UNIVERSITY OF SOUTHAMPTON

FACULTY OF ENGINEERING, SCIENCE & MATHEMATICS

School of Ocean and Earth Sciences

Pressure and Temperature Effects on Planktonic Stages of Benthic Invertebrates with Regard to their Potential for Invasion of the Deep Sea

by

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has been produced under the supervision of the following persons

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To my parents, to my husband and to my daughter Alice.

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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PRESSURE AND TEMPERATURE EFFECTS ON PLANKTONIC STAGES OF BENTHIC INVERTEBRATES WITH REGARD TO THEIR POTENTIAL FOR INVASION OF THE DEEP-SEA

By Rosana Aquino de Souza

A pre-requisite for the colonization of deep seas by shallow-water benthic invertebrates is the ability of their dispersal stages to survive the physical and chemical conditions that prevail in the deeper waters to be colonized. Along the depth gradient, hydrostatic pressure increases and temperature decreases. These factors are known to have important effects upon several biological processes, including embryonic and larval development. This thesis investigates the embryonic and larval responses to changes in hydrostatic pressure and temperature in four species of shallow-water invertebrates.

Chapters 2 and 3 describe the results obtained for echinoid species. Results from *Psammechinus miliaris* are similar to previous work on echinoids. Despite the negative effects of hydrostatic pressures and low temperatures on embryonic development, larvae of *Psammechinus miliaris* were able to tolerate hydrostatic pressure and temperatures conditions that prevail far beyond the depth limit of the adult. Differences in temperature/pressure effects on dispersal stages between a northern and a southern population of *Echinus esculentus* were also investigated. The results suggest that the northern population is better adapted to low temperature.

Chapter 4 and 5 focus on Patellidae (Gastropoda) species. Increasing hydrostatic pressure and low temperature had strong negative effects on trochophores of *Patella vulgata* and *Patella ulyssiponensis*. These results do not conform to previous results in this field and show that many more taxonomic groups need to be investigated before we know how widespread, among shallow-water invertebrates, is the physiological potential for larval migration into deep sea. The implications of larval temperature tolerances for latitudinal distributions are also discussed.

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CHAPTER 1

General introduction

1.1. Why study the colonization of deep-sea?

The deep-sea comprises the greatest area in the marine environment and, contrary to the initial ideas about the biota in deep water (Gage & Tyler, 1991), it is now well known that it is inhabited by dense and diverse communities (Sandlers, 1968; Gage & Tyler, 1991; Grassle & Maciolek, 1992; Gage, 1996; Snelgrove & Smith, 2002). Despite many advances, the deep-sea fauna is still much less known than that of shallow-water communities (Gage & Tyler, 2001; Gage, 2004).

Based on current knowledge, researchers do not yet agree on the relative contribution of recent colonization to current deep-sea diversity (Kussakin, 1973; Hessler et al., 1979; Jacobs & Lindberg, 1998; Horne, 1999; Wilson, 1999; Gage, 2004). During the Palaeozoic and early Cainozoic there were several mass extinctions due to global changes of seafloor temperature as well as disoxic events (Horne, 1999). Some authors have stated that due to these extinctions, the modern deep sea fauna originated from recent invasions from shallow-water populations (Menzies et al., 1973; Stock, 1986; Jacobs & Lindberg, 1998; Gage, 2004). However, many authors have argued that some of the ancient deep-sea fauna have survived and speciated, and that these organisms represent a considerable component of the modern deep-sea fauna (Wilson, 1999).

It is certain that various colonization events from shallow habitats to deep sea have happened recently and will probably continue to occur (Wilson, 1980; Jacobs &

Lindberg, 1998; Wilson 1998; Gebruk, 1994; Gage, 2004; Raupach et al., 2004; Smith, 2004; Smith & Stockley, 2005). It has been suggested these may have occurred worldwide during the early Cainozoic when the water column was warm and isothermal throughout the world (Young et al., 1997), and more recently in regions where deep-water masses originate and the water column temperature has been homogenous, such as the polar region (Kussakin, 1973; Tyler et al., 2000) and the Mediterranean Sea (Tyler et al., 2000).

Once a shallow-water species expands its geographical distribution to deeper regions, inhabiting a depth range that comprises contrasting environments exerting differential selective pressures, a process of vertical allopatric speciation may occur. Many speculations have been made on how species diverge in the marine environment in general (Palumbi, 1994; Zane et al., 2000), and particularly along the vertical gradient (Hessler & Wilson, 1983; France & Kocher, 1996; Chase et al., 1998). Early investigations on genetic variability of marine organisms, using enzyme analysis, suggested that genetic variability in deep-sea species along the bathymetric gradient could be very great (e. g. Doyle, 1972). These findings have been contested by other authors because of the small size of samples analysed (Siebenaller, 1978). However, recent mtDNA analyses have shown similar differentiation. France & Kocher (1996) found that 16S-rRNA gene sequences of a deep-sea amphipod diverge primarily between populations from abyssal and bathyal (as defined by the authors, respectively, as > or < 3500 m) zones independently of location. These findings have been corroborated by mitochondrial genetic analysis of a bivalve species (Chase et al., 1998; Zardus et al., 2006), and an asteroid (Howell et al., 2004). At the generic level, Morita (1999) concluded that the first speciation event to take place in the fish genus *Coryphaenoides* was the differentiation of abyssal species.

The above findings show that the vertical gradient promotes genetic variability perhaps greater than that found between large-scale geographical zones. Therefore, deep-sea invasion leads to important changes in marine deep-sea biodiversity on evolutionary scales, as well as on ecological scales. Thus assessing the potential of species to carry on such migrations is major contribution to deep-sea ecology.

1.2. Why study larval temperature/pressure tolerances?

The success of colonization of any habitat by any species will depend on successful dispersal. In macrobenthic species, dispersal is typically carried out by a larval stage (Scheltema, 1986; Grantham et al., 2003). It is assumed that long-lived larvae can disperse long distances by drifting in ocean currents (Palumbi, 1994). This would allow larvae from different parts of the ocean to reach regions of the ocean where deep water is formed and from there invade deep-sea. But, successful dispersal into a new habitat will partially depend on the larval physiological tolerances to the physical conditions prevailing in the new environment, since intolerance to these conditions by larvae will result in low larval density, or no larvae, available for settlement. Knowing what are the major barriers to larval migration into the deep-sea helps understand the process of deep-sea colonization.

Concerning the colonization of deep-sea, the dispersing larvae cannot avoid increasing pressures and decreasing temperatures (Menzies, 1965; Tyler, 1995). Considering the known effects of pressure and temperature upon biological systems (Vernberg & Vernberg, 1972; Somero et al., 1983; Somero, 1998; Pörtner, 2002), these are potential barriers to larval dispersal into the deep sea. Therefore, larval

tolerance to low temperature and high pressure is a requirement for deep-sea colonization events to take place.

Larval pressure and temperature tolerances have been studied in shallow-water echinoids (Young et al., 1997; Tyler & Young, 1998; Tyler et al., 2000) and sea-stars (Benitez Villalobos et al., 2006). To date tests of susceptibility of shallow-water species to low temperature and high pressure during larval stages have been restricted to echinoderms. Studies so far have suggested that pressure and temperature do not represent a major barrier to dispersal into the deep sea. But because these experiments include relatively few species and are mostly concentrated in the Echinodermata, more species need to be tested to avoid overgeneralization.

1.3. Overall aim

The overall aim of my study is to broaden the number of taxa tested for larval pressure/temperature tolerance, and to investigate between-species and within-species latitudinal effects, in relation to hypotheses concerning the colonization of the deep seas.

The physiological potential for deep-sea invasion by embryonic and larval stages was tested in *Psammechinus miliaris*, considering hydrostatic pressure and temperature, in order to compare these results with previous results from other echinoderms. These results are shown in chapter 2.

In chapter 3, *Echinus esculentus* was used as a test species to investigate intraspecific latitudinal variation in larval pressure and temperature tolerances by including data from a northern and a southern population. Analysing these data

enables the assessment of whether the northern population is adapted to low temperature and has more potential for invading the deep-sea.

Patella vulgata and *Patella ulyssiponensis* represent shallow-water patellogastropod species with different latitudinal distributions, therefore allowing a intrageneric analysis of the effects of latitudinal distribution on the larval physiological potential to withstand deep-sea pressure and temperature conditions. In chapter 4 a method to obtain a great number of larvae in laboratory from *Patella* species was developed, enabling laboratory experiments on larval ecology to be done. Experiments in chapter 5 are probably the first to include a shallow-water mollusc in the study of pressure effects on larval development, adding a new phylum to this field of research.

In chapter 6, the hypotheses concerning larval migration into the deep-sea and future research in this field are discussed in the light of my results.

1.4. Specific objectives

1.4.1. *Psammechinus miliaris* study (chapter 2)

The specific objectives of this chapter were to test the following hypotheses:

I. Developmental rates and the incidence of abnormality in *Psammechinus miliaris* embryos cultured under deep-sea pressure/temperature conditions are not different compared to embryos cultured under shallow-water pressure/ temperature conditions.

II. Larval survival rates in *P. miliaris* are not different at deep-sea pressure/temperature conditions compared to shallow-water pressure/temperature conditions.

1.4.2. *Echinus esculentus* study (chapter 3)

The specific objectives of this chapter were to test the following hypotheses:

I. Developmental rates and the incidence of abnormality during embryonic development at different hydrostatic pressures and temperatures, in the NE Atlantic species *Echinus esculentus* are not different between a northern and a southern population.

II. Developmental rates during larval stages at different hydrostatic pressures and temperatures in *E. esculentus* were not different between a northern and a southern population.

1.4.3. *Patella* study

Gamete maturation and fertilisation (chapter 3)

The specific objectives of this chapter were:

I. To test the hypothesis that a pH rise in seawater affects the maturation of *Patella vulgata* and *Patella depressa* oocytes.

II. To test the hypothesis that oocyte alkalinization increases fertilisation rates in these species.

III. To determine the optimum sperm concentration for fertilisation of alkalinized and non-alkalinized oocytes in these species?

Deep-sea invasion potential (chapter 4)

The following hypotheses were tested:

I. Trochophore larvae of the cold-temperate northern species *Patella vulgata* are more tolerant to cold temperatures than trochophore larvae of the warm southern species *P. ulyssiponensis*.

II. Trochophore larvae of the cold-temperate northern species *Patella vulgata* develop faster than trochophore larvae of the warm southern species *P. ulyssiponensis*.

III. In both species, the trochophore larvae are able to survive and develop normally at temperature/pressure conditions found at 0.5-1.0 Km of depth in the NE Atlantic, when compared to shallow-water conditions

CHAPTER 2

Early development and larval survival of *Psammechinus miliaris* under deep-sea temperature and pressure conditions

Embryos of *Psammechinus miliaris* were subjected to different combinations of pressure and temperature for 3, 6 and 12 hours. The percentage of embryos at each stage and the percentage of embryos developing abnormally were determined. Larvae at the gastrula and prism stages were subjected to pressure (1 atm, 50 atm, 100 atm. 150 atm e 200 atm) and temperature (5°C, 10°C, 15°C and 20°C) combinations for 24 hours and the larval survival was calculated measuring the percentage of swimming larvae. The results showed that both embryos and larvae could survive at far greater depths than the known adult depth limits. The larvae showed a much greater potential than the embryos for surviving deeper, with approximately 100 % of both gastrulae and prisms surviving up to 200 atm at 5 °C. These results are compared to data on other organisms from shallow and deep waters, including species within the same family.

2.1. Introduction

There is considerable debate on the origins and antiquity of the deep-sea species (Kussakin, 1973; Hessler et al., 1979; Jacobs & Lindberg, 1998; Horne, 1999; Wilson, 1999; Gage, 2004). It is clear, however, that shallow-water benthic

organisms have colonized the deep sea during several geological periods (Wilson, 1980; Jacobs & Lindberg, 1998; Wilson 1998; Gebruk, 1994; Gage, 2004; Raupach et al., 2004; Smith, 2004; Smith & Stockley, 2005). Invasion of the deep-sea is still occurring as suggested by the shallow-water species that have extended their depth distributions to the deep sea such as the seastar *Stichastrella* spp. (Howell et al., 2002) and the sea urchin *Echinus acutus* (Tyler et al., 1995). Although there is evidence that these invasions can take place, it is not clear how widespread among shallow-water invertebrates is the potential to extend depth ranges.

For a species to invade the deep-sea its dispersal stages must have the physiological ability to withstand the physical properties of this environment. Many species found in coastal habitats have a restricted depth distribution; this may be a reflection of the inability of their dispersal stages to survive under the physical conditions that prevail in the deep sea. But it might also be a consequence of many other factors such as biological interactions or the requirement of recruits or adults for a specific type of habitat or food. It has been shown in the echinoderms *Ophiura ljungmani* (Gage & Tyler, 1981 a and b), *Ophiocten gracilis* (Sumida et al. 2000) and *Luidia sarsi* (Howell et al. 2002) that the juvenile depth distribution may extend beyond the maximum depth of the adult, but this is not enough to establish generalizations about the tolerance of shallow-water species dispersal stages to deep-sea physical conditions. Unfortunately, dispersal and juvenile stages are rarely collected from deep-sea sampling and are not always easily identifiable (but see Gage & Tyler 1981 a and b). Nevertheless, it is possible to assess embryonic and larval tolerances to deep-sea conditions through the simulation of these conditions in the laboratory.

From shallow coastal waters to abyssal depths, various physical and chemical changes in the environment occur such as oxygen availability, light availability, sediment type, temperature and hydrostatic pressure (Menzies, 1965; Tyler, 1995). A relationship has been suggested between depth zonation of invertebrates and the limits of the permanent thermocline, which appear to establish important faunal boundaries (Menzies et al., 1973; Gage et al., 1985; Billett, 1991; Howell et al., 2002; Carney, 2005). Unlike temperature, pressure varies constantly along the depth gradient (increasing 1 atm for every 10 m depth increase in the water column) and its role in determining species depth distribution is not clear. However, both temperature and hydrostatic pressure affect biological systems at all organizational levels (Vernberg & Vernberg, 1972; Somero et al., 1983; Somero, 1998; Pörtner, 2002). Therefore, in order to assess the physiological ability of a species to invade the deep sea, it is important to test the ability of its dispersal stages to survive changes in these two environmental factors.

Along the depth gradient, temperature decreases while pressure increases. Therefore laboratory experiments to assess deep-sea invasion potential are more realistic when they reflect this by employing temperature and pressure combinations, instead of addressing these factors separately. Embryos or larvae of six strictly shallow-living sea urchin species have been submitted to temperature and pressure tests so far. These are *Arbacia punctulata* (Marsland, 1950), *Paracentrotus lividus*, *Arbacia lixula* and *Sphaerechinus granularis* (Young et al., 1997), *Echinus esculentus* (Tyler & Young, 1998), and *Sterechinus neumayeri* (Tyler et al., 2000). Other studies on echinoids have focused on hydrostatic pressure alone (Marsland, 1938; Young et al. 1995). Including another echinoderm class in this field of study, Benitez Villalobos et al. (2006) have performed studies on the embryos and larvae of

the seastars *Asterias rubens*, which extends from the intertidal to 900 m, and *Marthasterias glacialis*, a strictly shallow-water species.

To date, these studies have suggested 1) that high hydrostatic pressure can inhibit cleavage or lead to abnormal development, especially under low temperature and 2) that dispersal stages, particularly later larval stages, can survive in pressures higher than those encountered within the depth range of the adult. This has led to the general assumption that the potential for the invasion of the deep sea is widespread among invertebrates living in shallow water. Unpublished data on the limpet *Patella ulyssiponensis* (see chapter 4) showed that under low temperatures, trochophores cannot tolerate slightly raised pressures, equivalent to 500 m depth. These data contradict the results from previously studied species and shows that more pressure/temperature tests are needed.

The present study investigated the effects of various combinations of pressure and temperature, similar to those found along the depth gradient, on embryos and larvae of *Psammechinus miliaris*. *P. miliaris* is found on a variety of seashores including rocky shores, among algae and on muddy sand and down to 70 m. As a broadcast spawner with external fertilisation and planktotrophic development *P. miliaris* disperses during its embryonic and larval stages and its large-scale dispersal is influenced by water flow. This species occurs in the Eastern Atlantic, from Morocco to Norway. In this region, embryos and larvae have the potential to be transported through the thermohaline circulation and carried to the deep sea during deep water formation.

The genus *Psammechinus* is a close relative to *Echinus*, in which both shallow and deep-sea species can be found, and adaptation to develop under high hydrostatic pressure has been revealed (Tyler & Young, 1998). The current

investigation will support or refute general assumptions made by past researches on sea urchins, including *Echinus*. If *P. miliaris* larvae can survive deep-sea conditions, this investigation will provide further support for the suggestion that the physiological potential for larval migration into the deep sea is widespread among shallow-water invertebrates and that this migration can happen within one generation. Otherwise, along with the results on *Patella* (chapter 5), it will show that high pressure and low temperature can represent important barriers for bathymetric migration by shallow-water species and a strong determinants of the deep-sea fauna composition.

2.2. Materials and methods

2.2.1. Experiments on early stages of development

Individuals of *Psammechinus miliaris* were collected from Batten Bay, Plymouth, in the southwest coast of England and kept in the aquarium of the National Oceanography Centre, Southampton, UK. The animals were induced to spawn by intracoelomic injection of approximately 2 ml of 0.5M KCl. Approximately 3 ml of active sperm from at least 2 males were added to a 2L beaker containing the eggs obtained from at least 3 females. After 5 minutes, a drop of the egg suspension was analysed under the microscope to check fertilisation success. When all the eggs were fertilised, the egg suspension was used in the experiments. Spawnings and fertilizations were carried out at approximately 12 ^{o}C. A different fertilization event, using different individuals was performed for each experiment.

The zygotes were distributed among plastic scintillation vials which were placed inside steel pressure vessels (described by Young et al., 1995) containing water at ambient temperature. Each plastic scintillation vial contained approximately 200 zygotes. The vessels were subjected to different combinations of hydrostatic pressures (1 atm, 50 atm, 100 atm, 150 atm and 200 atm) and temperatures (5 °C, 10 °C, 15 °C and 20 °C). Three experiments were performed, using different incubation periods (3 hrs, 6 hrs, and 12 hrs). Pressurization of the vessels was achieved using a manual ENERPAC pump (model 111000). Temperature was controlled by placing the vessels in incubators and water baths. After depressurisation, embryo development was stopped by adding drops of formaldehyde to scintillation vials. A hundred embryos from each replicate were then analysed under a compound microscope to measure the percentage of embryos at different developmental stages, as well as abnormal embryos.

2.2.2. Experiments on larval stages

Zygotes obtained as described for the experiments above, were left to develop until larval stages, and then subjected to different combinations of hydrostatic pressures (1 atm, 50 atm, 100 atm, 150 atm and 200 atm) and temperatures (5 °C, 10 °C, 15 °C and 20 °C). After 24 hours, a hundred larvae from each replicate were analysed under a stereomicroscope to measure the survival rate (% of swimming larvae). Two experiments were performed, using larvae at different stages (gastrula and prism). A different fertilization was performed for each experiment. All experiments in this chapter were performed within a week.

2.3. Statistical analysis

A two-way ANOVA was performed to analyse the effects of pressure and temperature on abnormality rates during the development of embryos and on the survival of larval stages. A post-hoc Sheffé test was performed for specific comparisons between selected pairs of samples.

2.3. Results

2.3.1. General results

The analyses revealed pressure and temperature effects, as well as interaction effects between these factors, in determining embryonic abnormality rates (tables 1.1-1.3, figs. 1.1-1.3) and larval survival (tables 1.4 and 1.5, figs. 1.4 and 1.5). In all occasions, temperature had a stronger effect on the variables analysed. This means that variations in temperature have more severe consequences for embryonic development and larval survival than variations in hydrostatic pressure.

2.3.2. Pressure/temperature effects on zygotes after 3 hours of incubation

At 5 °C embryos did not show any cleavage in any of the pressures analysed. At 10 °C embryos had started to cleave at 50 and 100 atm, but no cleavage was observed at higher or lower pressures, at this temperature. At 15 °C and 20 °C development had begun in pressures up to 150 atm, the fastest developmental rates observed were at pressures up to 100 atm (fig. 1.1). ANOVA showed significant

pressure and temperature effects on abnormality rates ($p<0.01$), as well as an interaction component ($p<0.01$) (table 1.1).

2.3.3. Pressure/temperature effects on zygotes after 6 hours of incubation

ANOVA revealed strong effects of both pressure and temperature on abnormal developmental rates ($p<0.01$) as well as a strong interaction ($p<0.01$) (table 1.2). At low temperature (5 °C) no development was observed under high pressures (>50 atm), whereas at 1 atm abnormal development was observed. At 10 °C embryos were abnormal at pressures of 100 atm and above, and developed normally up to 50 atm. At 15 °C, approximately 90% of the embryos developed abnormally at pressures from 150 atm while a similar proportion were at the normal 64-cell stage at lower pressures. At 20 °C, in all pressure treatments tested, more than 50% of the embryos showed abnormal cleavage (fig. 1.2).

2.3.4. Pressure/temperature effects on zygotes after 12 hours of incubation

ANOVA results for the 12-hour experiment were similar to the 3-hour and 6-hour experiments; there was a strong effect of both pressure and temperature ($p<0.01$) on abnormal developmental rates, as well as a strong interaction component ($p<0.01$) (table 1.3). There were similar high rates of arrested zygote development under 5 °C at some pressures treatments, as in the shorter incubations. 5 °C zygotes either did not cleave or cleaved abnormally, at all pressures.

At 10 °C more than 90 % of embryos had developed to at least the blastula stage at pressures lower than 100 atm, whereas at higher pressures development did

not occur or resulted in unviable embryos. At 15 °C approximately 40 % of embryos developed normally up to 100 atm; development was 100 % abnormal at higher pressures. At 20 °C more than 80% of embryos showed abnormal development regardless of pressure (fig. 1.3).

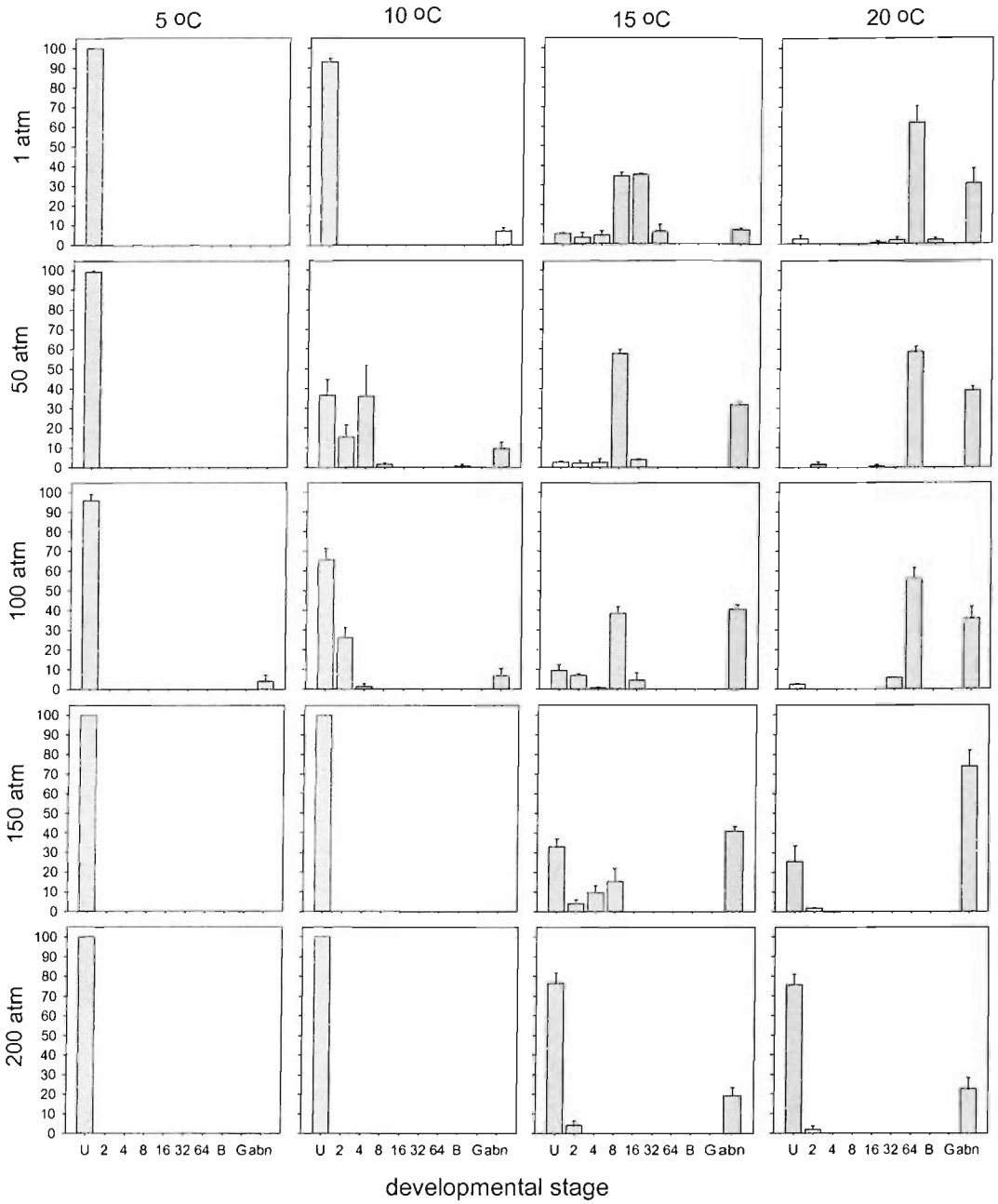


Figure 1.1. Percentage of *Psammechinus miliaris* embryos at different stages after being subjected to different combinations of temperature and pressure for 3 hours of incubation. Symbols: U- uncleaved; 2, 2-cells; 4, 4-cells; 8, 8-cells; 16, 16-cells; 32, 32-cells; 64, 64-cells; B, blastulae; G. gastrulae; abn, abnormal embryos. Abnormal embryos were not classed by stage. Bars represent standard error.

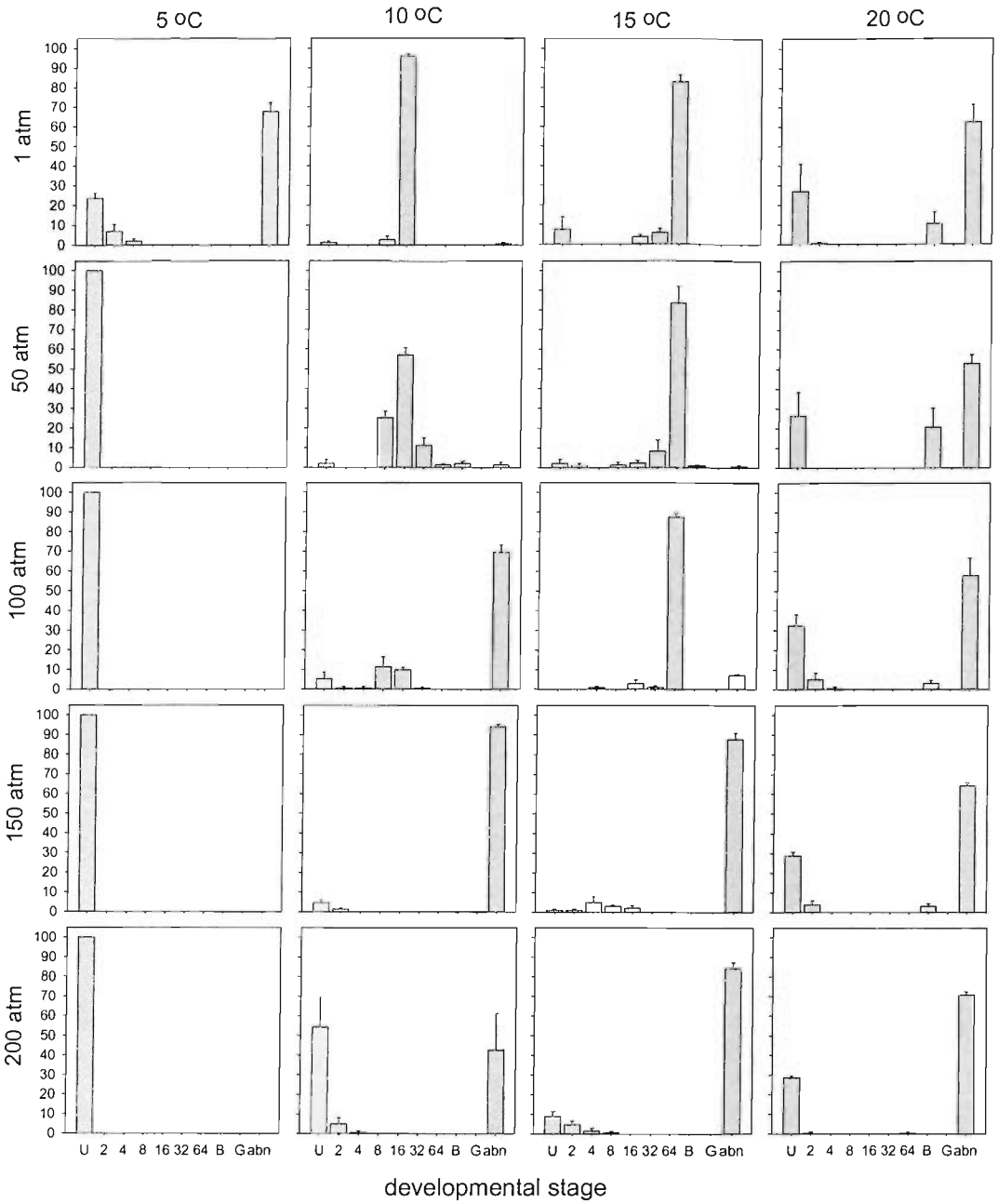


Figure 1.2. Percentage of *Psammechinus miliaris* embryos at different stages after being subjected to different combinations of temperature and pressure for 6 hours of incubation. Symbols: U- uncleaved; 2, 2-cells; 4, 4-cells; 8, 8-cells; 16, 16-cells; 32, 32-cells; 64, 64-cells; B, blastulae; G, gastrulae; abn, abnormal embryos. Abnormal embryos were not classed by stage. Bars represent standard error.

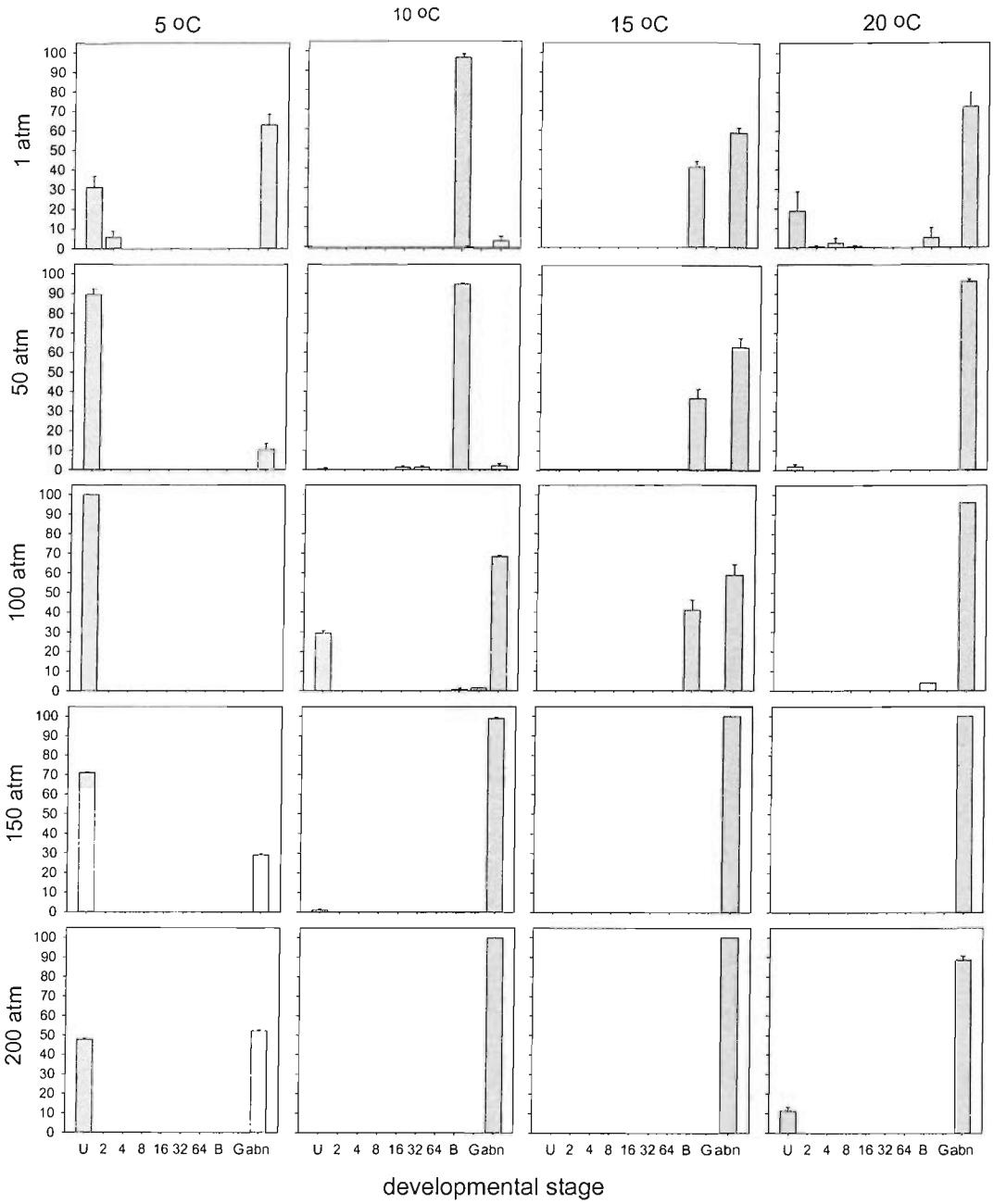


Figure 1.3. Percentage of *Psammechinus miliaris* embryos at different stages after being subjected to different combinations of temperature and pressure for 12 hours of incubation. Symbols: U- uncleaved; 2, 2-cells; 4, 4-cells; 8, 8-cells; 16, 16-cells; 32, 32-cells; 64, 64-cells; B, blastulae; G, gatrulae; abn, abnormal embryos. Abnormal embryos were not classed by stage. Bars represent standard error.

Table 1.1. Two-way ANOVA on percentages* of abnormal embryos of *Psammechinus miliaris* after 3 hours of incubation at five pressures (1 atm to 200 atm) and four temperatures (5 °C to 20 °C).

	df	ss	ms	F	p
Pressure	4	0.392	0.098	12.270	< 0.001
Temperature	3	4.329	1.144	180.354	< 0.001
Interaction	12	0.848	0.070	8.839	< 0.001

* Transformed to arcsin(√%)

Table 1.2. Two-way ANOVA on percentages* of abnormal embryos of *Psammechinus miliaris* after 6 hours of incubation at five pressures (1 atm to 200 atm) and four temperatures (5 °C to 20 °C).

	df	ss	ms	F	p
Pressure	4	2.811	0.702	61.013	< 0.001
Temperature	3	3.876	1.297	112.163	< 0.001
Interaction	12	7.689	0.640	55.624	< 0.001

* Transformed to arcsin(√%)

Table 1.3. Two-way ANOVA on percentages* of abnormal embryos of *Psammechinus miliaris* after 12 hours of incubation at five pressures (1atm – 200 atm) and four temperatures (5 °C to 20 °C).

	df	ss	ms	F	p
Pressure	4	4.50	1.127	232.6	< 0.001
Temperature	3	5.50	1.834	378.5	< 0.001
Interaction	12	5.23	0.436	90.0	< 0.001

* Transformed to arcsin(√%)

2.3.5. Pressure/temperature effects on gastrulae survival

ANOVA showed significant ($p<0.01$) pressure and temperature effects on survival (swimming) rates of gastrulae, as well as a significant interaction component ($p<0.001$) (table 1.4). High pressures either did not affect survivorship of gastrula or had positive effects, depending on temperature (fig. 1. 4). The 5 °C and 15 °C treatments at 1 atm, were the only ones where mean survival rates were lower than 80%, but these differences were not statistically significant ($p>0.05$). At the highest pressures analysed (150 and 200 atm), development was significantly lower ($p<0.05$) at 15 °C than at 5 °C or 10 °C.

2.3.6. Pressure/temperature effects on prism larvae survival

ANOVA showed significant temperature ($p<0.01$) and pressure ($p<0.05$) effects on survival (swimming) rates, as well as interaction effects ($p<0.01$) (table 1.5). At all pressures, survivorship of prism-stage larvae was significantly lower

($p<0.05$) at 15 °C than at 10 °C. At 5 °C, survivorship rates were not different from survivorship at 10°C ($p>0.05$), at any pressure. (Fig. 1.5).

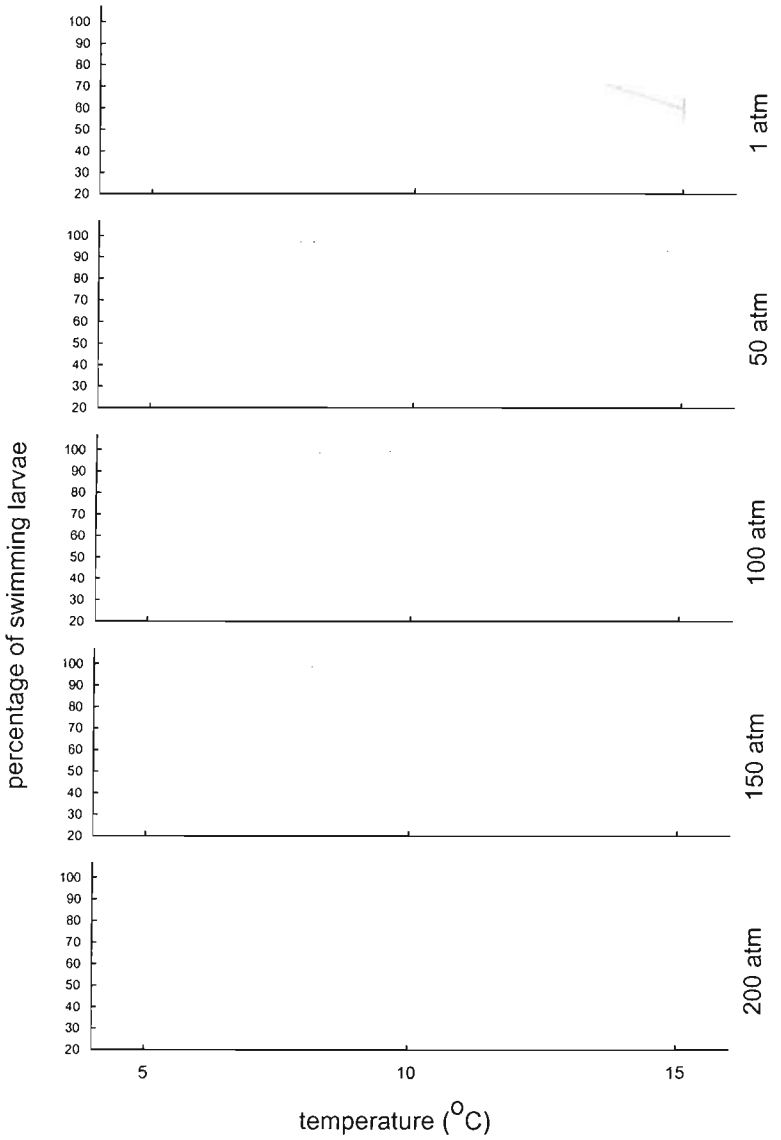


Figure 1.4. *Psammechinus miliaris.* Effects of pressure and temperature on the survival (swimming) rate of gastrulae, after 24-hours incubation. Bars represent standard errors.

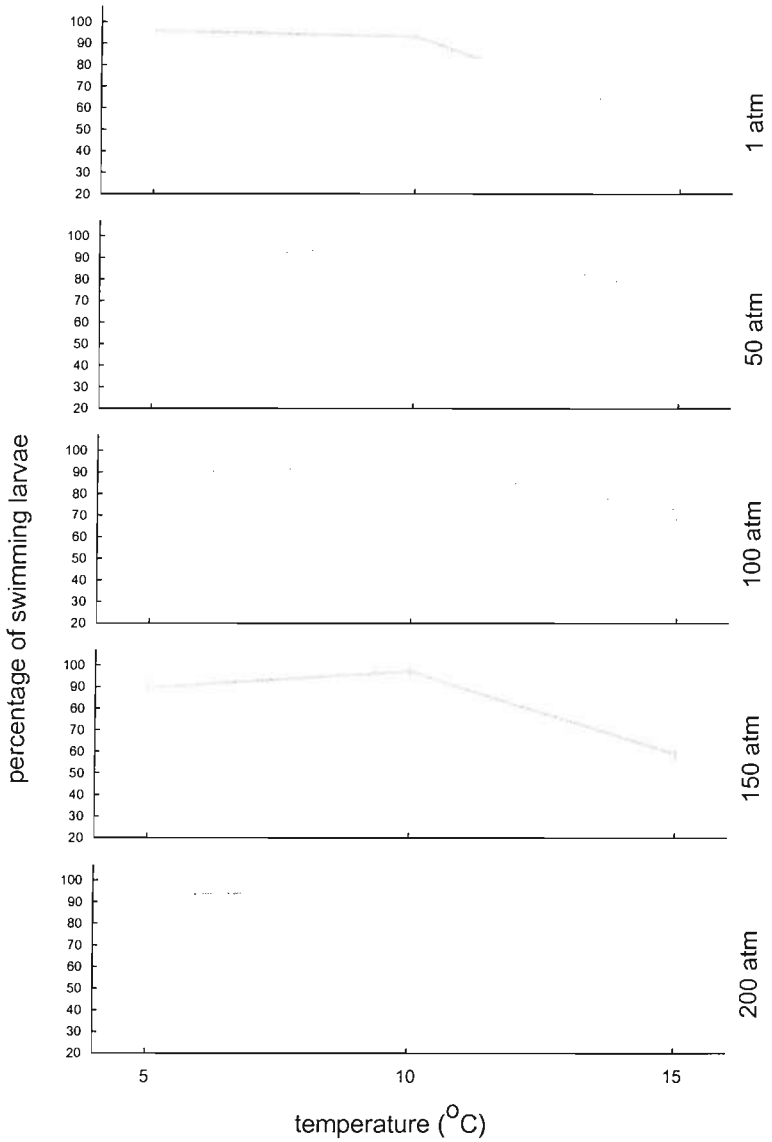


Figure 1.5. *Psammechinus miliaris.* Effects of pressure and temperature on the survival (swimming) rate of prism larvae, after 24-hours incubation. Bars represent standard errors.

Table 1.4. Two-way ANOVA on percentages* of swimming gastrulae of *Psammechinus miliaris* after 24 hours of incubation at five pressures (1 atm to 200 atm) and three temperatures (5 °C to 20 °C).

	df	ss	ms	F	p
Pressure	4	0.543	0.135	15.67	<0.001
Temperature	2	0.694	0.347	40.09	<0.001
Interaction	8	0.444	0.055	6.41	<0.001

*Transformed to arcsin(√%)

Table 1.5. Two-way ANOVA on percentages* of swimming prism larvae of *Psammechinus miliaris* after 24 hours of incubation at five pressures (1 atm to 200 atm) and three temperatures (5 °C to 20 °C).

	df	ss	Ms	F	p
Pressure	4	0.057	0.014	1.66	0.018
Temperature	2	1.922	0.961	111.9	<0.001
Interaction	8	0.263	0.032	3.832	<0.003

*Transformed to arcsin(√%)

2.4. Discussion

Embryos of *Psammechinus miliaris* can develop normally at pressures up to 100 atm, at 15 °C, but this was not observed at lower temperatures, revealing an interaction between temperature and pressure. This interaction acts by either inhibiting development or leading to abnormal embryos under "deep-sea" conditions. Therefore, the overall results indicate that the potential for deep-sea invasion at the

embryonic stage in this species is limited. After 12 hours of incubation, most of the embryos were developing abnormally at pressures higher than 100 atm. Furthermore, no embryos developed normally at 5 °C at any pressure treatment. At 10 °C embryos could survive and develop normally up to 50 atm. Taking into account the distribution limit of adults at 100m, these results suggest that pressure/temperature tolerances in the embryonic stages should not be regarded as limiting factors for this species to invade regions deeper than the habitats the adult occupies today, although it would not survive as deep as 1000 m. However, an egg fertilised within shallow water, being carried deeper into the ocean, will be in a more advanced developmental stage than the stage tested (zygote) in this experiment.

Having developed to a swimming stage, the larvae were able to survive in all conditions tested. This demonstrates that high pressures and low temperatures may prevent embryos from dispersing and surviving at depths greater than 500m, but do not prevent larvae from migrating to these depths and surviving. While embryonic stages last only a few hours, larval stages can last for weeks depending on temperature, thus larval stages are more important for dispersal than early embryonic stages, particularly for deep-sea invasion. The larval stages are the most likely to migrate vertically and invade the deep sea, since the embryo may stay in shallow-water during its short embryonic period, until it is brought to an area of deep-sea water formation and is dispersed into deep sea.

The data presented in this chapter suggest that 1) depth distribution of *P. miliaris* cannot be explained by physiological restrictions of its dispersal stages in relation to pressure and temperature, 2) in terms of pressure and temperature tolerances, *P. miliaris* has the physiological potential for deep-sea invasion and 3) this potential varies ontogenetically, being greater during larval stages than during

embryonic development. These findings corroborate past data from similar experiments on other species of the order Echinoida (Young et al., 1997; Tyler & Young, 1998; Tyler et al., 2000).

Past work includes data from the genus *Echinus* (Tyler & Young, 1998), which has shallow-water and deep-sea species (Tyler et al., 1996). This genus is believed to have originated in shallow water, in the NE Atlantic, where deep-sea water formation takes place (Tyler et al., 1996). Shallow-water species of *Echinus* have a high larval tolerance to high pressure and low temperature (Tyler & Young, 1998). *Sterechinus neumayeri* (Tyler et al., 2000) has also shown high larval tolerance to pressure and low temperature. This genus is restricted to Antarctica, where the ocean's deepest water is formed, and includes both deep-sea and shallow-water species (Brey & Gutt, 1991; Tyler et al., 2000). *Echinus* and *Sterechinus* may have undergone bathymetric migration and speciation from a shallow-water ancestor under similar conditions: 1) the formation of deep-water masses and 2) an extraordinary larval tolerance to high pressure and low temperature. This combination of factors may have been fundamental to the success of these genera in colonizing deep water.

The present study shows that a potential for larval dispersal into the deep-sea occurs also in *P. miliaris*, in a similar way to what has been found for *Paracentrotus lividus* (Young et al., 1997), and both *Psammechinus miliaris* and *Paracentrotus lividus* inhabit areas near deep-water formation. These species are very closely related to species of *Sterechinus* and *Echinus*. The genera *Psammechinus, Paracentrotus, Sterechinus* and *Echinus* all belong to the same clade or to sister clades, and their common lineage is believed to have diverged less ~30 Mybp (Littlewood and Smith, 1995; Smith, 1988; Smith, 2005), indicating that larval

tolerance to high pressure/low temperature in this group may be linked to their common ancestry. Despite the physiological potential for deep-sea invasion and favourable hydrographic conditions, both genera (*Psammechinus* and *Paracentrotus*) are restricted to shallow-water. Further research on later larval stages and phylogenetic investigations are necessary to understand the reasons why *Psammechinus* and *Paracentrotus* do not have deep-sea representatives.

There seems to be a critical value for the development of echinid embryos between 50 atm and 100 atm. It acts as a lower bathymetric limit for embryos of shallow-water species and an upper bathymetric limit for deep-sea species. *Paracentrotus lividus* (Young et al., 1997), *Echinus esculentus* (Tyler & Young, 1998), and now *Psammechinus miliaris,* showed markedly increased levels of abnormal embryonic development at pressures higher than 100 atm. However, this value is fifty times greater than the maximum hydrostatic pressure found in their habitat, since their depth distribution rarely extends deeper than 200 m. At the same time, 100 atm coincides with the minimum required pressure for successful embryonic development of the deep-sea echinoid *E. affinis* (Young & Tyler, 1993), which is only slightly lower than the pressure corresponding to its minimum recorded depth (120 atm). Results found in the deep-sea starfish *Plutonaster bifrons* (Young et al. 1996) were similar to the results in *E. affinis*.

Many physical and chemical factors undergo sharp vertical changes within the top 1000 m in the ocean and remain fairly constant beyond this depth. This is the case of important physiological factors such as temperature, oxygen concentration, salinity, and light availability, but is not the case of hydrostatic pressure (Menzies, 1965; Carney & Rowe, 1983; Tyler, 1995). Hydrostatic pressure increases at the same rate for all depths. Therefore deep-sea species moving upwards will encounter

few changes in the water column along the way, this may allow them to go as far as their tolerance to low pressure will permit. On the other hand, shallow-water species migrating downwards may be limited by temperature or lack of oxygen before they can even reach the maximum pressure they are able to tolerate. This could explain why there is a relationship between embryonic tolerance to pressure and the upper distribution limit in deep-sea species while no relationship is observed between embryonic pressure tolerances in shallow-water species and their bottom distribution limits.

The tolerance of larval stages to changes in pressure is much greater than for the embryonic stages. The pressure threshold found for embryos in this paper coincides with the threshold found for dehydrogenases from shallow-water organisms across various taxa (Somero, 1998). This threshold is situated between 50 atm and 100 atm. The mortality of embryos at 100 atm, observed in this study and others (e. g. Young et al., 1997) may be a consequence of the denaturation of proteins under similar pressures. However, whatever is the mechanism preventing embryos of *Psammechinus miliaris* from surviving higher pressure, it does not take place during the larval stages, as my work demonstrates. Ontogenetic changes were also observed in temperature tolerance. 10 °C was the optimum temperature for both embryos and gastrulae at 1 atm. But as the larvae develop into the prism stage, there is no effect of low temperature (5 °C) on larval survival. These ontogenetic changes in physiological tolerances are fundamental because they enable the larvae of *P. miliaris* to survive a wide range of environments, including habitats at low temperature and high hydrostatic pressure, providing larvae with a potential for deep-sea invasion.

CHAPTER 3

Embryonic and larval tolerances to temperature and pressure in two populations of *Echinus esculentus* from different latitudes

This chapter reports the effects of temperature and hydrostatic pressure on embryos and larvae from two populations of *Echinus esculentus* located at distant latitudes and reproducing under different temperature regimes. Embryos from a southern (Plymouth, UK) population developed normally at 15 °C, but not at 4 °C. Results were the opposite for a northern (Bergen, Norway) population, suggesting that northern populations have embryonic development adapted to low temperatures. Harmful effects of temperature on embryos, in both populations, were made worse by increased hydrostatic pressure. Northern population embryos and larvae generally developed faster than those from the southern population, under the same conditions. The implications of these results for the potential of deep-sea invasion by *E. esculentus* are discussed.

3.1. Introduction

Temperature is a main influence upon species latitudinal distributions both in marine and terrestrial environments (Orton, 1920; Hutchins, 1947; Ekman, 1953; Hall, 1964; Pörtner, 2002). In the ocean, temperature varies vertically, as well horizontally (Menzies, 1965; Tyler 1995), therefore it is not surprising that many

authors believe that temperature plays an important role also in determining species bathymetric distributions (e.g. Menzies et al., 1973; Gage et al., 1985; Billet, 1991; Howell et al., 2002). The role of temperature in determining marine faunal zonations is well established (Orton, 1920; Hutchins, 1947; Ekman, 1953; Hall, 1964; Engle & Summers, 1999; Pörtner, 2002; Peck, 2005). In the vertical dimension, however, it is more difficult to establish a direct relationship between temperature and distribution because other factors, such as pressure, also covary with depth and have similar thermodynamic effects.

Species distributions depend partially upon their dispersal capability. In benthic invertebrates with planktonic stages dispersal is often high (Thorson, 1950; Roberts, 1997; Shanks et al., 2003; Kinlan & Gaines, 2003; Kinlan et al., 2005). The wide dispersal of embryos and larvae of benthic invertebrates may subject these stages to a broad range of temperatures. An ability to develop and settle under a broad temperature range during planktonic stages should increase the potential for wide horizontal distributions. High temperature tolerance during planktonic stages should also increase the potential for a benthic species to broaden its depth distribution, as long as these stages can be carried into deeper regions of the ocean and tolerate high hydrostatic pressure.

A series of studies on sea urchins (Marsland, 1938; Marsland, 1950; Young et al. 1995; Young et al., 1997; Tyler & Young, 1998; Tyler et al., 2000; Aquino-Souza et al., in prep.) have shown that, at least for this group, planktonic stages tolerate pressures over a much greater range than the adults encounter naturally. In contrast, studies on early embryogenesis and larval development suggest that temperature tolerances of species and populations are commonly narrow during these stages (Fugisawa, 1989; Chen & Chen, 1992; Stanwell-Smith & Peck, 1998;

Doroudi et al., 1999). These narrow temperature tolerances suggest that temperature might be more likely to limit species bathymetric distributions than pressure.

The distribution of *Echinus esculentus* extends from northern Norway to southern Portugal. Thermal tolerance of embryonic and larval stages has been studied in southern Norway (Tyler & Young, 1998) were it reproduces from February to June (Lönning, 1976) when surface temperatures vary from 4 °C to 11 °C (ICES database). This species is very common in SW England where it reproduces from March to June when temperatures vary from 8 to 13 °C (Nichols et al., 1985).

We used new and published data (Tyler & Young, 1998) to compare the development of embryos and larvae of *E. esculentus* between a population from southern England and another from Norway, in order to test whether local adaptation with temperature occurs in these populations. Temperature variations were combined to variations in hydrostatic pressure, since pressure not only affects embryos and larvae directly but can also influence the effect of temperature. This is the first time combined effects of temperature and hydrostatic pressure upon embryos and larvae have been tested in horizontally separated populations of the same species.

Differences in developmental rates or in the incidence of abnormal development, at similar temperatures, would indicate adaptation to local temperature in *E. esculentus*. Local adaptations to temperature combined with pressure tolerance, in species of the NE Atlantic, suggest that populations located near their northern limits are more likely to be the sources of larvae for present-day migrations into the deep sea.

3.2. Material and methods

3.2.1. Collection of specimens

Individuals of *Echinus esculentus* were collected from a depth of 14 m by scuba diving in the Plymouth area, in SW England (fig. 2.1). The specimens were kept in the aquarium of the Marine Biological Association in running seawater for a maximum of 2 weeks before being used. Data obtained from these specimens were compared with data obtained earlier with speciemens collected by dredge from a water depth of 16m off Bukken Island, in the Bergen area, in Norway (fig. 2.1), and kept in running seawater at the Espergrend laboratory (see Tyler & Young, 1998 for details).

Figure 2.1. Geographic location of collection areas for *Echinus esculentus*: Southern site (Plymouth area, England) and Northern site (Bergen area, Norway).

3.2.2. General methods

spawning was induced with intra-coelomic injection of 0.55M KCl. Sperm and eggs were collected into separate finger bowls containing seawater. A dilute sperm suspension containing sperm from various males was added to a suspension of eggs containing eggs from various females. After successful fertilisation (detectable by the appearance of a fertilisation membrane), zygotes in suspension were pipetted into 8 ml scintillation vials that were filled to overflowing seawater and capped. Three replicate vials were assigned to each pressure/temperature combination. Each vial contained approximately 200 zygotes.

Blastulae, gastrulae and 4-armed plutei were cultured at 10 °C, the same temperature described in Tyler & Young (1998) in their study of a population from Bukken Island, near Bergen (Norway). At each stage, three replicate cultures were assigned to each pressure/temperature combination.

Constant temperature was achieved using cold rooms and water baths. The vials were pressurized using steel vessels (described by Young et al., 1995) and an Enerpac hydraulic manual pump. Each experiment in this chapter was performed from different parents.

3.2.3. Experiments on embryonic development

The zygotes were distributed among plastic scintillation vials and subjected to different combinations of hydrostatic pressures (1 atm, 100 atm) and temperatures (7 °C, 11 °C, 15 °C) and left to develop for 6 hours. After depressurisation, embryo development was stopped by adding drops of formaldehyde to scintillation vials. A

subsample from each replicate was then analysed under a compound microscope to measure the percentage of embryos at different developmental stages, as well as abnormal embryos. The results were compared to results obtained 7 years ago for a population of the Bergen area in Norway.

Two other experiments were performed using incubation periods of 12 hours and 24 hours. In these experiments, temperatures used were 4 °C, 7 °C and 11 °C. This was done to match the exact temperature treatments used by Tyler & Young (1998), for each incubation period.

3.2.4. Experiments on larval stages

Blastulae, gastrulae and 4-armed larvae were subjected to temperatures of approximately 4 °C, 7 °C and 11 °C (for details see tables 2.4-2.6) and pressures of 1 and 100 atm. After 24 hours, steel vessels were depressurized, a subsample of each replicate was analysed under a dissecting microscope and the stage attained was noted. The results were compared with data from the experiments of Tyler & Young (1998). While fertilized zygotes from Norway took 12 hours to reach the swimming stage at 10 °C, zygotes from Plymouth took around 24 hours to attain the swimming stage at the same temperature. Therefore, blastulae from Plymouth used in the experiment were 12 hours older than those used during the Norway experiments, in order to reflect the same developmental stage. For the same reasons, gastrulae were older during the Plymouth experiments than during the Norway experiments.

3.2.5. Statistical analysis

Three-way ANOVAs were performed to analyse the effects of pressure, temperature and population on occurrence of abnormality during the development of embryos for each species, at the end of each experiment. Post-hoc Sheffé tests were performed for comparisons between selected pairs of samples.

3.3. Results

3.3.1. Effects of pressure, temperature and population origin on embryonic development

Three-way factorial ANOVAs (factors: pressure, temperature and population) applied to data for abnormality rates of developing embryos from the 6-hour experiment (table 2.1), found significant ($p<0.05$) temperature and pressure effects. It also found pressure-temperature, temperature-population and pressure-temperature-population interaction effects ($p<0.05$). Data from the 12-hour experiment (table 2.2) found significant ($p<0.05$) temperature, pressure and population effects, as well as interaction effects between all factors. For the 24-hour experiment, a three-way ANOVA found significant ($p<0.05$) pressure, temperature and population effects, as well as temperature-population and pressure-temperature-population interactions (table 2.3).

The embryonic development of embryos from Plymouth (UK) often diverged from the data obtained previously from the analysis of embryos from Bergen (Norway). The two populations often showed very different rates of abnormality

under similar pressure and temperature conditions (fig. 2.2) and early development, up to the blastula stage, was slower in Plymouth than previously described for the Norwegian population (figs, 2.4-2.6).

6-hour experiment

At 7 °C, almost all embryos from Norway were normal 4-cell stage embryos, at both pressure treatments (fig. 2.4). In contrast, more than 50% of embryos from Plymouth were abnormal at 1 atm and abnormality rate was significantly ($p<0.05$) increased by pressure, with almost all embryos from Plymouth developing abnormally at 100 atm (fig 2.2).

At 11 °C, almost all embryos from Norway were between the 8-cell and 16-cell stage (fig. 2.4) and developing normally (fig. 2.2), at both pressure treatments. Most embryos from Plymouth (> 70%) were also developing normally (fig 2.2). Development was slower in Plymouth, with almost no embryo reaching 16-cell stage at this temperature (fig. 2.4).

At 15 °C, all embryos from Norway were cleaving abnormally at both pressure treatments (fig. 2.2). This demonstrates that 15 °C is not tolerated by zygotes from the Norway population. The opposite results were found in the Plymouth population; embryos were developing normally at both pressure treatments and had reached the 64-cell stage at 1 atm, and the 34-cell stage at 100 atm. (figs. 2.2-2.4).

12- hour experiment

At the lowest temperature (4 °C) embryos from Norway were developing normally at both pressures tested (fig. 2.2) and had reached the 4-cell stage at 1 atm (fig. 2.5). In contrast, a significantly ($p<0.05$) higher proportion of embryos from Plymouth (60%) were abnormal at 1 atm (figs. 2.2-2.3), and pressure negatively

affected ($p<0.05$) normal development (fig. 2.2). Normal embryos from Plymouth were developing slower than embryos from Norway and either did not cleave or performed only 1 cleavage (fig. 2.5).

At 8 °C, almost all embryos from Norway were developing normally (fig. 2.2) and more than 90% had reached the 16-cells stage at both pressures tested (fig 2.5). In contrast, embryos from Plymouth showed abnormal development and it was significantly ($p<0.05$) higher at high pressure (fig. 2.2).

At 11 °C, almost all zygotes from Norway had developed into normal blastulae at both pressure treatments (fig. 2.5). Similar to the embryos from Norway, almost all embryos from Plymouth were developing normally (fig 2.5), but development was slower, with no embryo reaching the blastula stage (fig. 2.5).

24- hour experiment

At 4 °C/1 atm, approximately all embryos from Norway were normal, while all embryos from Plymouth were abnormal (fig. 2.2). However, at 4°C/100 atm, the abnormality rate was high also for embryos from Norway (fig. 2.2). These results are consistent with the results from the 12-hour experiment and demonstrate that 4 °C is lethal for embryos from the Plymouth population regardless of pressure (fig. 2.2).

At 8 °C, approximately 80% of zygotes from Norway at both pressure treatments had developed into normal blastulae (fig. 2.6). At 1 atm, the results for embryos from Plymouth were similar to those for embryos from Norway. Similar to the results from the 6-hour (7°C) and 12-hour experiments (8°C), the abnormality rate was significantly higher ($p<0.05$) at 100 atm than at 1 atm, for embryos from Plymouth, but not for embryos from Norway (fig. 2.2).

At 11 °C, almost all embryos from Norway were normal blastulae at both pressure treatments. Embryos from Plymouth showed similar results (figs. 2.2 and

2.6). Approximately 30% of embryos from Plymouth were developing abnormally under 100 atm of pressure, but this was not significantly different ($p>0.05$) than at 1 atm (fig. 2.2); this was consistent through all three experiments, suggesting that pressure does not affect abnormality at 11 °C.

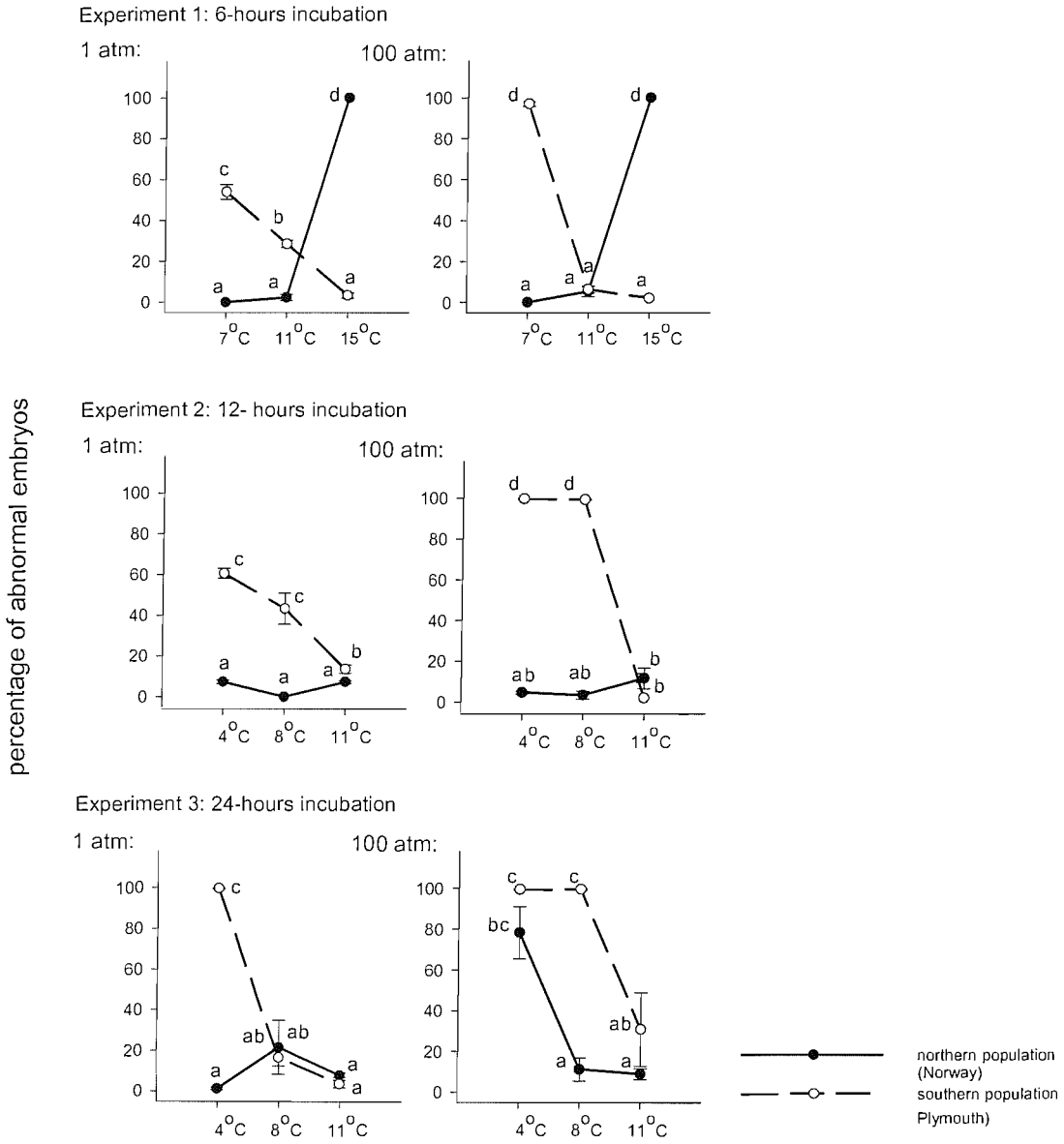


Figure 2.2. Percentage of abnormalities in developing embryos of *Echinus esculentus*, from a northern (Bergen, Norway) and a southern (Plymouth, UK) population, after being subjected to different combinations of temperature and pressure for 6, 12 and 24 hours. Same letters mean no significant difference within an experiment (incubation time). Bars represent standard errors.

Table 2.1. Three-way ANOVA on percentages* of abnormal embryos *of Echinus esculentus* from two populations (Plymouth and Norway) incubated for 6 hours at three temperatures (7 °C, 11 °C and 15 °C) and two pressures (1 and 100 atm).

	Ss	df	ms	F	p
pressure	0.026	1	0.026	8.412	0.007
temperature	1.990	2	0.995	312.43	<0.001
population	0.003	1	0.003	1.18	0.288
pressure*temperature	0.003	2	0.130	40.81	<0.001
pressure*population	0.004	1	0.004	1.306	0.264
temperature*population	9.866	2	4.933	1548.46	<0.001
pressure*temperature*population	0.376	2	0.188	117.996	<0.001

* Transformed to arcsin(√%)

Table 2.2. Three-way ANOVA on percentages* of abnormal embryos *of Echinus esculentus* from two populations (Plymouth and Norway) incubated for 12 hours at three temperatures (7 °C, 11 °C and 15 °C) and two pressures (1 and 100 atm).

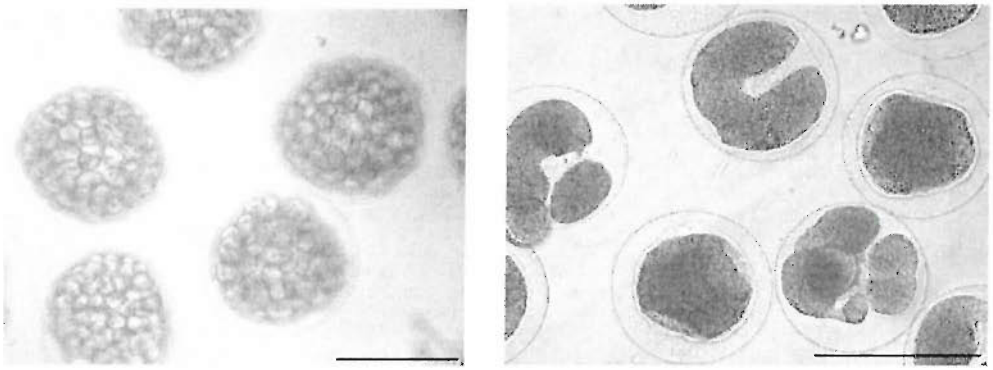
	ss	df	ms	F	p
pressure	0.45457	1	0.454	80.353	<0.0001
temperature	1.26587	2	0.632	111.881	<0.0001
population	3.84997	1	3.849	680.541	<0.0001
pressure*temperature	0.48255	2	0.241	42.649	<0.0001
pressure*population	0.27322	1	0.273	48.295	<0.0001
temperature*population	2.20400	2	1.102	194.796	<0.0001
pressure*temperature*population	0.46815	2	0.234	41.376	<0.0001

* Transformed to arcsin(√%)

Table 2.3. Three-way ANOVA on percentages* of abnormal embryos *of Echinus esculentus* from two populations (Plymouth and Norway) incubated for 24 hours at three temperatures (7 °C, 11 °C and 15 °C) and two pressures (1 and 100 atm).

	ss	df	ms	F	p
pressure	1.47253	1	1.47253	42.422	<0.0001
temperature	3.37830	2	1.68915	48.662	<0.0001
population	2.59217	1	2.59217	74.677	<0.0001
pressure*temperature	0.17274	2	0.08637	2.488	0.1042
pressure*population	0.09969	1	0.09969	2.872	0.1030
temperature*population	1.12142	2	0.56071	16.153	<0.0001
pressure*temperature*population	2.04607	2	1.02304	29.472	<0.0001

* Transformed to arcsin(√%)



(a) (b)

Figure 2.3. Embryos of *Echinus esculentus* from adults collected in Plymouth. (a) Submitted to 15 °C for 6 hours at normal hydrostatic pressure (1atm). (b) Submitted to 4 °C for 12 hours, at normal hydrostatic pressure (1atm). Scale bars: 200 μm.

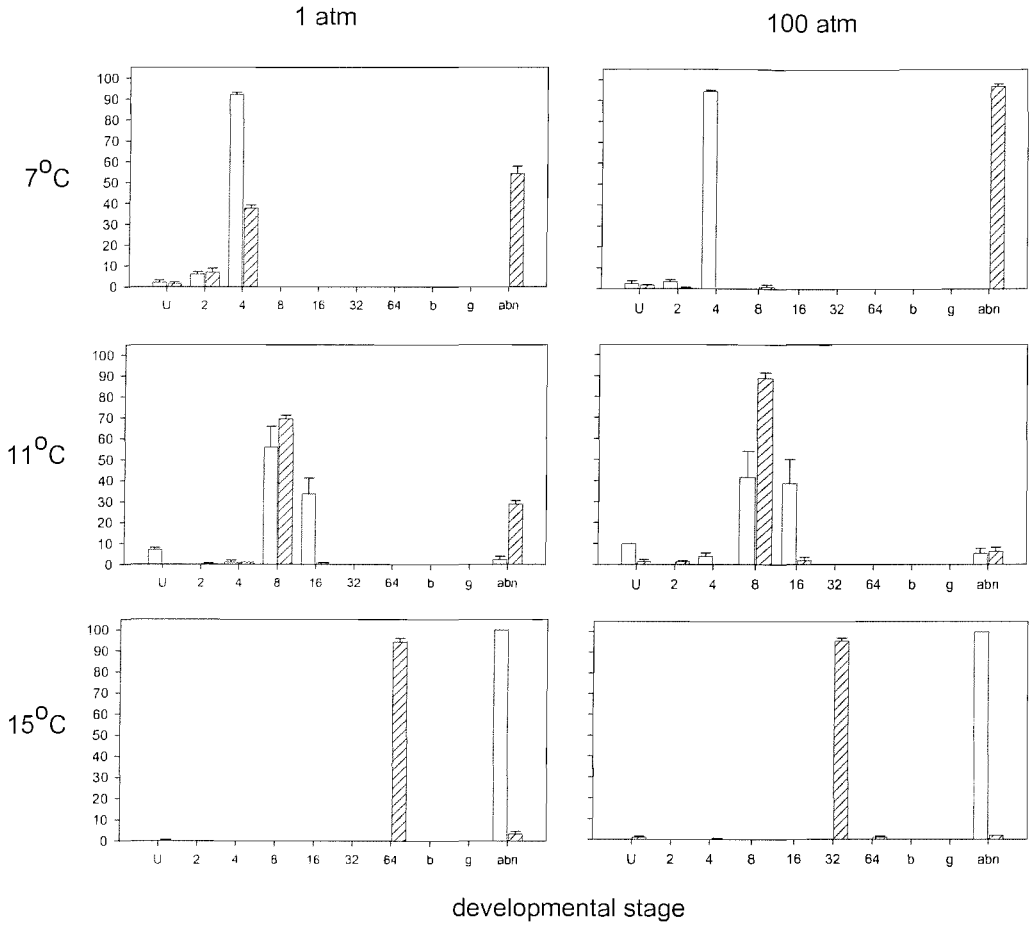


Figure 2.4. Percentage of embryos of *Echinus esculentus* at different stages after being subjected to different combinations of temperature and pressure over 6 hours. Symbols: u, uncleaved; 2, 2-cell; 4, 4-cell; 8, 8-cell; 16, 16-cel; 32, 32-cell; 64, 64-cell; b, blastula; g, gastrula; abn, abnormal embryos. Abnormal embryos were not classed by stage. Hatched columns represent Plymouth (southern) population; unhatched columns represent Bergen (northern) population.

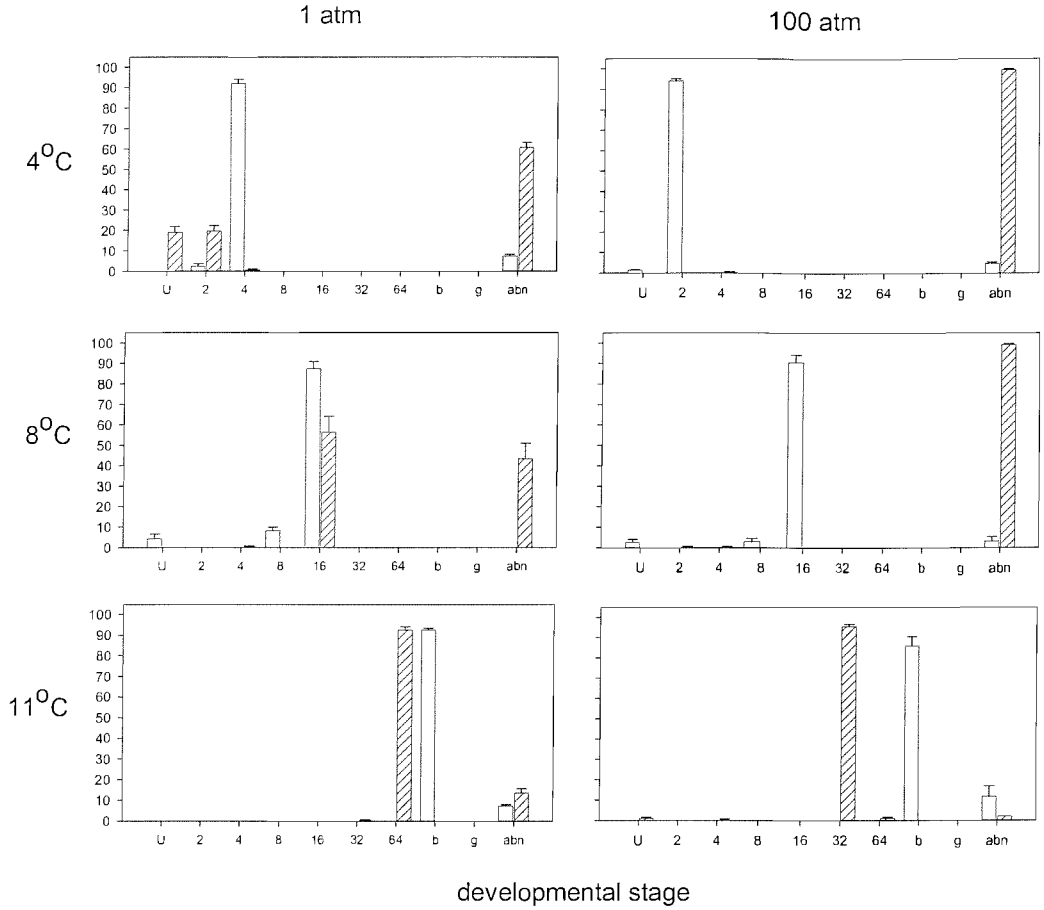


Figure 2.5. Percentage of embryos of *Echinus esculentus* at different stages after being subjected to different combinations of temperature and pressure over 12 hours. Symbols: u, uncleaved; 2, 2-cell; 4, 4-cell; 8, 8-cell; 16, 16-cel; 32, 32-cell; 64, 64-cell; b, blastula; g, gastrula; abn, abnormal embryos. Abnormal embryos were not classed by stage. Hatched columns represent Plymouth (southern) population; unhatched columns represent Bergen (northern) population.

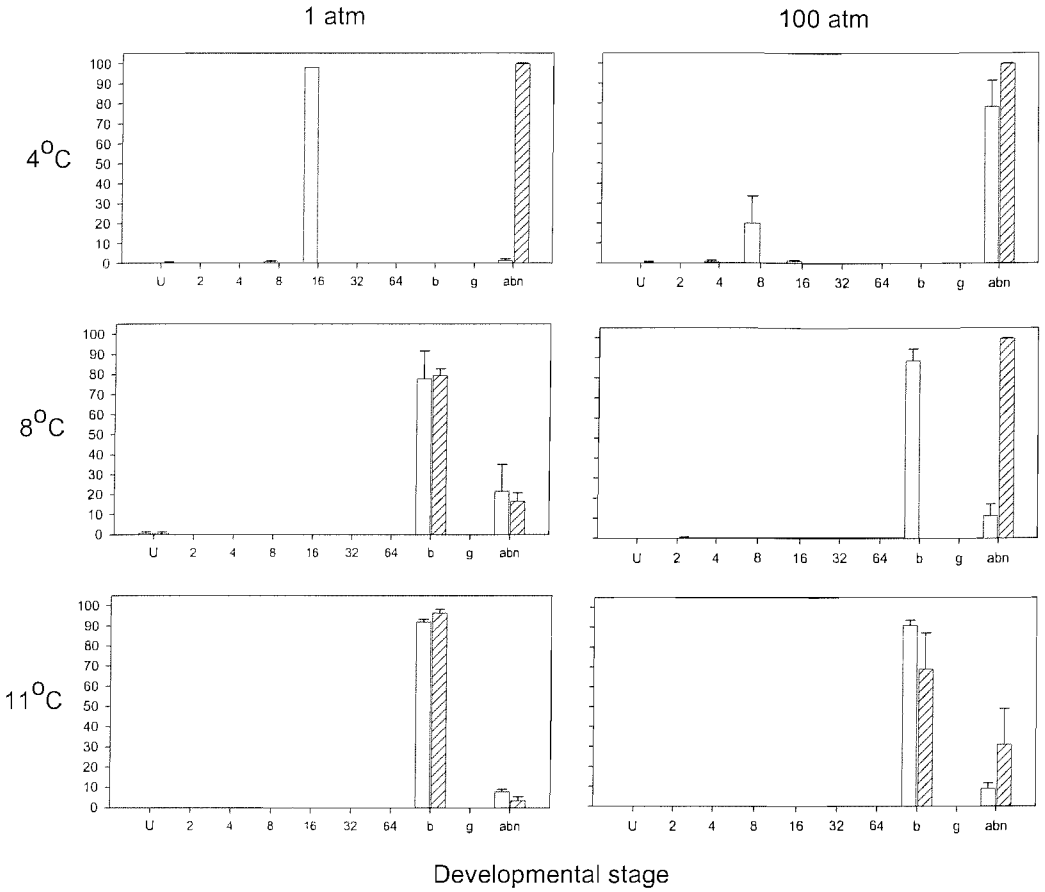


Figure 2.6. Percentage of embryos of *Echinus esculentus* at different stages after being subjected to different combinations of temperature and pressure, over 24 hours. Symbols: u, uncleaved; 2, 2-cell; 4, 4-cell; 8, 8-cell; 16, 16-cel; 32, 32-cell; 64, 64-cell; b, blastula; g, gastrula; abn, abnormal embryos. Abnormal embryos were not classed by stage. Hatched columns represent Plymouth (southern) population; unhatched columns represent Bergen (northern) population.

2.3.2. Effects of pressure, temperature and population origin on larval development

Swimming blastulae

Swimming blastulae from Norway incubated at 5 °C developed into early gastrulae at both pressures tested. Swimming blastulae from Plymouth showed similar results, except that about a third of the larvae had not started gastrulation. After 24 hours at 7 °C, results were similar to those found at 5 °C, but gastrulae from Norway incubated under 100 atm seemed to be in a more advanced stage of gastrulation. Swimming blastulae from both populations incubated at 10 °C were fully developed gastrula after 24 hours, at both pressure treatments. (Table 2.4).

Gastrulae

At 4 °C, gastrulae from Norway developed into prism or 4-armed larvae at both pressures. Gastrulae from Plymouth incubated at 4 °C were still at the gastrula stage with only a few larvae showing a complete gut, no 4-armed larvae were observed in samples of larvae from Plymouth. At 7 °C, gastrulae from Norway had developed into 4-armed larvae while those from Plymouth were still at the prism stage. All larvae from Norway were in an early 4-armed stage after 24 hours at 11 °C while those from Plymouth were at the prism stage with only a few starting arms formation. (Table 2.5).

Echinopluteus

All larvae remained at the 4-armed stage at both pressures and at all temperatures tested (table 2.6), throughout the trial.

Table 2.4. Developmental stages of swimming blastulae of *Echinus esculentus* from Norway and Plymouth cultured at 10°C and 1 atm submitted to different pressure/temperature combinations for 24 hrs. B: blastula; EG: early gastrula; G: gastrula.

Pressure (atm)	Temperature (°C)					
	5		7		10	
	Northern	Southern	Northern	Southern	Northern	Southern
1	EG	B/EG	EG	B/EG	G	G
100	EG	B/EG	G	B/EG	G	G

Table 2.5. Developmental stages of gastrulae of *Echinus esculentus* from Norway and Plymouth cultured at 10 °C and 1 atm submitted to different pressure/temperature combinations for 24 hrs. G: gastrula; P: prism; E4-arm: early 4-armed pluteus; 4-arm: 4-armed pluteus.

Pressure (atm)	Temperature (°C)					
	4		7		11	
	Northern	Southern	Northern	Southern	Northern	Southern
1	4-arm	G	4-arm	P	4-arm	P/E4-arm
100	P	G	E4-arm	P	4-arm	P

Table 2.6. Developmental stages of 4-armed pluteus of *Echinus esculentus* from Norway and Plymouth cultured at 10 °C and 1 atm submitted to different pressure/temperature combinations for 24 hrs.

Pressure (atm)	Temperature (°C)					
	4		7		11	
	Northern	Southern	Northern	Southern	Northern	Southern
1	4-arm	4-arm	4-arm	4-arm	4-arm	4-arm
100	4-arm	4-arm	4-arm	4-arm	4-arm	4-arm

3.4. Discussion

Generally, the results show that specimens of *Echinus esculentus* are adapted to reproduction under local temperatures. The main results include: 1) abnormal embryonic development under temperatures different from local temperatures during fertilisation periods, for each population and 2) slower development in Plymouth (Southern population) than in Bergen (Northern population) at any given temperature and pressure combination.

3.4.1. Developmental rates and temperature compensation

Differences in developmental rates among populations tested were small but consistent. Whenever there was a difference in the developmental stage between larvae from different localities under the same treatment, larvae from Norway were always in an advanced stage. Differences were not noticed where larvae from both localities had reached at least the gastrula stage, but because later stages last longer, longer incubation times would be required to demonstrate differences in developmental rates at these stages. Faster development under the same conditions in the northern population was also detectable during some of the experiments using embryos. On the other hand, larvae or embryos from Plymouth were never faster growing than those from Norway, under the same treatment. Additionally, Tyler & Young (1998) report that embryos from Norway, incubated at 10 ^{o}C, were swimming blastulae, after 12 hours of incubation; while blastulae from Plymouth did not reach the swimming stage until at least 24 hours after fertilisation, at the same temperature. Therefore, we conclude that larvae of *Echinus esculentus* from Bergen develop faster

than larvae from Plymouth when subjected to the same conditions, at least within the temperatures and pressures tested. These results suggest that partial temperature compensation occurs in this species.

Altogether, there is strong evidence for local temperature adaptation of developmental rates in northern populations of *E. esculentus* during planktonic stages. Assuming that faster development may bring advantages such as less exposure to predation , the results suggest that larvae of *E. esculentus* from northern latitudes would be more predictable to succeed in the deep sea. However, larvae from both localities developed normally at all treatments.

Faster developmental rates in embryos from colder regions are discussed by Thorson (1950) who predicted that northern populations would develop faster at a given temperature to compensate for the cold environment. My results reveal partial temperature compensation. Similar results were found in between-species comparisons using echinoderms (Fox 1936) and copepods (McLaren, 1969; Halsband-Lenk et al., 2002). However, many other studies have not confirmed this prediction (Patel & Crisp, 1960; Emlet et al. 1987; Johnston, 1990; Hoegh-Guildberg & Pearse, 1995), showing no temperature compensation in northern latitudes. These studies compare species with different geographic distributions, rather than populations of the same species from different origins. Within-species comparisons of the influence of geographic origin on developmental rates are not numerous and show contrasting results. Drent (2002) found slower developmental rates in larvae of *Malcoma baltica* collected from high latitudes (Norway) than in those from lower latitudes (France) at the same laboratory conditions. This is in contrast to the results for *E. esculentus* found in this study. Dehnel (1955) working on gastropods, Palmer (1994) working with *Nucella*, and Halsband-Lenk et al. (2002) working with

copepods, in agreement with our results in *E. esculentus*, found faster rates of development in higher latitude populations than in lower latitude ones, when subjected to the same experimental temperature. However, no research so far has demonstrated total temperature compensation because at local temperatures northern populations or species always developed slower than southern populations or congeneric species.

This is the first time the combined effects of temperature and pressure have been tested on developmental rates in different populations of the same species, no consistent effect of latitude on the influence of pressure on developmental rates seemed to occur. Much more needs to be done regarding this aspect of deep-sea invasion.

3.4.2. Embryonic abnormalities and deep-sea invasion

Embryonic abnormality rates were high in larvae from Bergen reared at 15 °C, and in larvae from Plymouth reared at 4 °C. These opposite results suggest an adaptation to develop at local temperatures. Similar adaptation to local temperature was found for populations of *Acanthaster planci* (Johnson & Babcock, 1994) in north-western Australia. Local adaptations may be genetic or as a result of adult acclimatization. Supplies of embryos for deep-sea invasion, in northern species, is more likely to come from populations located in the northern limits of the species distribution, if these populations are adapted for embryonic development at low temperatures. This is the case for *E. esculentus* based on our empirical observations, whether these adaptations are genetic or not.

Deleterious effects of extreme temperatures were amplified under hydrostatic pressure, and embryos from Bergen, which developed normally under 4 °C, showed abnormal development when low temperature (4 °C) was combined to 100 atm of hydrostatic pressure. On the other hand, at 7 °C and 11°C, embryos from Bergen were able to tolerate hydrostatic pressure. This shows that pressure interacts with temperature, and this interaction leads to no normal development under deep-sea pressure/temperature conditions. This does not make dispersal into the deep sea impossible, because by the time embryos achieve great depths, they will probably have already reached the larval stages, and in Tyler & Young's original experiments (1998) embryos from Norway tolerated pressures up to 50 atm at 4 °C, this pressure is equivalent to a depth of 0.5 Km.

Our results show that migration to the deep-sea, from populations located at the most southern areas of *E. esculentus* distribution (at least from the South-west of England to Portugal) will depend on embryos developing into larvae within this area before being dispersed into colder geographic regions of the oceans. On the other hand, migrations from northern areas can start at the embryonic stage, but development must reach the larval stage before arriving at depths where hydrostatic pressure becomes lethal to embryos, which is located between 0.5 km to 1 km. It is likely that, both populations would be sources of larval supply for vertical dispersal into the deeper areas of the ocean. A study on the vertical distribution of larvae and juveniles of this species is the only way to know whether this dispersal is already occurring.

CHAPTER 4

Artificial oocyte maturation and fertilisation in *Patella depressa* and *Patella vulgata*

Laboratory investigations of the environmental factors controlling fertilisation, embryonic development and larval survival of free-spawning marine invertebrates are often limited by methodological problems. One common limitation is the difficulty of inducing spawning under laboratory conditions in many species. In many cases species must be dissected in order to obtain the gametes from the gonads, often yielding unfertilisable immature eggs. This problem occurs in patellid gastropods. The role of pH on the maturation of oocytes is well known and is of particular importance in laboratory experiments. The artificial maturation of oocytes through alkalinisation was investigated in order to facilitate laboratory experiments on reproduction and developmental larval ecology of *Patella depressa* and *Patella vulgata*. Maturation was observed by analysing shape and chorion. Maturation was accelerated above pH 9.0. Alkalinising oocytes at pH 9.5 significantly enhanced *in vitro* fertilisation rate, especially at higher sperm concentrations. pH 10 is, however, harmful to eggs, probably causing death, which will lead to low fertilisation rates. Sperm dilution experiments using alkalinised oocytes suggest that both species require a similar optimum sperm concentration.

4.1. Introduction

Laboratory experiments have been valuable in investigating the controls of fertilisation success (Benzie & Dixon, 1994; Levitan, 1995; Sewell & Young, 1999; Baker & Tyler; 2001), embryonic development (Thorson, 1950; Sewell & Young, 1999; Bottger & McClintock, 2001) and larval survival (Davis & Calabrese, 1969; Rumrill, 1990; Tyler & Dixon, 2000) of free-spawning marine invertebrates. The biology and ecology of the early life-history stages of benthic invertebrates is of major importance in benthic ecology, since larval supply plays an important role in population dynamics of benthic invertebrates with consequence for community structure (Cameron & Rumrill, 1982; Gaines & Roughgarden, 1985; Gaines et al., 1985; Grosberg & Levitan, 1992; Kinlan & Gaines, 2003; Shanks et al., 2003; Kinlan et al., 2005). Larval biology and ecology is also essential for research in aquaculture of high value species (e. g. Gallardo & Sanchez, 2001; Ponis et al, 2003; Messinas et al., 2005). Despite its importance, laboratory experiments using larvae are often limited by methodological problems. In order to perform the experiments, large amounts of eggs, embryos or larvae are needed. These may be very difficult, or impossible, to collect directly from the plankton, even for very common shallow-water organisms. For instance, many larval forms are not known or are impossible to distinguish, and the only way to isolate them is through larval rearing.

Therefore, in most cases mature gametes must be obtained from ripe adult animals, not only for fertilisation studies, but also for investigations of early developmental ecology and larval survival. In some taxa this is not problematic, since spawning can be induced through different methods. In some mollusc species (Morse et al., 1977; Peña, 1986), including patellid representatives (Kay & Emlet,

2002) spawning can be induced by placing adults in seawater containing hydrogen peroxide. In echinoderms, laboratory spawning can usually be achieved by intracoelomic injections of a KCl solution (Bosch et al., 1987; Kelly et al., 2000) or other methods (Benzie & Dixon, 1994). Indeed much of our current knowledge on the fertilisation ecology of free-spawning invertebrates is based on results obtained with echinoderms (Lillie, 1915; Rupp, 1973; Pennington, 1985; Levitan, 1995) partly because of the ease of laboratory spawning and fertilisation. But for many species from other taxa artificial induction of spawning has not been successful. This is the case for some patellid limpets, in which it is necessary to dissect the gonads in order to obtain the gametes (Dodd, 1955; Baker, 2000). Many researchers that have performed artificial fertilisation experiments on gametes obtained by dissection have reported difficulties in fertilising eggs, and in the development and rearing of the larvae (Patten, 1868; Loeb, 1902; Wolfsohn, 1907; Smith, 1935; Dodd, 1955; Smaldon & Duffus, 1985; Baker, 2000). In spite of the difficulties, gonad dissection has been useful for describing morphological events of *Patella* development, by observing the few fertilisable oocytes obtained (Dodd, 1955; Smaldon & Duffus, 1985). However, the fertilisation rates obtained have generally been low and unsuitable for experimental studies of survival, larval longevity and settlement.

The immature oocytes in *Patella* include irregularly shaped cells, with a thick chorion, in contrast to the perfectly spherical-shaped and non-chorion-layered fertilisable oocytes (Wolfsohn, 1907; Dodd, 1955; Smaldon & Duffus, 1985; Baker, 2000). It has long been known that hydroxyl ions can induce maturation in starfish (Loeb, 1902) as well as an increase in the amount of fertilisable oocytes in starfishes (Loeb, 1902) and limpets (Wolfsohn, 1907). In *Patella vulgata* and other prosobranchs, a pH increase can release oocytes from meiosis arrest (Guerrier et al.,

1986; Catalan & Yamamoto, 1993) and a pH rise has been noted in bivalve oocytes undergoing hormone-induced maturation (Deguchi & Osanai, 1995). Artificial alkalinisation of oocytes has been used in shellfish aquaculture (Corpuz, 1981) and cytological research (Gould et al., 2001). However, the use of this technique, or any other artificial oocyte maturation procedure, is not commonly used by benthic ecologists to perform reproductive and developmental bioassays. Thus there are few methodical tests regarding this application. Smaldon & Duffus (1985) studied the effect of pH on fertilisation rates in *Patella vulgata*, but because of their experimental design they could not distinguish whether pH was acting upon the oocyte maturation process or during fertilisation. Harris and co-workers (Harris et al., 2002) have successfully used alkalinisation to mature and fertilise oocytes of the bivalve *Pteria sterna*, and suggested its use to produce larvae for repopulation of this exploited species. Hodgson (unpublished data), has improved fertilisation rates in *Patella ulyssiponensis* by exposing oocytes to NH_4OH.

In this chapter the potential use of artificial maturation of gametes through alkalinisation for subsequent application in fertilisation kinetics and larval biology studies is described. The effect of oocyte alkalisation prior to sperm contact on its fertilisability was tested for *Patella depressa* and *Patella vulgata*. Various pH values and contact times were tested. How sperm concentration influences fertilisation rates of pH-matured oocytes was also investigated in order to determine the optimum concentration for fertilisation.

The specific questions addressed in this paper are: in *Patella depressa* and *Patella vulgata*, does a pH rise in seawater affect the maturation of oocytes? Does oocyte alkalinization increase fertilisation rates in these species? What is the

optimum sperm concentration for fertilisation in these species, using alkalinized and non-alkalinized oocytes?

Further, to test whether embryos obtained by this method could yield to normal larvae, these were cultured in laboratory. Survival and normal development were quantified.

4.2. Materials and methods

4.2.1. Effects of pH on oocyte maturation

4.2.1.1. Material used and preliminary observations

Specimens were collected in Batten Bay, Plymouth (UK), during the breeding season for each species (Orton et al., 1956; Orton & Southward, 1961), and maintained in running seawater tanks in the Marine Biological Association, UK, for several weeks during the experiments. Sperm and oocytes were obtained by dissecting the foot and pipetting the gametes from the gonads into a clean beaker. Oocytes from 3-5 females were rinsed, pooled together and homogenised in a beaker prior to the experiments. This yielded to a suspension of approximately $2x10^5$ oocytes. ml^{-1}.

Seawater was manipulated by adding a few drops of dissolved NaOH to filtered seawater until the desired pH value was reached. On all occasions non-treated filtered seawater was used as a control.

Preliminary observations were made on the effect of different pH values (8.5, 9.0, 9.5, 10 and 10.5) on oocyte shape and presence/absence of chorion, in *P.*

depressa. An oocyte suspension was reduced to a minimum volume by decanting the oocytes and siphoning the water. This suspension was then distributed equally among glass bowls containing 150 ml of pH-manipulated seawater solutions (8.5, 9.0, 9.5, 10 and 10.5) and containing control seawater.

After different periods of time (0.5, 1.5, 2.5, 5.5 hrs), 100 oocytes from each bowl were analysed under a stereomicrospe to calculate the percentage of spherical oocytes and non-chorion-layered oocyte. The experiment was stopped after 5.5 hrs because the cell membranes started to break at the highest pH treatments.

A similar experiment was performed in *P. vulgata* except that only pH 9.5 was tested for this species.

4.2.1.2. Replicated experiments

Following the preliminary results, replicated experiments (n=3) were performed to confirm the effect of alkalinisation (pH=9.5) on oocyte maturation, using *P. depressa*. A two-way ANOVA was performed to test the effects and interactions of pH and time. LSD post-hoc tests were applied.

4.2.2. Effects of pH on fertilisation rates

Glass bowls containing *P. depressa* oocytes, submerged in alkalinised and normal seawater, were prepared as above. After different periods of time (1, 2.5, 5.5 hrs), 30 ml from each glass bowl (normal seawater, pH 8.5, pH 9.5 and pH 10) were filtered through a 30 μm mesh and resuspended in 30 ml of filtered seawater. Nine ml of this final suspension was then transferred to three glass vials. Sperm from 3

recently dissected males were diluted separately until a concentration of 10^8 spermatozoa. ml^{-1} was achieved. One ml from each sperm suspension was added to one vial containing the oocytes. The same fertilisation procedure was applied to oocytes immediately after spawning (artificially).

8 hours after sperm contact had started, fertilisation rates were categorised as being Low (<10%), Moderate (>10% and <50%) or High (>50%), by observing the relative number of developing embryos. For the 1 hour treatment, 100 oocytes from each vial was analysed under a stereomicroscope and a one-way ANOVA was performed to test the effect of oocyte treatment on fertilisation rates. LSD post-hoc tests were applied to identify the significant differences between treatments.

3.2.3. Effects of sperm concentration x alkalinization on fertilisation rates

Half the volume of an oocyte suspension was added to 250 ml of seawater alkalinised at pH 9.5, and the other half was diluted in the same amount of normal seawater. After 3 hours, the oocytes from both treatments were filtered and resuspended in 250 ml of normal seawater. 24 small glass vials were filled with 9 ml of the alkalinised oocytes suspension, and 24 with 9 ml from the non-alkalinised oocyte suspension. Sperm solutions at 7 different concentrations were prepared from one male and added to the vials, one ml from each sperm concentration was added to three vials of each oocyte treatment (normal and pH-matured). One ml of filtered seawater was added to three egg-only control vials for each oocyte treatment. After 8 hours, the percentage of developing embryos in a subsample of each replicate was measured under a stereomicroscope.

This experiment was performed both for *P. depressa* and *P. vulgata*. Two-way ANOVA was performed to test the effects and interactions of sperm concentration and pH-maturation on fertilisation rates. In *P. depressa*, there was no fertilisation in non-alkalinised oocytes at any sperm concentration, therefore a one-way ANOVA was performed for this species to test only the effect of sperm concentration upon fertilisation rates in pH-matured oocytes. *P. depressa* data was square-root transformed prior to ANOVA to meet the analysis pre-requisites. LSD post-hoc tests were applied for both species.

4.2.4. Rearing of the larvae

Oocytes from four females of *P. vulgata* were pooled together and matured artificially at pH 9.5. The artificially matured oocytes were then subjected to fertilisation in a sperm solution of 10^6 spermatozoa. ml^{-1}. Approximately $4x10^4$ eggs were obtained. After 5 hours, sperm was washed off, and eggs were evenly distributed in three 500 ml beakers filled with seawater and left to develop at 18 °C.

When the embryos reached the swimming trochophore stage, the normal swimming larvae were isolated from the abnormal ones. This was done by siphoning the upper layer of water, representing about 2/3 of the total volume from each beaker and filtering it using a mesh. All the normal larvae were resuspended and pooled together in a beaker containing 400 ml of seawater. The swimming larvae were isolated again whenever abnormal or dead larvae were detected at the bottom of the culture. When the larvae reached the veliger stage, the culture was filtered and refilled everyday, and a few drops of a rich culture of *Phaoelodactylum tricornutum* was added at this occasion. The total number of embryos at different stages of the

culture was estimated homogenising the culture and analysing a subsample (5 ml) under a microscope.

4.3. Results

4.3.1. Preliminary observations

The experiment using *P. depressa* was designed to establish which pH range was worth investigating in more detail. It is very clear (fig. 3.1) that higher pH improved the maturation process when compared to lower pH values. A stepwise change occurred at pH 9.5 between lower pH treatments and high pH treatments, but it was not possible to detect differences between the effect of pH 9.5, 10.0 and 10.5 upon shape.

After 0.5 hours, in lower pH treatments (control, 8.5 and 9.0) around 5% of oocytes observed were spherical, while in higher pH treatments (9.5, 10, 10.5) this value ranged between 35% to 49%. This difference was maintained during the monitoring period. After 1.5 hour, at higher pH, the percentage of spherical oocytes was always greater than 70%. In contrast, at low pH, the percentage of spherical oocytes was never greater than 40%.

By analysing chorion disintegration (fig. 3.2), only pH 9.5 enhanced maturation. After 0.5 hours, in all treatments, all oocytes still presented a chorion layer. At pH 9.5, 79% of the oocytes had lost the chorion layer after 2.5 hours, whereas in all other treatments the number of oocytes without chorion remained close to zero.

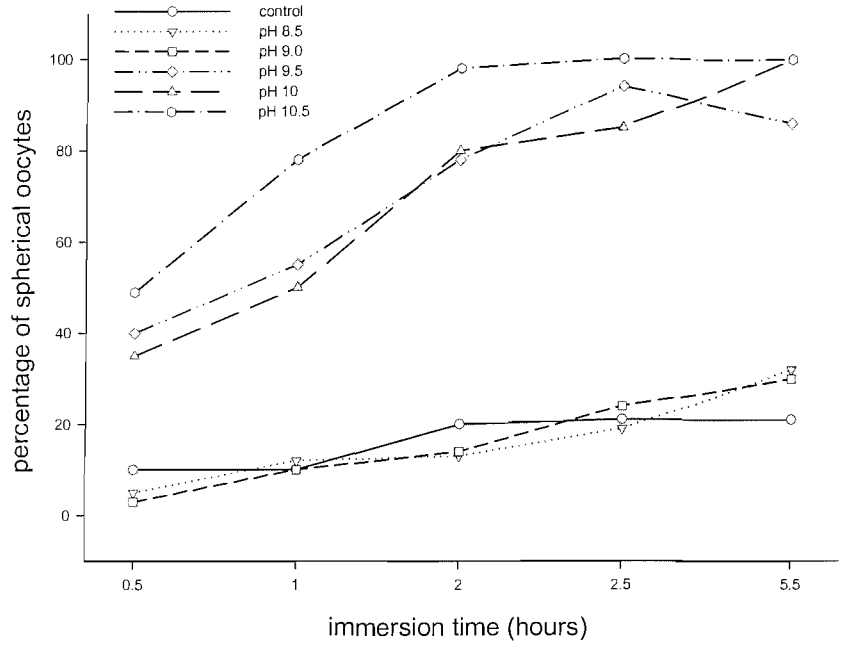


Figure 3.1. *Patella depressa*. Percentage of spherical oocytes, with differing immersion times and seawater pHs. All oocytes were artificially spawned, irregular in shape and chorion-layered before the test.

In *P.vulgata* the effect of pH 9.5 upon oocyte shape was very similar and consistent over the period studied. However, no conclusive statement can be drawn about the pH effect upon chorion disintegration (figs. 3.3 and 3.4).

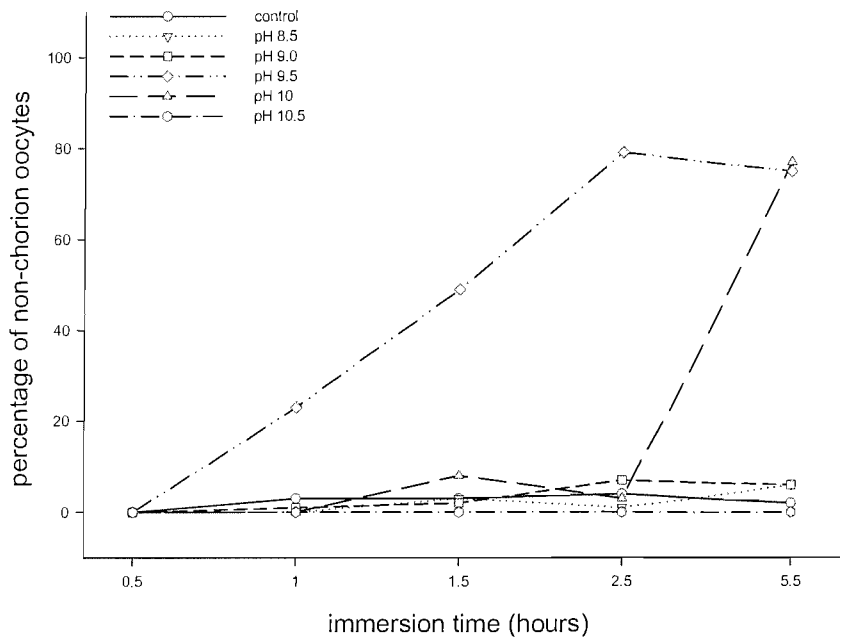


Figure 3.2. *Patella depressa*. Percentage of non-chorion oocytes, with differing immersion times and seawater pHs. All oocytes were artificially spawned, irregular in shape and chorion-layered before the test.

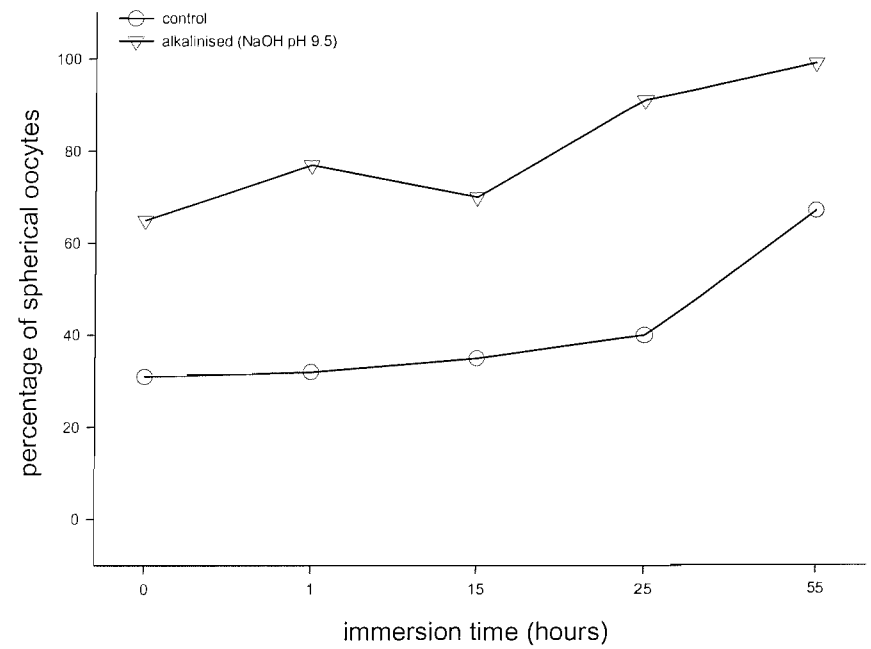


Figure 3.3. *Patella vulgata*. Percentage of spherical oocytes, with differing times and seawater pHs. All oocytes were artificially spawned, irregular in shape and chorion-layered before the test.

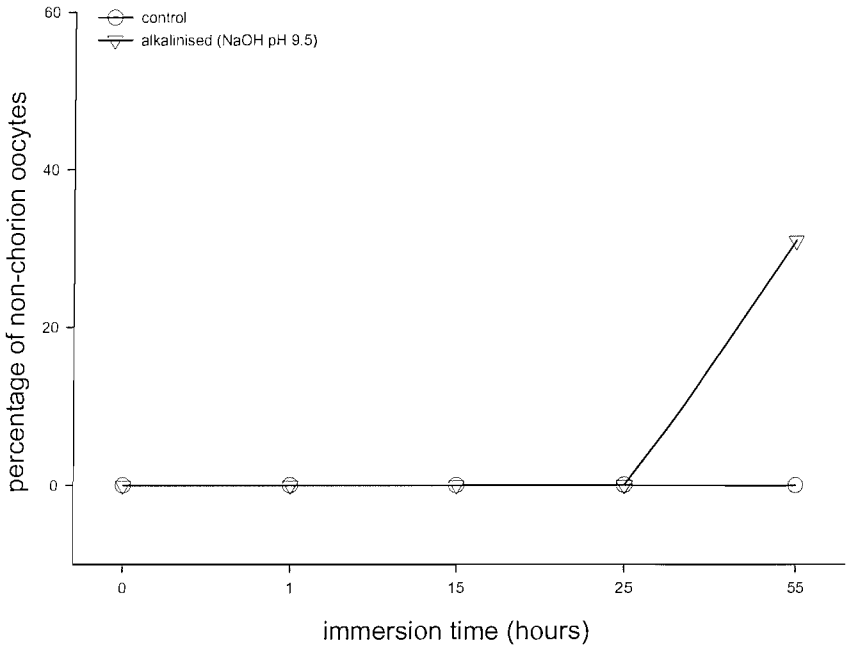


Figure 3.4. *Patella vulgata*. Percentage of non-chorion oocytes, with differing immersion times and seawater pHs. All oocytes were artificially spawned, irregular in shape and chorion-layered before the test.

4.3.2. Effects of alkalinisation on oocyte maturation

Significant ($p<0.05$) effects of pH and time of immersion on the rate of spherical oocytes were found. No significant interaction ($p<0.05$) was found between pH and time. Post-hoc tests revealed a percentage of spherical oocytes significantly higher ($p<0.02$) at pH 9.5 than in controls (Fig. 3.5), at all time treatments. The effect of pH and time of immersion upon the percentages of oocytes without chorion was significant ($p<0.05$) with a highly significant interaction ($p<0.01$) between pH and time of immersion. Post-hoc tests revealed a significant ($p<0.05$) effect of alkalinisation on the rate of oocytes without chorion, from 1.5 hour of immersion onwards (Fig. 3.6).

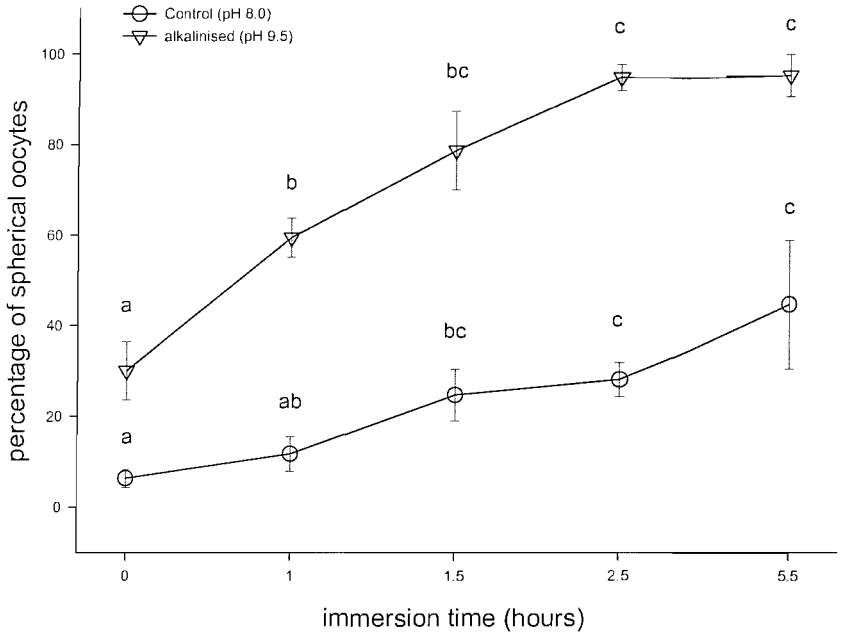


Figure 3.5. *Patella depressa*. Mean percentage of spherical oocytes, with differing times in control and alkalinised (pH 9.5) seawater. All oocytes were artificially spawned, irregular in shape and chorion-layered before the test. Bars represent the standard error. Significant difference (LSD test, $p<0.05$) between control and alkalinised treatments was found for all time treatments. Same letters indicate no sognificant differences (LSD test, $p<0.05$) between immersion times within a pH treatment.

Table 3.1. *Patella depressa*. Two-way ANOVA on percentages* of spherical oocytes in control (pH 8.0) and alkalinised (pH 9.5) samples for increasing immersion times (0.5, 1.0, 1.5, 2.5 and 5.5 hours).

	ss	df	ms	F	p
pH	2.706	1	2.706	113.035	<0.001
Time	1.767	4	0.444	18.450	<0.001
Interaction	0.213	4	0.053	2.227	0.1227

* Transformed to arcsin($\sqrt{\%}$)

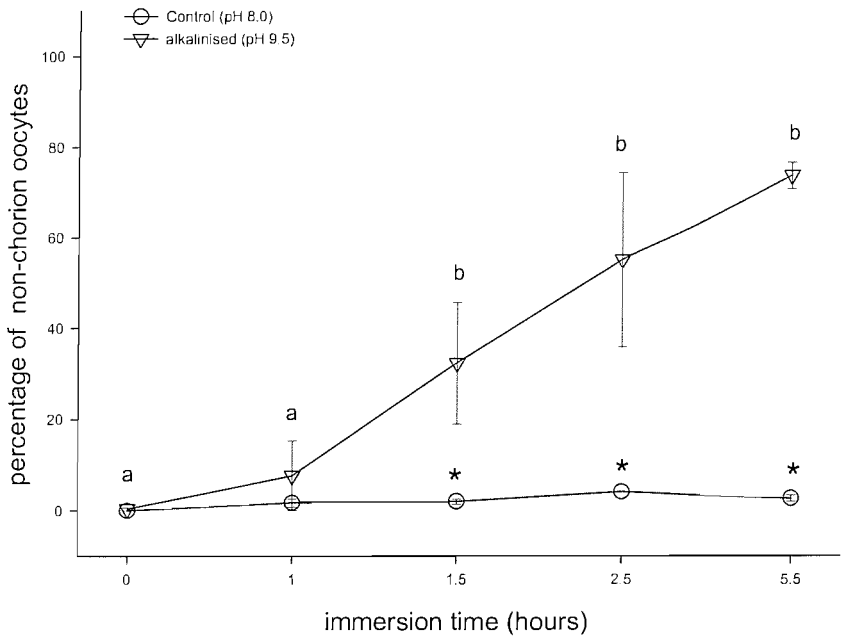


Figure 3.6. *Patella depressa.* Mean percentage of non-chorion oocytes, with differing immersion times in control and alkalinised (pH 9.5) seawater. All oocytes were artificially spawned, irregular in shape and chorion-layered before the test. Bars represent the standard error. * Significant difference (LSD test, $p<0.05$) between control and alkalinised treatments. Same letters indicate no significant differences (LSD test, $p<0.05$) between immersion times within a pH treatment.

Table 3.2. *Patella depressa.* Two-way ANOVA on percentages* of oocytes without chorion in control (pH 8.0) and alkalinised (pH 9.5) samples for increasing immersion times (0.5, 1.0, 1.5, 2.5 and 5.5 hours).

	Ss	df	ms	F	p
PH	1.242	1	1.242	39.870	<0.001
Time	1.458	4	0.364	7.045	<0.001
Interaction	0.789	4	0.197	6.3302	0.002

* Transformed to arcsin($\sqrt{\%}$)

4.3.3. Effects of alkalinisation on fertilisation rates

Oocytes alkalinised at pH 9.5 yielded better fertilisation rates. Prolonged immersion periods in alkaline mediums did not lead to higher rates of fertilisation. Exposure to pH higher than 9.5 reduced fertilization rates. There was no variation between the different males used within a treatment, and therefore only one symbol had to be used for each treatment in table 3.3.

Samples from the one-hour treatment were subjected to quantitative analysis and the percentages from each male were used as replicates (fig. 3.7). This confirmed an optimum pH at 9.5, with a highly significant difference ($p<0.01$) of fertilisation success between oocytes alkalinised at pH 9.5 and oocytes alkalinised at pH 10.

4.3.4. Effects of sperm concentration x alkalinisation on fertilisation rates

Sperm concentration significantly ($p<0.05$) affected fertilisation rates of alkalinised oocytes of both species (figs. 3.8 and 3.9). The optimum sperm concentration in *P. depressa* was around $6x10^5$ spermatozoa. ml^{-1}; as the sperm diluted, fertilisation success decreased rapidly (fig. 3.9). Similar concentrations also proved to be critical for *P. vulgata* (fig. 3.8). For alkalinised oocytes in *P. vulgata*, there was a steep rise in the fertilisation rates up to a concentration of $3x10^5$ spermatozoa. ml^{-1}; and from there on, fertilisation continued to increase, but at a much slower rate (fig. 3.8). The fertilisation success was on average 32% at $3x10^4$ spermatozoa. ml^{-1}; at $3x10^5$ spermatozoa. ml^{-1} it showed a high rise to 58%; and at $3x10^6$ spermatozoa. ml^{-1}, only a small rise to 66% was observed. Alkalinisation had

highly significant effect ($p<0.0001$) on fertilisation success. Post-hoc tests revealed significant differences at all concentrations from $3x10^4$ sperm. ml^{-1} ($p \leq 0.02$).

Table 3.3. *Patella depressa*. Fertilisation rates of recently shed oocytes (control 1), oocytes immersed into untreated water (control 2) and into alkalinised seawater mediums of different pHs (8.5, 9.0, 9.5 and 10). Symbols: L, < 10%; M 10%-50%; H, >50%. Three replicates were used for each treatment but, because there was no difference between replicates within a treatment, only one letter is shown for each treatment.

Immersion period	Control 1	Control 2	pH=8.5	PH= 9.5	PH=10
no immerson	L	-	-	-	-
1 hour	-	L	L	H	M
2.5 hours	-	L	L	H	L
5.5 hours	-	L	L	H	L

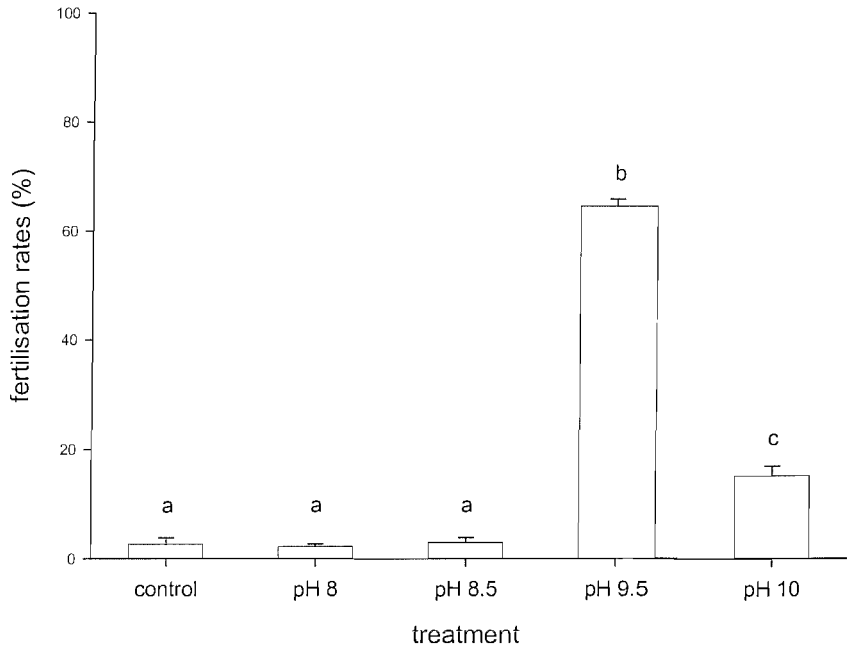


Figure 3.7. *Patella depressa*. Mean fertilisation rate of oocytes recently shed (control 1), immersed into normal seawater for 1 hour (pH 8), and immersed in alkalinised seawater for 1 hour at various pHs (8.5, 9.5 and 10). Bars represent standard error. Different letters indicate significant differences (LSD test, $p<0.05$). This test was performed using samples from the same experiment described in table 3.3, but quantifying the exact percentage of fertilised oocytes, which was completed only 1 hour after the start of the experiment.

Table 3.4. *Patella depressa*. One-way ANOVA on fertilisation rates* of oocytes recently shed (control 1), immersed in normal seawater (pH 8.0), and submitted to different alkalinisation treatments (pHs 8.5, 9.5 and 10) for one hour.

	ss	df	ms	F	P
pH	1.366	4	0.341	171.626	<0.001

* Transformed to arcsin($\sqrt{\%}$)

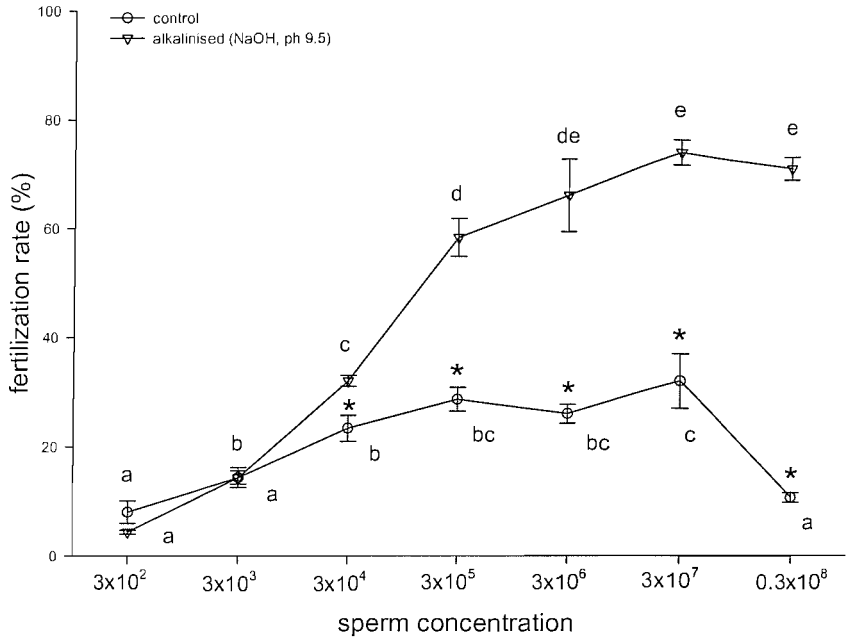


Figure 3.8. *Patella vulgata*. Mean fertilisation rate at different sperm concentration, in alkalinised (pH 9.5) and non-alkalinised oocytes. Oocytes were artificially spawned. Bars represent the standard error. Significant difference between alkalinised and non-alkalinised treatments (LSD test, $p<0.05$) is designated by *. Same letters indicate no significant differences (LSD test, $p\geq0.05$).

Table 3.5. *Patella vulgata*. Two-way ANOVA on fertilisation rates* of alkalinised (pH 9.5) and non-alkalinised oocytes at different sperm concentrations ($3x10^2$-$3x10^8$).

	Ss	df	ms	F	p
sperm concentration	0.737	6	0.737	224.03	<0.001
alkalinization	1.550	1	0.258	78.482	<0.001
Interaction	0.647	6	0.107	32.791	<0.001

* Transformed to arcsin($\sqrt{}$%)

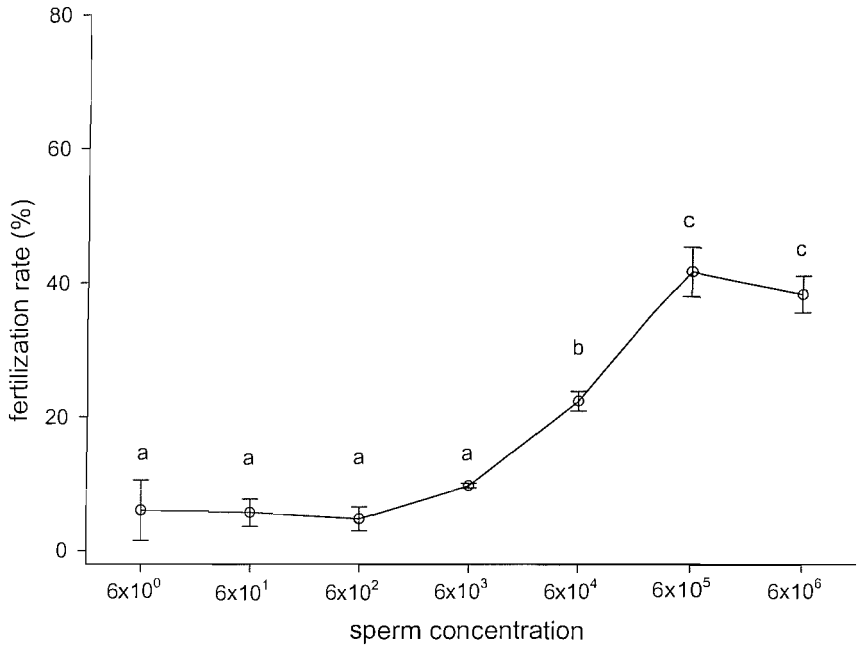


Figure 3.9. *Patella depressa*. Mean fertilisation rate at different sperm concentration. Oocytes were artificially spawned and matured at pH 9.5. Bars represent the standard error. Different letters represent significant difference (LSD test, $p<0.05$).

Table 3.6. *Patella depressa*. One-way ANOVA on fertilisation rates* of alkalinised (pH 9.5) and non-alkalinised oocytes at different sperm concentrations ($6x10^0$-$6x10^6$).

	ss	df	ms	F	p
sperm concentration	1.060	6	1.151	20.768	<0.001

* Transformed to arcsin($\sqrt{\%}$)

4.3.5. Rearing of the larvae

After 10 hours all embryos had reached at least the 32-cells stage. After 24 hours all trochophores had reached the swimming stage. Pre-torsional veligers were obtained 48 hours after fertilisation. Operculums were visible at 72 hours, and eyes at 96 hours. From the $4x10^4$ early trochophores, obtained from 4 females, approximately $8x10^3$ became normal late trochophores. Approximately 50% of those developed into normal veligers and underwent torsion. The culture terminated at 140 hours after fertilisation, when a few larvae had acquired sedentary habit, crawling on the bottom of the beaker. Thus the length of planktonic life at temperatures similar to 18 ^oC was approx. 6 days.

4.4. Discussion

4.4.1. The effect of pH on maturation

Many previous studies have already demonstrated that alkalinisation can induce oocyte maturation (Wolfsohn, 1907; Guerrier et al., 1986; Catalan & Yamamoto, 1993; Villain et al., 1993). Through the analysis of external cellular characteristics I have quantified how alkalisation can improve artificial maturation and fertilisation of stripped-spawned oocytes obtained by dissection in two species of *Patella*. This has practical implications for laboratory experiments on the environmental control of reproductive success in these species. Besides demonstrating the use of alkalinisation techniques in *Patella* species, this work also

investigated the optimum pH to be used. The effect of sperm concentration upon fertilisation rate was also examined.

The results suggest the most effective pH is 9.5. Lower pHs are likely to have no effect on shape, chorion and fertilisation rate. On the other hand, care must be taken not to alkalinise seawater too much because pH 10 partially prevented oocytes from being fertilised or from undergoing cleavage. Although pH 10 or above seemed to be effective in inducing oocyte shape change, it did not seem to induce chorion disintegration. Our observations indicate that pH 10 may improve fertilisation rates if the oocytes are not submerged to the alkalinisation medium for more than one hour, but even then the results were not as complete as in pH 9.5. These results appear to be contrary to the recommendation by Duffus & Smaldon (1985) that "seawater must be carefully maintained at 7.9, the normal seawater pH", in their study on the effect of pH on maturation and fertilisation of gametes in *Patella vulgata*. In their experiments, eggs were left for 1 hour in different flasks under different pHs in which later fertilisation occurred by adding a few drops of sperm. Regular adjustments of pH were made during fertilisation and embryonic development. Therefore it is not possible to state whether the results observed were a consequence of pH action on 1) the oocyte maturation process, 2) sperm activity, 3) fertilisation process or 4) cleavage stages. In our experiments, after maturation, oocytes were rinsed and fertilisation always occurred under normal pH conditions. Therefore it has been possible to account for the effect of pH on maturation only. And it is possible to deduce that improved fertilisation rates were a consequence of the maturation process of oocytes triggered by a pH rise prior to fertilisation.

The pH value suggested in this chapter is similar to that used by Corpuz (1981) to induce oocyte maturation in the patellid *Cellana exarata* in the laboratory.

However the author does not say on what this decision was based, and whether he tested different pHs. Hodgson (unpublished data) used pH 9.0 to induce maturation in *Patella ulyssiponensis*, but also did not test different pHs and used NH_4OH instead of NaOH. These preliminary results achieved for *P. ulyssiponensis* are similar to those reported here for *P. depressa and P. vulgata*. Catalan & Yamamoto (1993) analysed the effect of pH on the percentage of oocytes undergoing Germinal Vesicle Breakdown (GVBD), in *Cellana nigrolineata*. That study also found an optimum pH, which was from 9.0 to 9.5. Higher pH had detrimental consequences, similar to the observations presented here for *P. vulgata*. *C. nigrolineata* is similar to *P. vulgata* in terms of meiosis arrests and stage at which fertilisation occurs. The similarities regarding the optimum pH for artificial maturation process might reflect the natural mechanism of meiosis reactivation process, in which pH may play an important role. Harris et al. (2002) found pH 8.0 and 8.5 to be effective in promoting GVBD in the bivalve *Pteria sterna*, but they did not test higher pH, and like Hodgson (unpublished), used methods other than adding NaOH to alkalinise seawater.

4.4.2. The effect of sperm concentration on fertilisation rates

The fertilisation ecology of free-spawners was reviewed by Levitan (1995). Most research suggests that sperm dilution is an important limiting factor for fertilisation success in free-spawning marine invertebrates with a steep increase in fertilisation rates as a function of sperm concentration (e.g. Pennington, 1985; Benzie & Dixon, 1994; Baker & Tyler, 2001). This is also true for *Patella* species. The optimum sperm concentration for fertilisation success in *P. vulgata* had already been estimated by Baker (2000). She found the best fertilisation success at a sperm

concentration of $4.4x10^6$ spermatozoa. ml^{-1}, followed by a decrease in fertilisation success at higher sperm concentrations. Our experiments suggest a rapid increase in fertilisation success up to $3x10^5$ spermatozoa. ml^{-1}, followed by minor improvements in both alkalinised and non-alkalinised oocytes. A rapid decrease in fertilisation rates in concentrations > $3x10^7$ spermatozoa. ml^{-1} was observed in this work in non-alkalinised oocytes, as in Baker's experiments (2000). This did not happen in alkalinised oocytes within the sperm concentration range of our experiment. Our work suggests that the response of *P. depressa* alkalinised oocytes is very around that of *P. vulgata*, with a rapid increase in fertilisation rates from sperm concentrations similar to 10^4 spermatozoa. ml^{-1} up to sperm concentrations similar to 10^6 spermatozoa.ml^{-1}, followed by minor improvements. This general pattern of increasing fertilisation rates with increasing sperm concentration up to a certain sperm concentration value, is similar to what is found in many other free-spawning marine invertebrate species (Levitan, 1995). Although we have not quantified the proportion of abnormally developing embryos, it is well known that abnormality rate increases with sperm concentration as a result of polyspermy (Levitan, 1995). To avoid or to minimise polyspermy we recommend a sperm concentration not higher than 10^6 spermatozoa. ml^{-1}, since above this value no major improvement in fertilisation rates were observed.

4.4.3. Rearing of the larvae

From four females, it was possible to obtain 4,000 veligers undergoing normal development, in *Patella vulgata*. Despite the great number of non-developing oocytes and abnormal development in *Patella*, scientists have been motivated to

achieve results on these species (Wolfsohn, 1907; Dodd, 1955; Smaldon & Duffus, 1985; Baker, 2000) because of its great importance in European rocky-shore habitats (Lewis, 1964; Southward, 1964; Hawkins & Hartnoll, 1983; Hawkins & Hartnoll, 1985). These previous publications do not state the proportion of normal larvae obtained and therefore it is not possible to compare our work with past attempts in *Patella* species. 'D' larvae of the bivalve *Pteria sterna* were reared from alkalinised oocytes, but no quantification of survival during the rearing stages is presented by the authors (Harris et al., 2002). A survival of 50% of larvae from the trochophore to the late veliger stage is not much lower than the 68% observed in cultures of the patellid *Cellana exarata,* using naturally mature eggs (Corpuz, 1981). There is no reason to assume that this difference is a consequence of the use of artificially matured eggs.

Although it is not possible to compare exactly how effective our method is in comparison to other studies, a considerable quantity of normal larvae was obtained from only a few individuals. Our overall results, and the observations made by other authors who performed artificial fertilisations on *Patella*, show that this would not have been possible through the use of untreated oocytes obtained by excision.

4.5. Conclusion

The main aim of this work was to test the use of alkalinisation for fertilisation enhancement and its use in ecological laboratory experiments in *Patella* species. I demonstrated that it works very effectively and can be used on fertilisation ecology experiments. We found that the best results are attained when oocytes are alkalinised at pH 9.5 and recommend the use of a sperm concentration between 10^5

spermatozoa. ml^{-1} or 10^6 spermatozoa. ml^{-1}. It was found that through this technique a considerable amount of trochophores and veligers can be obtained from a few individuals, making the study of larval ecology in the laboratory feasible for these species. In our laboratory, *Patella* larvae obtained by this technique were successfully used to analyse larval survival and developmental rates under different physical conditions (chapter 5).

CHAPTER 5

Larval pressure and temperature tolerances of *Patella vulgata* and *Patella ulyssiponensis* with regard to their potential for colonization of cold and deep habitats

Trochophore larvae of *Patella vulgata* and *Patella ulyssiponensis* were incubated at different combinations of temperature (5 °C, 10 °C, 15 °C and 20 °C) and pressure (1 atm, 50 atm and 100 atm) for 24 hours. After incubation trochophores were analysed to determine: 1) survivorship, 2) percentage of veligers and 3) incidence of normal development. Larval tolerance to low temperature was higher in *P. vulgata* than in *P. ulyssiponensis*. Despite slow development, *P. vulgata* showed high larval survivorship and low incidence of abnormal development at very low temperature conditions (5 °C). This might play an important role in maintaining its populations in the extreme north of its distribution. In *P. ulyssiponensis*, the capability of trochophores to survive and develop normally under cold temperature (5 °C) was completely absent. Based on the present results, we conclude that the physiological potential in trochophores from *P. ulyssiponensis* for colonization of the northern parts of Norway is low. Results from both species suggest that, although the trochophore larvae can survive high pressure within their optimal temperature, its potential to migrate to deeper water would be limited by their inability to develop normally under high pressure/low temperature conditions. The present results do not corroborate the idea that larval stages of shallow-water organisms are generally

tolerant to pressure and physiologically capable of migration into deep seas. This does not seem the case in patellogastropods.

5.1. Introduction

The dispersal of planktonic propagules in marine sessile or sedentary species is essential for habitat colonization (Gaines & Roughgarden, 1985; Kinlan & Gaines, 2003; Shanks et al., 2003; Kinlan et al., 2005). In benthic invertebrates with indirect development long-distance dispersal is typically carried out during the larval stages (Scheltema, 1986; Grantham et al., 2003). Therefore, the potential of a species to colonize a specific habitat depends partially on its larval physiological tolerance to the physical characteristics of that habitat. In order to understand the mechanisms that control the colonization of deep-sea habitats, there has been a large amount of experimentation on the larval tolerances to pressure/temperature in shallow-water echinoderms. These experiments (Young et al., 1995; Young et al., 1997; Tyler & Young, 1998; Tyler et al., 2000; Aquino-Souza et al., in prep.) have shown that very often the limits tolerated by the dispersal stages are far wider than the distribution limits of the adults. However, care must be taken before generalizing that this high physiological potential for larval migration into the deep sea is widespread. Since these studies only include echinoderm species, it is possible that in other taxonomic groups the physiological potential for deeper water colonization is not widespread.

In order to broaden the taxonomic range of species tested for larval presssure/temperature tolerance we carried out experiments with two patellid limpet species (Gastropods: Patellogastropoda: Patellidae). Most patellid limpets release both eggs and sperm in the water column, where fertilization takes place and zygotes

develop into trocophore larvae which develop into veligers before settlement (Dodd, 1995). Therefore, from fertilisation until settlement, pattellid species live in the water column and during this period they can be transported by ocean currents to distant places, and if they are carried to the polar regions, which are the main areas of deep-sea water formation and deep-sea invasions, they could reach deep-sea habitats. The overall aim of this chapter was to examine whether the trochophores of *Patella vulgata* and *Patella ulyssiponensis* are tolerant to high pressures and low temperatures, similar to deep-sea conditions, as has been demonstrated in many shallow-water echinoderms studied to date.

Patella is a well distributed and diverse patellid genus in northeastern Atlantic shores. With the taxonomic split of *P. aspera* into *P. ulyssiponensis* (for continental areas and British islands) and *P.aspera* (for Macronesia islands), the genus now comprises 9 recognized species, distributed from Senegal to northern Norway (see Ridgeway et al., 1998; Koufopanou et al., 1999; Weber & Hawkins, 2005). Three species occur around the British Isles: *Patella depressa* is restricted to south-west England and Wales, *P. ulyssiponensis* is absent in the southeast, and *P. vulgata* occur wherever there is available substratum (Southward et al., 1995). *Patella vulgata*, a boreal cold temperate species, is distributed from southern Portugal (Fischer-Piette, 1953) to the Arctic (Vader, 1975). In contrast, *Patella ulyssiponensis*, a more southern species, only extends northwards up to southern Norway and the Shetlands; and it extends its distribution as far south as North Africa (Fischer-Piette, 1935, Southward et al., 1995) (fig. 4.1).

Regarding the breeding cycles of *P. vulgata* and *P. ulyssiponensis* in Europe, it has been observed that they vary both geographically and annually (Orton et al., 1956; Baxter, 1983; Bowman & Lewis, 1986; Guerra & Gaudencio, 1986; Garwood,

1987). However, in both species, spawning always occurs earlier in the North and East than in the South and West (Bowman & Lewis, 1986).

Together, the species distributional and reproductive patterns suggest that *P. ulyssiponensis* and *P. vulgata* might have, during some development stage, distinct thermal requirements from each other, which may explain their distributional differences. It has been suggested that the latitudinal distributions of *P. ulyssiponensis* and *P. vulgata* are set by temperature control of gonad activity or by spat susceptibility to temperature (Bowman & Lewis, 1977; Bowman & Lewis, 1986; Lewis, 1986). According to the settlement-timing hypothesis (Todd & Doyle, 1981), there is an optimum time for the larvae to settle. Species reproductive adapatations will, whenever possible, be adapted so that the larvae are at the settlement stage when conditions to settle are optimum. This could explain the latitudinal differences in the breeding cycles, within species, in *Patella,* as well as differences between *Patella* species in the same geographical area. Additionally, different temperature tolerances during reproduction stages could also determine latitudinal limits of both species.

However, by focussing only on temperature control of gonad activity or spat suceptibility, past works on temperature control of reproduction on these species have ignored the planktonic phases between gamete production and larval settlement. It is possible that temperature limits latitudinal distributions by affecting the dispersal stages rather than the benthic phases of *Patella* species. If the dispersal stages of *P. ulyssiponensis* are not able to tolerate temperatures as low as those of *P. vulgata*, it would be reasonable to speculate that this is currently preventing *P. ulyssiponensis* from colonizing higher latitudes. Furthermore if larval temperature tolerance is

controlling the northern limit of *P. ulyssiponensis*, it could also reduce its potential to colonize deep environments.

This paper will investigate the following hypotheses:

1) Trochophore larvae of *P. vulgata* are not more tolerant to cold temperatures than trochophore larvae of *P. ulyssiponensis*.

2) In both species, the trochophore larvae are able to survive and develop normally at temperature/pressure conditions found at 0.5-1.0 Km depth in the NE Atlantic.

If the first is accepted, it will show that differences in latitudinal distributions between *P. vulgata* and *P. ulyssiponensis* cannot be attributed to differences in larval temperature tolerances. If the second is accepeted it will reinforce the suggestions that the physiological potential to colonize deep-sea environments is widespread among invertebrate larvae.

5.2. Materials and methods

5.2.1. Specimens collection, fertilisation and embryonic development

The specimens were collected from different sites within Plymouth Sound (fig. 4.1), kept in running seawater and fertilized following the technique described in chapter 4. Embryos from approx. 4 females and 3 males were left to develop in three 500 ml beakers, containing seawater and kept at 15 ^{o}C, with a density of approx. 4.10^4 embryos per beaker. At the trochophore stage, the abnormal larvae have a very limited ability to swim in contrast with the normal larvae. The normal larvae were then isolated by siphoning and filtering the upper layer of water,

representing about 2/3 of the total volume, from each beaker, and re-suspending the larvae in 400 ml. This led to a suspension of about 20 larvae. ml^{-1}.

5.2.2. Experimental design

36 plastic tubes were filled with 8 ml from a larval suspension (of about 20 larvae.ml^{-1}) and covered with a lid. Three tubes were assigned for each treatment. The pressurizing method used was the same described for *Psammechinus miliaris* (chapter 2). Three pressure treatments (1 atm, 50 atm and 100 atm) and four temperatures (5 °C, 10 °C, 15 °C and 20 °C) were tested. All pressures were combined with all temperatures. After 24 hours, the vials were depressurised, and a subsample from each replicate was analysed under the microscope to determine the proportions of larvae: 1. live and dead, 2. normal and abnormal and 3. trochophore and veliger.

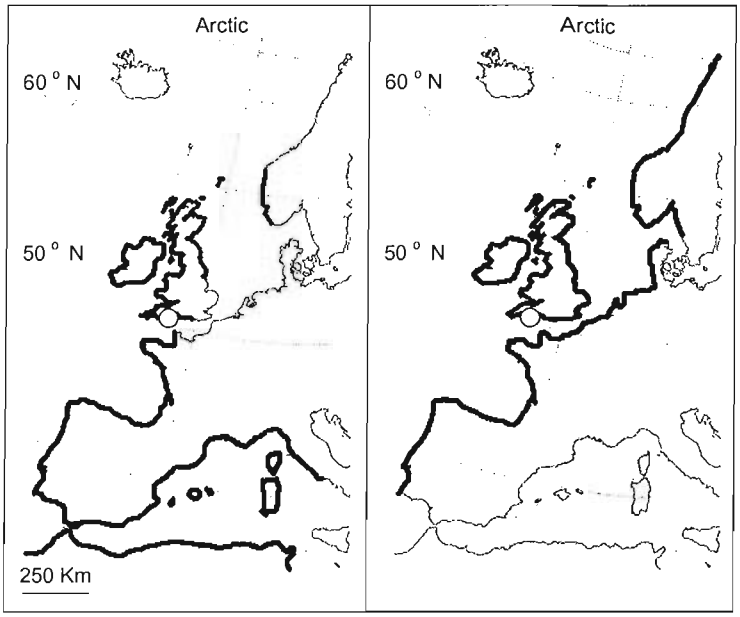


Figure 4.1. Distributions of *Patella vulgata* and *Patella ulyssiponensis* in the NE Atlantic. The circles show the approximate location of Plymouth Sound, the origin of all the specimens used in this study, for both species. (After Southward et al., 1995 and Hawkins, pers. com.).

5.2.3. Statistical analysis

The effects of temperature and pressure upon larval survival, abnormality rates, and percentage of larvae attaining the veliger stage at the end of each experiment were tested using two-way factorial ANOVAs. Scheffé post-hoc tests were used to identify homogenous groups. Prior to ANOVAs, percentages were transformed accordingly to meet the requirement of homogeneity among variances.

5.3. Results

5.3.1. General results

ANOVAS showed an effect ($p<0.05$) of pressure and temperature, as well as interaction ($p<0.05$), on determining survivorship, developmental rates and percentages of normal development, in *Patella ulyssiponensis* (tables 4.1-4.3) and *Patella vulgata* (tables 4.4 -4.6). By the end of the experiments, most larvae from both species were still alive at all temperatures (figs. 4.2 and 4.5), but those from *P. ulyssiponensis* were mostly abnormal at extreme temperatures (5 °C and 20 °C) (fig. 4.4), which would eventually lead to high larval mortality. In both species, larvae were still at the trochophore stage at the lowest temperature (5 °C) (figs. 4.3 and 4.6); nevertheless, larvae from *P. vulgata* were alive (fig. 4.5) and developing normally (fig. 4.7). For both species, pressure affected especially the rate of abnormality (figs. 4.4 and 4.7; tables 4.3 and 4.6). It also affected the rate of survival at extreme temperatures (figs. 4.2 and 4.5), but explained little variation in developmental rates, particularly in *P. vulgata* (fig. 4.6 and table 4.5).

5.3.2. *Patella ulyssiponensis*

5.3.2.1. Larval survival

Survival was high (>75%) at all temperatures tested, at 1 atm, but survival at 5 ° C was significantly ($p<0.05$) lower than at 10°C. As pressure increased, survival remained high at mid-temperatures (10°C-15°C) and decreased significantly ($p<0.05$)

at extreme temperatures (20°C and 5°C), reaching values under 20 % at 100 atm. These results show that low temperatures, similar to 5°C: i) negatively affects the survivorship of larvae from *Patella ulyssiponensis* even at normal pressures and that ii) this effect is made more severe by pressure.

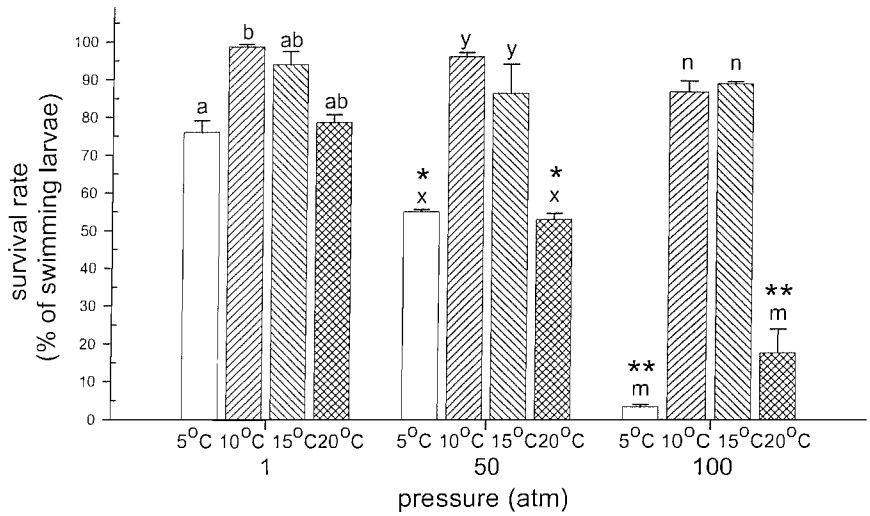


Figure 4.2. *Patella ulyssiponensis*. Survival rates of larvae incubated during 24 hours at the trochophore stage at different pressures and temperatures. Bars show standard error. Same letters show no significant difference between temperature treatments within a pressure treatment. * means a significantly lower percentage than at 1 atm under the same temperature. ** means a significantly lower percentage than at 50 atm under the same temperature.

5.3.2.2. Developmental rates

No *P. ulyssiponensis* larvae developed into the veliger stage at 5 °C, at any pressure. At higher temperatures (10 °C, 15 °C and 20 °C) larvae developed well at 1 atm, with more than 85 % reaching the veliger stage. Developmental rates had significantly ($p<0.05$) dropped to 15% at 50 atm, at 10 °C; and to approx. 25 % at

100 atm, at 10 °C. Developmental rates remained high at all pressures only at 15 °C, where nearly 100% of the larvae reached the veliger stage regardless of pressure.

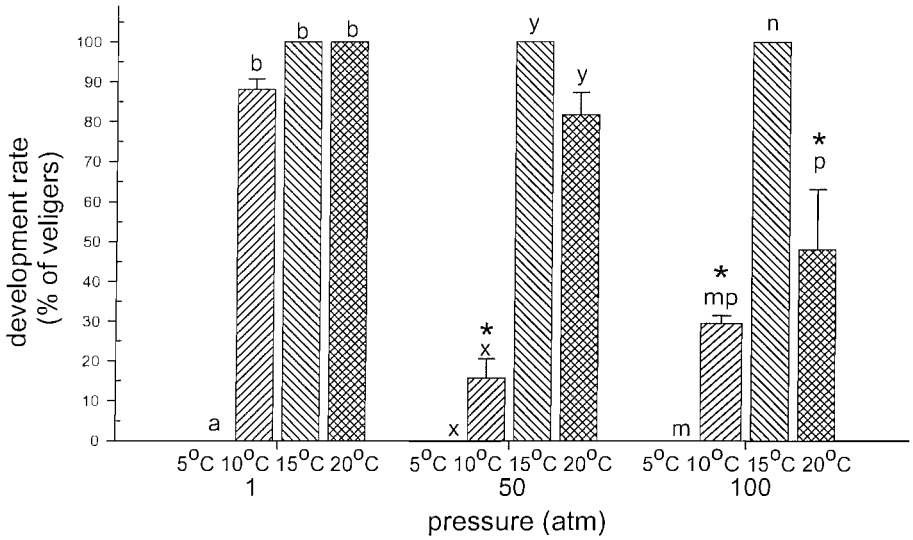


Figure 4.3. *Patella ulyssiponensis*. Percentage of trochophore larvae reaching the veliger stage after 24-hours incubation at different pressures and temperatures. Bars show standard error. Same letters show no significant difference between temperature treatments within a pressure treatment. * means a significantly lower percentage than at 1 atm under the same temperature.

5.3.2.3. Incidence of abnormality

Extreme temperatures (5 °C and 20 °C) were very deleterious to larvae, with less than 20 % developing normally even at 1 atm, which was significantly lower ($p<0.05$) than at 10 °C and at 15 °C. At 10 °C and 15 °C more than 65 % developed normally at 1 atm. Rates of normal development, at 10 °C and 15 °C significantly dropped to less than 40 % under 50 and 100 atm. Under hydrostatic pressures, the occurrence of normal developing larvae showed no significant difference ($p<0.05$) among temperatures (except between 5 °C and 15 °C at 100 atm) and were always under 40 %.

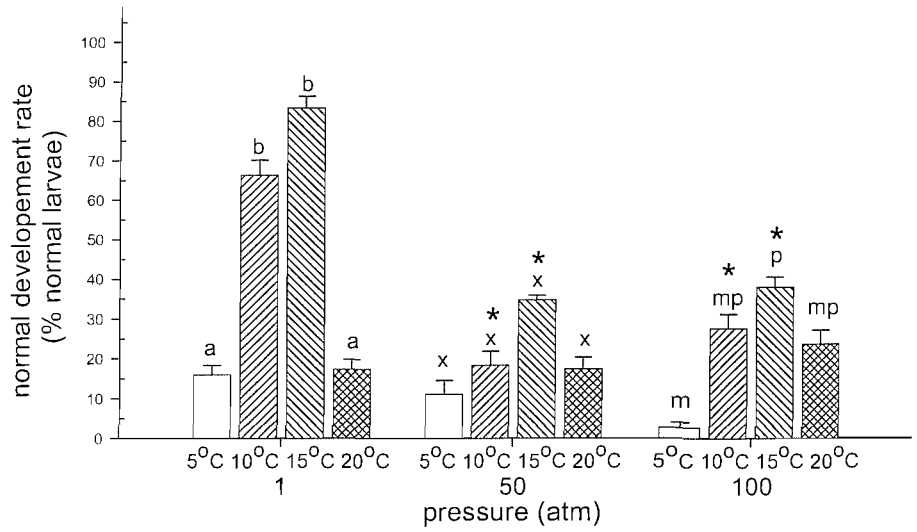


Figure 4.4. *Patella ulyssiponensis*. Percentage of normally developing larvae, analysed after 24-hours incubation of trochophores at different pressures and temperatures rates. Bars show standard error. Same letters show no significant difference between temperature treatments within a pressure treatment. * means a significantly lower percentage than at 1 atm under the same temperature.

Table 4.1. *Patella ulyssiponensis*. Two-way ANOVA on larval survival (% swimming)* 24 hours after trochophore incubation at three pressures (1, 50 and 100 atm) and four temperatures (5°C, 10°C, 15°C and 20°C).

	df	Ms	F	p
Temperature	3	1.029	124.68	<0.001
Pressure	2	0.743	90.09	<0.001
Interaction	6	0.108	13.16	<0.001

* Transformed to arcsin($\sqrt{\%}$)

Table 4.2. *Patella ulyssiponensis*. Two-way ANOVA on developmental rate* (% of veligers after 24 hours) of trochophores incubated at three pressures (1, 50 and 100 atm) and four temperatures (5°C, 10°C, 15°C and 20°C).

	df	Ms	F	p
Temperature	3	16979.34	279.24	<0.001
Pressure	2	2313.19	38.043	<0.001
Interaction	6	1145.72	18.842	<0.001

* Transformed to arcsin(√%)

Table 4.3. *Patella ulyssiponensis*. Two-way ANOVA on the percentage* of normal larvae 24 hours after trochophore incubation at three pressures (1, 50 and 100 atm) and four temperatures (5°C, 10°C, 15°C and 20°C).

	df	Ms	F	p
Temperature	3	0.467	83.689	<0.001
Pressure	2	0.301	53.997	<0.001
Interaction	6	0.074	13.275	<0.001

* Transformed to arcsin(√%)

5.3.3. *Patella vulgata*

5.3.3.1. Larval survival

Trochophores showed >80% survival rate at all temperatures tested under normal (1 atm) pressure. Unlike larvae from *P. ulyssiponensis*, there was no significant difference among any temperatures at 1 atm. At 5 °C and 20 °C, survival decreased gradually as pressure increased, with less 15 % survival at 100 atm which was significantly lower ($p<0.05$) than at 1 atm. At 10 °C, survival rates remained high up to 50 atm, but was significantly lower ($p<0.05$) at 100 atm, with approximately 25 % survival. At 15 °C survival remained high in all pressure treatments.

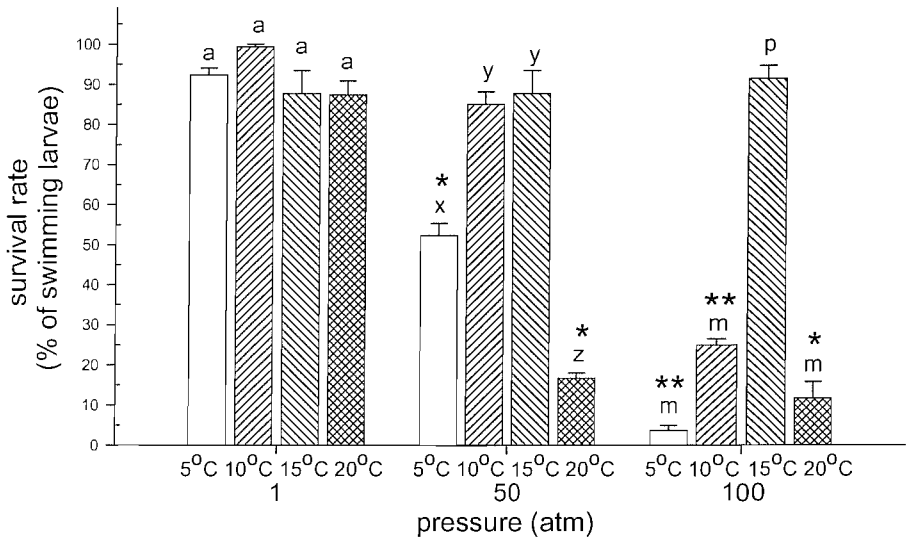


Figure 4.5. *Patella vulgata*. Survival rates of larvae incubated at the trochophore stage during 24 hours at different pressures and temperatures. Bars show standard error. Same letters show no significant difference between temperature treatments within a pressure treatment. * means a significantly lower percentage than at 1 atm under the same temperature. ** means a significantly lower percentage than at 50 atm under the same temperature.

5.3.3.2. Developmental rates

At temperatures above 5 °C, survival showed no significant difference between temperature/pressure treatments. Similarly to larvae from *P. ulyssiponensis*, there were no veliger larvae at 5 °C at any pressure treatments.

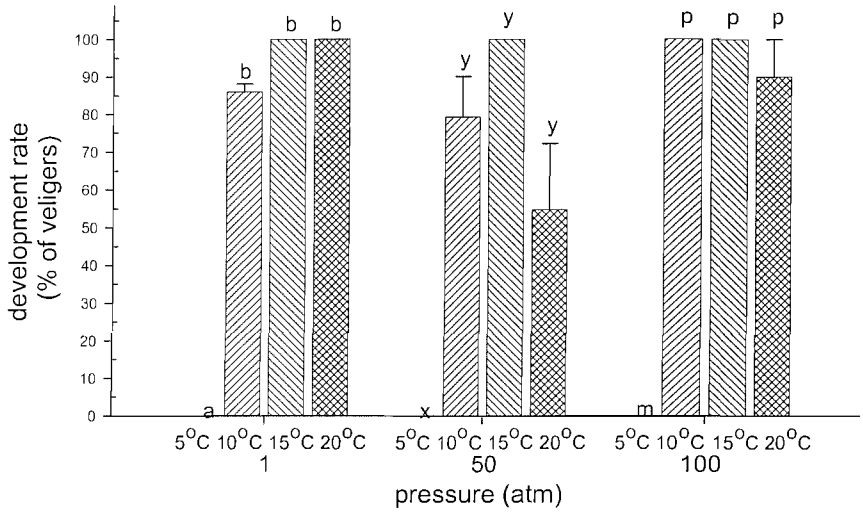


Figure 4.6. *Patella vulgata*. Percentage of trochophore larvae reaching the veliger stage after 24-hours incubation at different pressures and temperatures. Bars show standard error. Same letters show no significant difference between temperature treatments within a pressure treatment.

5.3.3.3. Incidence of abnormality

Unlike in *P. ulyssiponensis*, development normality/abnormality showed no significant difference among temperatures at 1 atm. Pressure significantly ($p<0.05$) affected normal development at 5 °C, where nearly all larvae developed abnormally from 50 atm onwards; and at 10 °C, where less than 10 % of larvae developed normally at 100 atm.

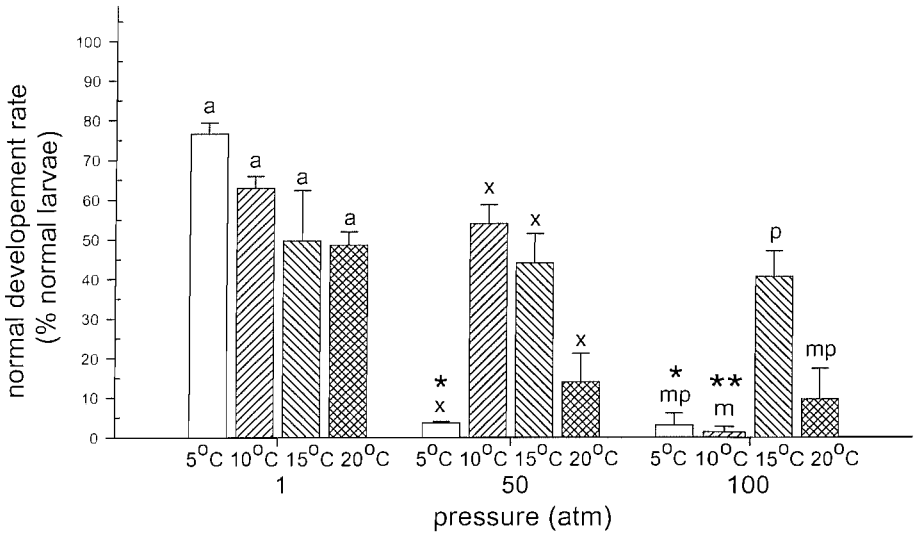


Figure 4.7. *Patella vulgata*. Percentage of normally developing larvae, analysed after 24-hours incubation of trochophores at different pressures and temperatures. Bars show standard error. Same letters show no significant difference between temperature treatments within a pressure treatment. * means a significantly lower percentage than at 1 atm under the same temperature. ** means a significantly lower percentage than at 50 atm under the same temperature.

Table 4.4. *Patella vulgata*. Two-way ANOVA on larval survival* (% swimming) 24 hours after trochophore incubation at three pressures (1, 50 and 100 atm) and four temperatures (5 °C, 10 °C, 15 °C and 20 °C).

	df	ms	F	p
Temperature	3	0.684	65.039	<0.001
Pressure	2	1.599	152.03	<0.001
Interaction	6	0.254	24.150	<0.001

* Transformed to arcsin($\sqrt{}$%)

Table 4.5. *Patella vulgata*. Two-way ANOVA on developmental rate* (% of veligers after 24 hours) of trochophores incubated at three pressures (1, 50 and 100 atm) and four temperatures (5 °C, 10 °C, 15 °C and 20 °C).

	df	ms	F	p
Temperature	3	18539.00	141.22	<0.001
Pressure	2	651.23	4.96	<0.01
Interaction	6	423.37	3.22	<0.01

* Transformed to arcsin(√%)

Table 4.6. *Patella vulgata*. Two-way ANOVA on the percentage* of normal larvae 24 hours after trochophore incubation at three pressures (1, 50 and 100 atm) and four temperatures (5 °C, 10 °C, 15 °C and 20 °C).

	df	ms	F	p
Temperature	3	0.141	6.622	0.001
Pressure	2	1.984	46.204	0.001
Interaction	6	0.219	10.290	0.001

* Transformed to arcsin(√%)

5.4. Discussion

5.4.1. Larval tolerance to temperature and the potential for latitudinal migration

The effects of temperature on recruitment in *Patella vulgata* was investigated by Vader (1975) who related poor recruitment to low air temperature, and by Bowman and Lewis (1977) who found evidence that spat intolerance to low temperature could lead to poor recruitment in NE England. Bowman & Lewis (1986) found that the breeding periods of *P. vulgata* and *P. ulyssiponensis* occurs earlier in cold regions than in warm regions and suggested that this may be a mechanism to ensure optimal temperature during settlement and survival of new spat. These studies suggest that low temperature negatively affects recruitment and can set the northern limit in *Patella* species. Probably because of methodological problems, these authors did not investigate the species planktonic phases. However, low survival rates during stages prior to settlement is likely to reduce recruitment success and potentially limit a species distribution.

The distribution of *Patella vulgata* extends to the extreme north of Norway while *P. ulyssiponensis* is restricted to the Southern area of Norway. This research aimed to investigate whether the thermal tolerance in trochophore larvae may play a role in establishing the northern limit of these species, and to determine the physiological potential of *P. ulyssiponensis* to colonize northern areas.

It was observed that: 1) trochophores from *P. vulgata* show similar survival rates at all temperatures with no statistical differences, whilst trochophores from *P. ulyssiponensis* showed significantly lower survival rates at 5 ^{o}C than at 10 ^{o}C; 2) At 5

°C development of *P. vulgata* was normal and that of *P. ulyssiponensis* was abnormal.

Overall, these results suggest that larval tolerance to low temperature in *P. vulgata* is higher than in *P. ulyssiponensis*. Despite slow development under 5 °C for both species, a large larval population of *P. vulgata* can survive and develop normally at this temperature, in contrast to *P. ulyssiponensis*. This might play an important role in maintaining its populations in the extreme north of its distribution, in Finnmark, where temperature rarely reaches 10 °C (ICES database). In *P. ulyssiponenis*, the very high rates of abnormalities at 5 °C coupled with slow development at this temperature, suggest that no larval supply is available for colonization of cold environments by this species. Based on the present results, it is suggested that the physiological potential for northwards migration in *P. ulyssiponensis* during the early stages of larval development is very low. Such migration would only be likely to happen as a consequence of rising sea temperatures. This is happening in the southern part of England (Hawkins & Moschella, unpublished data).

5.4.2. Larval tolerance to low temperature/high pressure and the species potential for colonization of deeper waters

Previous data (chapter 2) showed that southern populations of *Echinus esculentus* (Echinodermata) cannot develop normally at 4 °C, whereas northern populations undergo normal development at similar temperatures, making it more feasible for embryos from northern populations to initiate migration to deep-sea sites than embryos from southern populations. The present study examined adaptations to

cold temperature in *Patella* embryos and larvae using interspecific analysis, and investigated the influence of pressure on the effect of low temperatures.

The results suggest that although the trochophore larvae from both species can survive high pressure within their optimal temperature, their migration to deeper water would be greatly limited by the inability of the larvae to survive and develop normally in low temperatures (*P. ulyssiponensis*), or when low temperature is combined to high pressure (*P. vulgata*). There is a possibility that in the Mediterranean, where the bottom waters are warmer than in the N Atlantic , and where there is also deep water formation, larval stages from *P. ulyssiponensis* might be able to migrate to the deep sea and a small proportion survive and develop normally.

The results for *Patella* species are different from those found for the intertidal echinoid species *Psammechinus miliaris* (chapter 2), in which pressure generally showed no effect on normal development and survival of larvae (except at 15°C for the prism stage). All other shallow-water organisms studied show similar results to *P. miliaris* (Young et al., 1995; Young et al., 1997; Tyler & Young, 1998; Tyler et al., 2000; Benitez Villalobos et al., 2006). One of the objectives of this study was to use *Patella* species as models to test the validity of the general assumption that larvae from shallow-water species can tolerate temperature and hydrostatic pressure conditions typical of deep-sea habitats. This assumption follows a series of studies using echinoderms as test organisms (Young et al., 1995; Young et al., 1997; Tyler & Young, 1998; Tyler et al., 2000; Benitez Villalobos et al., 2006, and chapter 2 and 3). The present results do not corroborate the proposal that larval stages of shallow-water organisms are generally tolerant to pressure. This work shows that more species from varied taxa must be examined before knowing if the intolerance found

in *Patella* to high pressure/low temperature is a common phenomenon, an exception among invertebrates, or a taxon-related characteristic.

CHAPTER 6

General discussion

6.1. General discussion

The mechanism of dispersal of a shallow-water benthic invertebrate into the deep-sea will depend on each species, since different species disperse in the ocean by different adaptations. Many species do not have a larval form and must disperse by floating or rafting. But most nonbrooding benthic invertebrate species produce larvae, which constitute their principal or their only mechanism of dispersal. Which ever is the mode of dispersal, the dispersing larva, juvenile or adult will most likely be transported to deep-sea passively, from the geographical areas where deep-sea water masses are formed.

Deep-sea water formation takes place mainly in the polar regions, through the thermohaline circulation. Warm waters are transported to high latitudes where they become denser, sink, and spread throughout the oceanic floor (Smethie et al., 2000). One of the most important pathways by which this process occurs is the formation and spreading of North Atlantic Deep Water (NADW) in the Norwegian Sea, which is a geographical area where the species from this study occur. There is also recent evidence that there may be a variety of other sites of deep-water formation in the NE Atlantic (Pickart et al., 2003).

The present research aimed to study the physiological potential of embryos and larvae of a few selected species from the NE Atlantic to disperse into the deep-sea. These species are used as models, their larvae are not more likely than that of

other species in the NE Atlantic to be carried by the thermohaline circulation into the deep-sea. The echinoids have been selected because it allowed comparisons to other echinoids of other studies. On the other hand, *Patella* species are examples of a group without deep-sea representatives and belongs to a phylum (Mollusca) not yet included in this specific field of larval research, which has so far been restricted to echinoderms. The species selected are external fertilizers and are expected to disperse mostly throught their larval stages, adults are large and not expected to migrate long distances. Dispersal during early juvenile stages might be possible in the species tested, however more time would be needed to achieve reasonable culture technique for the production of juveniles at suitable quantity. For this reason juvenile stages were not tested.

During this research, temperature and pressure were assumed as major potential barriers to long distance vertical larval dispersal. But other factors could also limit the dispersal of larvae and embryos from shallow- to deep-sea waters in nature, as many environmental variables vary along the bathymetric gradient. For example, oxygen concentration decreases with depth, there is no light availabitity below 1000m, and primary production becomes more dependent on the production from surface (Menzies, 1965; Tyler, 1995; Carney, 2005).

However, temperature and pressure were chosen because changes in these factors have the potential to directly affect the physiology of larvae and embryos from any species. Adaptations to high pressure in deep-sea fauna is evident (Somero et al., 1983; Somero, 1998; Carney, 2005), both pressure and temperature can cause protein denaturation and affect membrane fluidity (Hazel & Williams 1990; Hazel, 1995; Siebenaller & Garrett, 2002). Additionally, temperature is always low in any geographical area of the deep-sea except at hydrothermal vent sites, and pressure

always increases in a precise and predictable pattern along the bathymetric gradient (Menzies, 1965; Tyler, 1995; Carney, 2005).

During this and other research on the potential for deep-sea colonization (e.g. Young et al., 1997; Tyler and Young, 1998; Tyler et al., 2000), embryo and larvae tested may be able to survive and develop normally under high pressure and low temperature and yet not be able to metamorphose and settle under these conditions. Further, later feeding stages may be alive but in no condition to feed. It is not suggested in this study that larval survival and normal development at low temperature and high pressure necessarily discard the possibility that these factors act as barriers to larval dispersal into the deep-sea by the species tested. All aspects of larval pressure and temperature ecology should be studied, ranging from biochemical analysis to behaviour experiments. My research focused on one aspect: the ability of larvae to survive and develop normally during dispersion along the bathymetric gradient.

Embryos and larvae of the echinoids tested during my research are able to survive, in the laboratory, at pressure and temperature combinations typical of depths far beyond the limits of the adult distributions. These findings were similar to data from other species in the same class (Young et al., 1996; Young et al., 1997; Tyler and Young, 1998 Tyler et al., 2000), and from asteroids (Benitez Villalobos et al., 2006). This means that the ability to withstand deep-sea temperature and pressure conditions during the larval stages may be widespread in this phylum, making it highly capable of invading the deep sea, since the most potential barriers to deep-sea colonization can be overcome by the dispersal stages. Once dispersal of larvae into the deep-sea can happen in high densities, other factors, such as adult tolerance,

ability to reproduce and fertilise successfully, feeding patterns and habitat preferences, could be gradually adapted over generations.

Scientists should continue to study tolerance to pressure and temperature in echinoderms, including representatives of the Holothurioidea and Ophiuroidea, in order to establish whether or not the ability of the larvae to withstand low temperature/ high pressure is indeed widespread in this phyla. Also, increasing the number of studies comparing the depth distributions of juveniles with that of the adults can show whether larval settlement beyond the adults distribution depth limits, as demonstrated in *Ophiocten gracilis* (Sumida et al., 2000) is a common phenomenon within this phyla.

This study and previous research (Young et al., 1997; Tyler and Young, 1998; Tyler et al., 2000; Benitez Villalobos et al., 2006) have demonstrated that, in echinoderms, larval stages are more tolerant to pressure and temperature than embryonic stages. This demonstrates ontogenetic changes in species tolerances to pressure and temperature. Some stages are therefore potentially more able to pursue migration to deep-sea sites. But all stages must be able to develop under deep-sea conditions in order to establish a population that is not dependent on continous larval input from shallow-water. However, it was demonstrated in *Echinus acutus* that tolerance to high pressures was higher in embryos from deep-sea populations than in embryos from shallow-water populations (Tyler and Young, 1998), showing that embryo development can be gradually adapted to deep-sea conditions. On the other hand, for deep-sea colonization to take place a life stage able to be dispersed by water currents must be pre-adapted to deep-sea conditions. In a deep-sea colonization by a species with planktotrophic development, this stage is expected to be a larval stage. Future research should focus in studying even later stages, beyond the 4-arm

stage and up to settlement stages. This requires different technologies than the one used in this research because, once pressurized, vials cannot be manipulated and larvae cannot be easily fed. This can be overcome by using lecithotrophic species.

Additionally, to understand the ontogenetic changes in pressure tolerance, more research is needed at the molecular level. It has been demonstrated that pressure can affect the fluidity of biomembranes and the activity of enzymes, and can slow metabolism (Carney et al., 1995). Ontogenetic shifts in pressure tolerance could be explained if it is demonstrated that short-term adaptations to pressure in molecular systems, in order to maintain adequate functioning, are more likely to occur during particular life stages. Potential subjects for research are changes in the lipid composition of membranes to maintain its fluidity and aminoacid substitutions in enzymes to preserve their three-dimensional structure. Experiments on these topics designed to perform ontogenetic comparisons have not been carried out to date. These ontogenetic changes are important because data from this and other researches, despite being short-term experiments, demonstrate a clear trend for higher temperature and pressure tolerance during larval stages as compared to embryonic stages, in echinoids. This phenomenon enables larval dispersal in this group into a wider range of environments, potentially including deep-sea habitats.

Our data on *Patella* are the first on pressure and temperature larval tolarance results on a species not belonging to Echinodermata, and the results contrast previous research. *Patella* species tested in this research have low larval tolerance to deep-sea temperature and pressure conditions. This is very different from results of studies on larvae of echinoderms tested in this and previous research (Young et al., 1997; Tyler and Young, 1998; Tyler et al., 2000; Benitez Villalobos et al., 2006). This suggests that some phylogenetic groups might have a higher larval potential to deep-sea

colonization and results from echinoderms obtained to date might reflect a group with strong potential for deep-sea invasion. Indeed, echinoderms have been particularly successful in deep-sea, being the most conspicuous element of deep-sea megafauna (Tyler, 1980; Billett, 1991; Gage & Tyler, 1991).

My research investigated molluscs that belong to the genus *Patella*, which does not have any representative beyond the continental shelf. In fact, no species belonging to the same family (Patellidae) occur in the deep-sea. Although some patellogastropods are known to inhabit deep-sea chemosynthetic habitats, many belong to families that are endemic to this kind of habitat (Fretter, 1990; McLean, 1990; Beck, 1996; Warren & Bouchet, 2001; Sasaki et al., 2003) and their evolutionary relationships with shallow-water species are not well known (Harasewych & MacArthur, 2000).

Future work in this field should examine the hypothesis that the success of a group in colonizing the deep-sea is related to its tolerance to deep-sea temperature/pressure conditions. It is therefore necessary to identify genera and families that have recently invaded the deep-sea, and continue to compare the larval pressure and temperature tolerances in shallow-water species belonging to these groups with the larval tolerance of shallow-water species from taxa that have not colonized the deep sea.

Differences were detected in temperature and pressure tolerances between northern and southern *Patella* species and between northern and southern *E. esculentus* populations, during this research. This could mean that larvae migrating to and settling in deep-sea waters of the NE Atlantic, are mainly constituted of larvae from northern species and northern populations.

We hope that future genetic studies, including various shallow-water and various deep-sea populations of the same species, along a latitudinal gradient in the NE Atlantic, will assess this question. Currently, such studies do not exist. Species such as *Echinus acutus* are suitable for these studies because its depth distribution extends from Northern Africa and the Azores to Bear Island (North of Norway) and has been recorded from 20 m to 1300 m. Such study could also contribute to understand whether larval physiological adaptations to deep-sea pressure conditions in this species may be leading to speciation, as suggested by Tyler & Young (1998).

Finally, long-term experiments subjecting larvae to a gradual change in temperature and pressure, should be attempted. Long-term experiments would add new and useful data to this area of deep-sea biology research, taking into account the poossiblity of acclimation.

6.2. Main conclusions

I. Embryos of *Psammechinus miliaris* are negatively affected by high pressures and low temperatures, but can resist pressure and temperature conditions typical of up to 500 m. On the other hand, larvae of *Psammechinus miliaris* can survive pressure/temperature combinations similar to 2000 m. This shows that, like in other echinoderms, this species larvae probably can survive passive dispersal into deep sea.

II. Embryos and larvae of *Echinus esculentus* Bergen (Norway) population develop faster, at a given temperature and pressure, than those from Plymouth (Southern UK) population. Further, embryonic development of

this species seems to be adapted to the local temperature; northern embryos cannot develop at 15 °C, whereas southern embryos cannot develop at 4 °C. Therefore, larvae from the northern population are predicted to better succeed during dispersal into the deep-sea.

III. Individuals belonging to the northern species *Patella vulgata* are more tolerant to low temperature than individuals belonging to the southern species *Patella ulyssiponenis* collected in the same area. Both species have low survival at 5 °C, but larvae of *P. vulgata* that survive this low temperature are able to develop normally, while those of *P. ulyssiponensis* do not. We conclude that it is not likely that *P. ulyssiponensis* can extend its geographic distribution, in the horizontal or bathymetric dimension, to colder areas.

IV. None of the species of *Patella* studied are capable of larval survival under high hydrostatic pressure, at low temperatures. This is new in larval pressure ecology because in all species previously tested larvae were able to survive high pressure. I propose that this may explain the absence of patellids in deep-sea habitats and, on the other hand, great larval pressure tolerance in echinoderms may be a key factor in the success of this group in recent deep sea colonization events. However an alternative explanation is that patellids may not be able to succesfully migrate deeper for other reasons which has led the larvae to adapt to local conditions of pressure and temperature.

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