fertilized and from these 22% reached stage III (4-cell) after 4 hours of incubation (Chapter 5, Fig.5.1.).

Table IV.iii. Kruskal-Wallis analysis of variance testing the effects of pressure (1, 50, 100, 200 and 300atm) on the proportion of abnormally developing embryos (Stage A) and on the proportion of unfertilized eggs (Stage O) of *Psammechinus miliaris* reared at different temperatures [*H* statistic (degrees of freedom, *N*=Number of replicates].

Temperature (ºC)	Stage A		Stage O	
	H (4, *N*=15)	*p*-value	*H* (4, *N*=15)	*p*-value
10	5.409	0.248	8.922	0.063
15	6.463	0.167	11.730	0.020
20	2.085	0.720	7.582	0.108

As for the 1 atm treatment, at 50 atm was observed an increase in the percentage of abnormally developing embryos with increasing temperature (Chapter 5, Fig.5.1.). The percentage of abnormally developing embryos was slightly higher for pressures of 100 and 200 atm. The effect of pressure on the proportion of abnormally developing embryos was not significant for all temperatures analysed (10, 15 and 20ºC; Table IV.ii.). In pressurized cultures, the abnormalities observed were the occurrence of irregular cleavages, cytoplasmic extrusions and the rupture of the cell-membrane, often in the polar body region (Fig. IV.ii.). The polar body is formed after fertilization and it is the result of the expansion of the cell-membrane in this area which probably looses strength and burst under the effect of pressure.

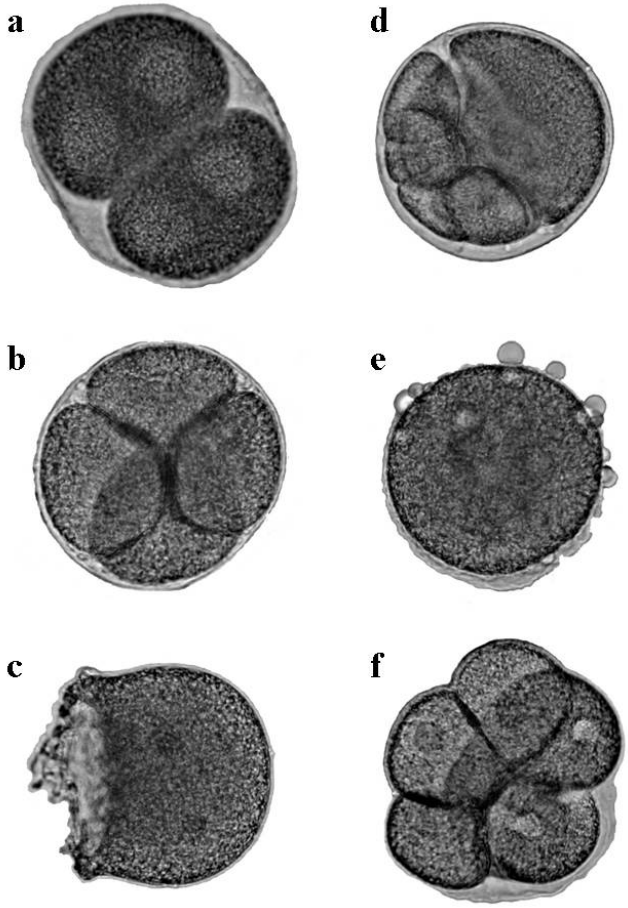

Fig. IV.ii. Examples of pressure effects on the morphology of *Psammechinus miliaris* embryos incubated for 4 hours, with fertilization occurring at incubation pressure (light microscope images). a) 2-cell stage (10ºC/50 atm); b) 4-cell stage (10ºC/100 atm); c) fertilized egg with rupture of the membrane in the polar body region (15ºC/100 atm); d) abnormal embryo with irregular cleavages (15ºC/100 atm); e) abnormal embryo with cytoplasmic extrusions over the surface of the cell membrane (15ºC/100 atm); f) abnormal embryo with 6-cells (15ºC/200 atm).

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