DOCTOR OF PHILOSOPHY

Role of oxygen in the control of embryonic growth and metabolism in the edible crab Cancer pagurus

McCleary, Stephen John

Award date:
2006

Awarding institution:
University of Wales, Bangor

Role of Oxygen in the Control of Embryonic Growth and Metabolism in the Edible Crab *Cancer pagurus*

by

Stephen John McCleary BSc

In candidature for a degree of Philosophiae Doctor

School of Biological Sciences
University of Wales
Bangor
Gwynedd
LL57 2UW

September 2006

Supervised by Dr. Nia Whiteley

Abstract

The edible crab *Cancer pagurus*, brood as many as three million embryos, twice the usual allometric relationship found in brachyuran crustaceans. The embryos are tightly packed on the abdomen where they grow and develop until hatching takes place 6-9 months later. Ovigerous females remain buried for greater than 25% of embryonic incubation, which exposes the embryos for prolonged periods to severe hypoxia ($\leq$ 2 kPa).

Synthetic correlates of growth and metabolism, and the influence of short term hypoxia on these were analysed at four key phases in embryogenesis: the blastula; limb formation; eye; and hatching phases, which were identified from a novel developmental scheme. Cellular responses to hypoxia were also analysed.

Whole embryo rates of oxygen uptake ($\dot{M}O_2$) scaled isometrically with body size during development, although values were lower than predicted by tissue mass. Rates of protein synthesis (k_s) showed a phase specific relationship over the same period at 10°C, reaching maximum mean values of 24.4 % day^{-1} at the eye phase, which coincided with the major growth period and increase in developmental rate. By hatching phase mean k_s had decreased to 6.3 % day^{-1} and were similar to the mean values recorded at limb formation. Generally there wasn't a close relationship between $\dot{M}O_2$ and k_s during development, resulting in variable metabolic costs, ranging between 131 mmol ATP g $protein^{-1}$ at the eye phase, to 469 mmol ATP. g $protein^{-1}$ synthesised at hatching phase.

The physiological effects of Hypoxia on embryonic metabolism were more pronounced on $\dot{M}O_2$ compared with k_s. $\dot{M}O_2$ at the early developmental phases were less sensitive to hypoxia, particularly the blastula, where metabolism was relatively quiescent. The eye and hatching phases were oxygen conformers, which appears to relate to an increase in metabolising tissue, an increase in growth, and restriction to oxygen uptake. Generally k_s values were reduced and stable in hypoxia at approximately 5 % day^{-1}. At the eye phase progressive hypoxia resulted in a 5-fold and rapid decrease to normal values, caused by changes in k_{RNA}, since RNA levels remained unchanged. Hypoxic k_s and $\dot{M}O_2$ values were directly correlated, generating a fixed metabolic cost for protein synthesis of 149 mmol ATP. g $protein^{-1}$.

Characterisation of the cellular response to hypoxia showed evidence of high basal levels of heat shock protein 70 and related homologues. Preliminary evidence was also suggestive of hypoxia specific protein expression patterns, some of which could also be part of the Hsp70 family. An indication of an arrest or extension to the cell cycle during development, may form another important element of the cellular defence utilised by embryonic *C. pagurus* against natural brooding hypoxias. Taken as a whole, the suppression to rates of protein synthesis and the cellular responses to hypoxia appear appropriate to the natural occurrence of hypoxia in the brooding egg mass.

Contents

Acknowledgements

I feel it is an understatement to say that this work would never have been possible without the help and support of so many people. To avoid an Academy Award type thank you list, I will reserve mention here to the fantastic few.

A special thank you to Dr Nia Whiteley whose sound counsel, ideas, support, and not least saintly patience, have been instrumental to the development and completion of my studies during my time at Bangor.

Big thanks to the ever reliable Alison Bell whose meticulous and enthusiastic approach, not forgetting excellence in histology, are also due very special mention.

Similarly, thanks also go to Andrew Davies for help with design and construction of apparatus, guidance with the Confocal microscope, in one word 'Genius'. In addition thanks to a number of other technical staff who have thrown me a paddle in times of need!

My gratitude also to my family, who all seem to have the 'Duracell' gene in abundance, managing to stay up and help me, not to mention put up with me, particularly during my darker hours. Particular thanks to my brother Paul for his expert IT skills, and my partner Susie for her love and support, and also the help provided by her father. Similarly, thanks go to the power systems bunch at Finnings for their printing efforts.

The Brambell building, albeit voted one of the ugliest buildings in the U.K has essentially been my home over the last four years. The people I have met and befriended, some of which who have spared their time and shared ideas with me also deserve my appreciation. Thanks go to 'Angry' Peter Wright for loan of his office and microscope facilities, which unfortunately carried a price, usually jibes at United. Jenny Rock my office and lab buddy, and the herpetological crowd for the Brambell banter.

Abbreviations

A_s	absolute rate of protein synthesis
ATP	adenosine-triphosphate
β-PEA	β-phenylethylamine
BSA	bovine serum album
Ca_2^{2+}	calcium ion
Ci	curie
DNA	deoxyribonucleic acid
DPM	disintegrations per minute
GHZ	giga hertz
Hsc(s)	heat-shock cognate(s)
Hsp(s)	heat-shock protein(s)
H+	hydrogen ion
HIF-1	Hypoxia inducible factor 1
HIF-1α	Hypoxia inducible factor 1 alpha unit
kDa	kilo Dalton
KHZ	Kilo hertz
k_{RNA}	rate of ribosomal activity
k_s	fractional rate of protein synthesis
[³H] Phe	L-[2-6 ^{3}H] phenylalanine
k_d	protein degradation
kPa	Kilo pascal
Mg^{2+}	magnesium ion
$\dot{M}O_2$	rates of oxygen uptake

mRNA	messenger ribonucleic acid
NaOH	sodium hydroxide
PAGE	polyacrylamide gel electrophoresis
PCA	perchloric acid
PCR	Polymerase chain reaction
Phe	phenylalanine
Pmol	pico mole
PO_2	potential pressure of oxygen
Q10	temperature coefficient
R	radius
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
Sa	specific radioactivity of the free-pool phenylalanine
Sb	specific radioactivity of protein-bound phenylalanine
SDS	sodium dodecylsulfate
SE	standard error
STPD	standard temperature and pressure
µm	micrometer
UV	ultraviolet
V	volume
2D	two dimensional

Chapter 1

1.1. General Introduction

The true crabs (Class Malacostraca: Order Decapoda; and Infraorder Brachyura) with their compacted body plan, abundant speciation and extensive colonisation represent the most successful group of decapodan crustaceans (Ruppert and Barnes, 1994). Adult brachyurans characteristically have a compressed cephalothorax, and a reduced and folded abdomen, representing structural adaptations for a predominantly benthic existence. The abdomen also possesses modified pleopods that are used in males as a copulatory appendage, and in females for egg brooding (Benninger et al., 1988). Almost all brachyurans brood their embryos by carrying them using the pleopods until they hatch as zoea larvae (Pearse, 1979). The pelagic zoea larva subsequently passes through a series of moult cycles before metamorphosing into the benthic juvenile crab. Although mainly aquatic, some brachyuran species have successfully invaded terrestrial environments, but almost all still rely on access to water for larval development and completion of the life cycle.

Much of the earlier work on brachyuran embryogenesis has been highly descriptive, characterising morphological changes of the embryos during development. Even with the addition of more recent investigations identifying biochemical changes, and examining metabolic rates during development,

information on the direct synthetic correlates of growth, such as protein synthesis, in brachyuran embryos is scarce (Subramoniam, 1991; Petersen and Anger, 1997). More is known of the growth and metabolism of both the larval and adult life phases. Characterisation of chemical growth correlates, such as the accumulation of nitrogen and carbon from lipid and protein stores generally show that brachyuran larvae have a high sensitivity to environmental variables (Anger and Dawirs, 1982; Anger and Hirche, 1990; Anger, 1998). However, direct measurements of growth and metabolism in crustaceans have only been completed on adult decapods and isopods, which undergo large elevations in rates of protein synthesis during the moult cycle, to coincide with the main growth periods (El Haj et al., 1996; El Haj and Houlihan 1987; Whiteley et al., 2001; Whiteley and Faulkner, 2005). The developing embryos are also known to moult, although little is known about whether moulting is associated with increased rates of protein synthesis and other correlates of growth, or indeed how these are related to the developmental period as a whole. Furthermore, many aspects of growth during embryogenesis are unknown, such as the timing and the control mechanisms involved, along with their sensitivity to environmental factors.

To better understand growth during brachyuran embryogenesis, it is important to consider the natural environment for embryonic growth and development, which in certain species raises several interesting questions (see below). All brachyurans produce lecithotrophic embryos, which they brood on their abdomen until they hatch as zoea larvae or in rare exception as juveniles. Embryo brooding is a common reproductive trait in decapods, and is generally considered to benefit the developing offspring by offering protection from predators and environmental stresses, for example protection from changes in temperature or salinity, which characterise the surface waters and, therefore, the stresses experienced by planktonic embryos (Shine, 1978; Strathmann et al., 2002). In addition, recent evidence suggests that brooding also allows maternal control over rates of embryonic development, since the embryos require ventilation for aerobic metabolism in the developing egg mass, caused by the restriction of natural oxygen diffusion, and competition for oxygen between siblings (Marois and

Croll, 1991; Booth, 1995; Cohen and Strathmann, 1996; Lee and Strathmann, 1998). This particular feature of brooding is important in species with large egg masses containing many tightly arranged embryos, which can further restrict oxygen supply and convective gas exchange. Recent data suggests that large brachyuran egg masses can be poorly ventilated and severely hypoxic during incubation (Naylor et al., 1999; Fernández et al., 2000; Baeza and Fernández, 2002). In addition, there is evidence that the sensitivity of embryos to hypoxia varies during development, which could influence patterns of embryonic growth and metabolism, and involve mechanisms of cellular protection, possibly through hypoxia inducible gene expression.

The edible crab, *Cancer pagurus,* was chosen as the species in which to study embryonic growth and development in brachyurans, since it possesses a number of interesting features with respect to reproduction and embryogenesis. Also studies have described certain aspects of the reproduction, including brooding behaviours and embryogenesis of *C. pagurus*, and other closely related Cancrid crabs (Naylor et al., 1997; Naylor et al., 1999; Fernández et al., 2000). Furthermore, much is known about the reproductive ecology of the adults due to the commercial importance of *C. pagurus*.

Female *C. pagurus*, migrate offshore into deeper waters during the autumn to spawn, where environmental conditions e.g. temperature, salinity, light and water currents are more stable, compared with intertidal habitats (Nichols et al., 1982). Females breed annually, or once every two years (Edwards, 1979). Spawning and attachment of the embryos (described later) usually takes place between November and January, with peak spawning observed in December. It is estimated that *C. pagurus* can spawn as many as three million embryos in one spawning event, representative of greater than 20% female body weight, which is double the usual allometric relationship found in brachyurans (Hines, 1982; Hines, 1991).

Following spawning, another interesting and salient feature of reproduction in *C. pagurus* is the burying behaviour of the ovigerous females. If undisturbed, ovigerous crabs can remain completely buried for up to two months or longer until they emerge in early spring. During this period female oxygenation of the embryo mass through abdominal flapping behaviour is impeded, therefore, the embryos are probably convectively limited of oxygen (Fernández et al., 2000). Consequently the incubation period in *C. pagurus* is relatively prolonged compared with other temperate brachyuran species distributed around the coasts of Britain, such as the shore crab *Carcinus maenas*.

The possibility of the embryos undergoing metabolic quiescence or diapause during development has also been considered, and is thought to be related to the changing patterns of oxygen provision during development (Naylor et al., 1999; Fernández et al., 2000; Fernández and Brante, 2003). Similar responses to hypoxia are also known in a number of hypoxia tolerant animals, which exhibit a pronounced decline in metabolic rate to save energy expenditure until normal oxygen conditions return (Watson and Smallman, 1971; Clegg, 1997; Caceres and Tessier, 2004; Smith et al., 1996; Fraser et al., 2001). However, in less hypoxia tolerant animals, metabolic rate is defended using anaerobic pathways until metabolism is compromised and cell death ensues. In addition, hypoxia can also induce genes that are responsible for further differentiation and development (Iyer et al., 1997; Kotch et al., 1999). It remains to be seen what effect hypoxia has on developing *C. pagurus* embryos.

1.2. Reproduction in Brachyuran Crustaceans

Brachyurans reproduce following copulation between a male and female crab. Males often fight tenaciously to claim a female nearing ecdysis, since this is the main opportunity for many species to reproduce during the lifecycle, and competition for breeding rights can be high (Hartnoll, 1969; Christy, 1987). Males place sperm filled capsules called spermatophores on to the ventral surface of a female, or in higher species the spermatophore can be delivered directly to the seminal receptacles using specialised copulatory pleopods (Benninger et al., 1988). The sperm is either used to fertilise immediately, or can be stored until a later time for fertilisation in species that possess seminal receptacles (Subramoniam, 1993).

Before fertilisation can take place the ovaries must be fully mature. This is achieved during vitellogenesis, and is responsible for much of the net gain in oocyte size during maturation (Adiyodi and Subramoniam 1983; Tsukimura, 2001).Vitellogenesis, or yolk formation, is highly correlated to both age and also the bioenergetics of an individual female crab, therefore age at maturation can be variable between populations of the same species (Wenner et al., 1974), due to biotic and abiotic factors, like the availability of food and competition between individuals and species within an environment (Pianka, 1970). Much of the energy gained and allocated for reproduction is mobilised from the hepatopancreas in the form of precursor molecules including; lipoproteins; ecdysteroids; ribosomal RNA; haemocyanin; plus many other pigments, which are packaged into the maturing oocytes prior to extrusion and fertilisation (Shapiro et al., 2000; Warrier et al., 2001, Gunamalai et al. 2003).

Fertilisation is often internal, and is believed to occur during spawning, whilst the oocytes (ovum) pass through the seminal receptacles where the male sperm is stored. The eggs are extruded through two gonopores located on the ventral surface of the thorax (Saigusa et al., 2002), and depending on species, millions of embryos can be released in one spawning event, which can have a number of important physiological effects on development (see below). However, even

when the effects of season and habitat are considered, the number of eggs spawned in brachyurans is strongly and positively correlated with female body size (Efford, 1969; Subramoniam, 1977; Hines 1982).

Each embryo is intimately attached to the pleopods on the abdomen through what has recently been termed the 'attachment system' (Saigusa et al. 2002). The means by which the newly extruded eggs attach to the setae on the pleopods has caused much debate, and consequently resulted in a number of different theories (see Saigusa et al. 2002). A recent investigation suggests that following extrusion the eggs collect in the abdominal space, which has been shown in an estuarine crab (*Sesarma haematocheir*) to remain close to the ventral surface of the thorax, to prevent the eggs from spilling away from the abdomen. Saigusa et al (2002) have shown that after approximately 30 minutes the egg membranes become adhesive, during which time they are gently mixed with incubation fluid (possibly seawater) and moved around by beating action of the pleopods. This causes the egg envelopes to expand and encapsulate part of an ovigerous hair, and consequently to form a flaccid membranous 'stalk like' structure, known as the funiculus. After approximately two hours, all the eggs in *S. haematocheir* are attached to the pleopods in this way, enabling the newly ovigerous mother to freely move around. The same attachment mechanisms have also been described following ultrastructural studies using a number of other decapods such as *C. maenas,* and the lobster, *Homarus gammarus* (Goudeau and Lachaise, 1980, Goudeau et al. 1987, Talbot and Goudeau, 1988).

Following copulation and attachment of the eggs, ovigerous female brachyurans embark on perhaps their greatest challenge, embryonic brooding. Brooding is typically linked to small body size in marine invertebrates. The reasons for this include the positive relationship between total egg generation and adult body size, and also the high mortality rate associated with broadcast spawning in the marine environment, although the relationship is not exclusive (Strathmann and Strathmann, 1982). Larger animals are associated with broadcast spawning because the morphological constraints of reproduction, namely physical space for

egg brooding, limit the total brood size more powerfully than they limit total egg generation (Strathmann and Strathmann, 1982; Hines, 1982; Hines, 1992). In brachyuran crabs, size constraints on reproductive output arise due to the limitation of physical space available for egg attachment to the pleopods, also similarly the available space for ovarian development within the cephalothorax. Recent data would also suggest that the capacity to oxygenate the embryos during brooding is an additional constraint to fecundity (Fernández et al., 2003). Contrary to such allometric relationships, all female brachyurans, regardless of size and environment develop their embryos by brooding (Pearse, 1979). Brood size in most families of brachyurans are thought to be limited to approximately 10% of female body weight, although certain families, such as the family Cancridae, have been reported to carry broods greater than double the normal allometric relationship in certain species (Hines, 1982; Hines, 1991).

1.2.1. **Brachyuran Embryogenesis**

After extrusion and attachment, brachyuran embryos grow and develop within the egg mass through a series of pre-determined developmental events. Traditional descriptive studies tended to categorise these developmental events into recognisable phases based on the formation of morphological structures such as the abdomen, and by the appearance of pigmented chromatophores, along with changes in the size and colour of the yolk (Broekhuysen, 1936; Taylor and Leelapiyanart, 2001; Pinheiro and Hattori, 2003; Guerrero and Hendrickx, 2006).

The earliest phase that can be distinguished by traditional means is generally the blastula, which follows fertilisation and a period of cell division during cleavage. At the blastula the blastoderm cell layer is formed which is comprised of cells of a common developmental fate (Wear, 1974; Vidal, 1980; Hoegh-Guldberg and Pearse, 1995; Moriyasu and Lanteigne, 1998). Subsequent phases, distinguished by morphological characteristics, are the naupliar phases (nauplius and metanauplius), where growth and differentiation proceeds to form the early body segments of the cephalothorax and abdomen, and continues with development of primordial limb structures, such as the maxillipeds and antennae-antennules

(Moriyasu and Lanteigne, 1998; Guerrero and Hendrickx, 2004; Muller et al., 2004; Guerrero and Hendrickx, 2006). Following the naupliar phases, brachyuran embryos appear to undergo the main period of morphological development, as they progress to the zoea phase. During this developmental progression the metanaupliar embryo passes through the protozoea and prezoea phases in development whilst still in the egg membranes. Most of the internal yolk is transformed into embryonic tissue during this period, coinciding with episodes of neurogenesis and organogenesis, to complete embryogenesis and form the zoea larva (see below). The wider implications, considered as ecological consequences, of brooding eggs until development reaches the pelagic larval phase is considered below, before the specific effects on the physiology of the embryos is covered

1.2.2. **Ecological Considerations**

Using a deterministic model for fecundity, brachyurans and decapods as a whole, are generally considered to be k-selection strategists, although this characteristic is not exclusive for the entire infraorder. This strategy maximises the energy allocated into each egg, and as a consequence favours the production of relatively large eggs, and highly competitive and developed offspring (Pianka, 1970; Smith and Fretwell, 1974; Stearns, 1992). Consequently, the lecithotrophic nature of brachyuran embryos has resulted in a compression of the embryonic development period. This has resulted in hatching being delayed until after the naupliar and prezoea phases, which are retained during embryonic development, and, therefore, consequently pose a number of important physiological constraints to development (see below).

There are essentially two main modes of brachyuran reproductive cycles. The continuous strategy, which is found in environments with a prolonged growth season (for example, the tropics) and the annual strategy, where the reproductive cycle is periodically synchronised to coincide with pelagic food resources of spring and summer months in temperate environments (Subramoniam and Gunamalai, 2003). These two strategies have important implications for

fecundity, and aspects of embryogenesis, such as duration and potential physiological exposure to environmental stress e.g. hypoxia. In addition, polar environments, characterised by extremely short periods of productivity and cold temperatures, amongst other environmental factors, have resulted in certain brachyurans being characterised by highly prolonged periods of embryonic development. For example, the Lithodids brood their embryos for up to two years, which comprises a number of dormant periods during the freezing winters similar to the Majidae crab family (Moriyasu and Lanteigne, 1998; Thatje et al., 2003).

Continuous strategists have the capacity to spawn multiple broods of embryos each year whenever resources permit and, therefore, can be characterised by a year round release of zoea larvae into the plankton (Giese, 1959). Annual strategists, e.g. most members of the family Cancridae, carry broods of embryos within a population at the same phase in development, and brood only once each year or every two years (Hines, 1991). Consequently, embryogenesis for continuous strategists is on the whole usually completed far more rapidly, in the order of weeks, compared with annual strategists, which range from the order of months to years (Moriyasu and Lanteigne, 1998; Harzsch et al., 1998; Naylor et al., 1999; Guerrero and Hendrickx, 2004; Guerrero and Hendrickx, 2006). Such differences in the duration and frequency of embryogenesis can be related to variations in environmental temperature at the time of development, the availability of resources for oogenesis, and species specific variations (Wenner et al., 1974). When these considerations are more favourable, brachyurans undergo rapid growth and development, resulting in a greater amount of eggs produced per female during a life time, even when scaled for body size (Giese and Kanatani, 1987). Another consequence is that the size of the egg is often smaller and the pelagic larval phases are also longer (Podolsky and Strathmann, 1996; McEdward, 1997; Krug, 1998 Subramoniam and Gunamalai, 2003).

The zoea and megalopa larval phases represent the only pelagic part of the brachyuran lifecycle, which is thought to improve the chance of reproductive recruitment by reducing exposure time to predators, especially marine filter

feeders (Pechenik, 1987). Despite this, several months are spent in the water column and chances of survival remain low, particularly compared with other decapods such as crayfish, which produce embryos that hatch directly as benthic juveniles. Although dispersal in marine environments is improved relative to freshwater environments, and competition for resources is reduced.

1.2.3. **Physiological Consequences**

Prior to the hatching period and the continuation of the lifecycle as the larval phases, the developing embryos of brachyurans face various challenges, some of which are similar to those experienced by planktonic embryos of marine invertebrates e.g. predation (Shields and Kuris, 1988). In general, brooded embryos benefit from much greater protection against predators and are also buffered from the extremes of environmental change by parental care (Hazlett, 1983; Thiel et al., 1997; Thiel, 2003). However, a crucial difference between embryos that are broadcast and those that are brooded is the availability of oxygen during development. Broadcasting is an effective and metabolically cheap strategy to provide embryonic phases with sufficient oxygen for growth and development (Strathmann and Strathmann, 1982). Development in large egg masses poses serious physical constraints on the supply of oxygen to the developing embryos present within the egg mass (Strathmann and Chaffee, 1984; Marois and Croll, 1991; Booth, 1995; Strathman and Strathman 1995; Steer et al. 2002).

The egg mass in brachyurans can range in size' reaching several centimetres both in diameter and in depth, and comprised of relatively few large embryos, or many small embryos tightly packed together (Hines, 1982; Taylor and Leelapiyanart, 2001). Collectively, each of these variables can influence the physical microenvironment of the developing embryos, so although oxygen level has a major regulatory influence on offspring success in developing egg masses, the effects are species specific. In brachyuran egg masses, species with high packing densities and small egg sizes can experience restricted water flow between the embryos, especially embryos in the centre of the egg mass (Fernández et al.,

2000; Fernández and Brante, 2003). In addition, inadequate maternal ventilation of the egg mass can further limit oxygen supply during development (Fernández et al, 2000; Baeza and Fernández, 2002; Fernández et al., 2003), which can result in the presence of severe hypoxia within the egg mass, e.g. < 2.1 kPa recorded in *C. pagurus*, and extended durations of embryogenesis (Fernández et al, 2000). This situation is, therefore, similar to that experienced by marine invertebrates that rely soley on passive oxygen diffusion for completion of embryonic development (Booth, 1995; Fernández et al, 2000). If environmental conditions are particularly unfavourable then it is also not uncommon for the growth and development of brachyuran embryos to arrest at a given developmental phase, and to enter a state of quiescence or diapause during the incubation period until favourable conditions return (Lovrich and Vinuesa, 1993; Petersen and Anger 1997; Moriyasu and Lanteigne, 1998; Taylor and Leelapiyanart, 2001).

Not all brachyurans produce embryos that experience hypoxia, although the fundamental requirement of oxygen provision for brachyuran development has resulted in ovigerous crabs showing a series of highly conserved brooding behaviours to overcome this potential problem (Wheatly, 1981; Naylor et al., 1999; Baeza and Fernández, 2002; Ruiz-Tagle et al., 2002; Fernández and Brante, 2003). These include abdominal flapping, maxilliped and pleopod beating, and cheliped probing of the egg mass (Fernández and Brante, 2003). Primarily these behaviours act to improve water flow and covective gas exchange to the developing brachyuran embryos, but additional functions may include detection of oxygen or metabolic waste levels, dislodging parasites, or propulsion of hatched larvae into the water column. All are potentially vital functions, since the embryos rarely survive away from the mother. Not surprisingly, each brooding function by the females is potentially costly, as resting metabolic rate is higher in ovigerous females, which might have a negative influence on fecundity (Fernández and Brante, 2003). Molecular oxygen can, therefore, be considered to be an important environmental variable that can be controlled by the brooding female crab to regulate the duration of embryogenesis and, therefore, possibly embryonic growth (Wheatly, 1981; Naylor et al., 1999; Baeza and Fernández,

2002; Ruiz-Tagle et al., 2002; Fernández and Brante, 2003). The role of oxygen in the control of embryo growth will be considered in Chapter 4.

In adult crabs, growth is restricted to the moulting period when crabs shed the old exoskeleton and increase in body size due to the increased uptake of water and the resulting increase in hydrostatic pressure (Dall and Smith, 1978; Mykles, 1980; Mangum 1992; Chang, 1993). Muscle growth, in particular, is associated with a dramatic increase in fractional rates of protein synthesis during early postmoult, before the new exoskeleton calcifies (El Haj et al., 1984; El Haj et al., 1995). A number of investigations have shown that moulting occurs during the embryonic development of decapod crustaceans, leaving a series of egg membranes or envelopes around the periphery of the egg at the time of hatching (Goudeau, 1976; Goudeau and Lachaise, 1983; Helluy and Beltz, 1991; S. J. McCleary, unpublished observations). Ectodermal cells of the naupliar phase embryo secrete an additional egg membrane, the first of four synthesised during the embryonic period in *C. maenas* and five in *H. americanus*. The second membrane, indicating the second moult cycle, is produced at the metanaupliar phase in *C. maenas*, following limb growth and early periods of neurogenesis (Harzsch et al., 1998). The remaining two membranes are secreted during the protozoea phase (eye and chromatophore phase, described in Chapter 2), and finally during the prezoea phase, in which the final embryonic membrane secretion reflects the formation of the zoeal cuticle. By definition, true ecdysis only occurs during the third moult cycle, where formation of the zoeal cuticle is followed by the shedding of the egg membranes during hatching (Chung and Webster, 2004).

Microscopic examination of newly secreted egg membranes reveals a high degree of invagination, which decreases as the membrane swells prior to the next moult cycle (Goudeau and Lachaise 1983). These observations suggest that the egg membranes accommodate growth and differentiation, but also osmoregulation during ontogeny. It is important to note that, with respect to the latter, recent studies in the euryhaline crabs *Hemigrapsus sexdentatus* and *Hemigrapsus*

crenulatus have shown a constant exchange of water and particularly salts across the egg membranes, punctuated by periods of elevated water absorption during development (Seneviratna and Taylor, 2006). Water uptake has also been shown in many decapod embryos (Pandian, 1970; Chung and Webster, 2004) and amino acid transport has been shown across embryonic membranes in a number of other invertebrate species (Manahan and Crisp, 1983; Jaeckle and Manahan, 1989; Shilling and Manahan, 1990; Pace and Manahan, 2006), demonstrating the exchange of nutrients, ions, and water between the embryos and the external environment. Rates of embryonic protein synthesis and, therefore, growth and differentiation in embryonic brachyurans could possibly correlate with periods of membrane secretion or embryonic moult cycles, similar to the moult-related growth characteristics of adults, although this relationship remains to be shown.

1.3. Protein Metabolism during Growth and Development

Animal growth, at any point in time, is a reflection of protein retention efficiency i.e. the difference between rates of protein synthesis (k_s) and protein degradation (k_d) (Millward et al., 1975; Houlihan, 1991; Morgan et al., 2000). For growth to take place, k_s must be greater than k_d. The relationship between the two is thought to be highly variable, being influenced by a number of abiotic and biotic factors, including cyclical changes in food availability, and or food quality (Houlihan et al., 1989; McMillan and Houlihan, 1988; Carter and Bradfield 1992; Conceição et al., 1997), optimal temperature ranges for protein metabolism (Fauconneau and Arnal, 1985; McCarthy et al., 1999), age and body size (Houlihan et al., 1986; Conceição et al., 1997; Varvra and Manahan, 1999), and species-specific differences (see below for examples).

Great effort has been undertaken to improve our understanding of growth in a variety of species, both vertebrate and invertebrate, during different phases of the lifecycle, and in different physiological conditions. To achieve this, rates of protein synthesis and protein deposition are typically studied as the

measurements are relatively straightforward, whereas the determinations of rates of protein degradation are fraught with problems because of the various pathways used to breakdown and excrete nitrogenous waste products. As crustacean growth is step-wise and dependent on the moult cycle, growth rates are difficult to estimate. Consequently, much more is known about the relationship between growth rate and rates of protein synthesis in fish, where growth rates are relatively easy to obtain, and research has been driven by commercial interests in the optimisation of growth rates and body size.

Protein retention efficiency and, therefore, growth can be very high in larval and juvenile life phases compared with adults (Fauconneau, 1985; Conceição et al., 1997; Morgan et al., 2000). Protein assimilation efficiency appears to reflect reduced rates of protein turnover (Conceição et al., 1997). For example, protein retention efficiency ranged between 30-70% during early to late larval phases in the turbot, *Scophthalmus maximus*, which is similar to the values reported for larval striped bass (Conceição et al., 1997). High retention efficiencies ranging between ~54 – 99% were also found in juvenile salmon, *Salmo salar (*Morgan et al., 2000), and in juvenile Atlantic halibut, *Hippoglossus hippoglossus* (Fraser et al., 1998). In contrast, adult fish are reported to have lowered rates of protein retention efficiency, such as Atlantic cod, *Gadus morhua* (~40%), and in rainbow trout, *Salmo gairdnerii* (~30%) (Fauconneau and Arnal, 1985; Houlihan et al., 1988). Thus as body size increases following growth and development, protein assimilation appears to decrease. Specific examples of this relationship have been shown in the rainbow trout, *Salmo gairdneri* (Houlihan et al., 1986) and in the mussel, *Mytilus edulis* (Hawkins et al., 1988).

Decrease in rates of protein synthesis and retention is not surprising when adult life phases are reached, since the maximum or optimum body size has often been reached, and energy investment is reorganised and prioritised for reproduction (Encina and Granado-Lorencio, 1997 and references within). In addition, as cell number increases during growth and development, so to does the total synthetic capacity and thus total protein accumulation capacity. Collectively these changes compensate for a decrease in average or cellular synthesis during the life cycle. Furthermore, Houlihan (1991) has discussed the effects of tissue specific

contributions to whole animal rates of protein synthesis. The connection between high rates of protein synthesis in larval fish, and the relatively large proportion of the body comprised by the gut is significant. Gut tissue proportion decreases during development, and is superseded by white muscle tissue as the largest tissue by proportion to body size. The latter has a much lower fractional rate of protein synthesis but a higher protein accumulation rate.

Nutritional status also has a powerful influence on protein retention efficiency (Carter and Bradfield, 1992; Conceição et al., 1997). Optimisation of dietary supplements is a crucial part of animal husbandry, and key for maximising the protein yield or deposition of an animal (McCarthy et al., 1999). For example, a recent study on juvenile shrimps, *Litopenaeus vannamei* by Mente et al (2002) demonstrated a 14% increase in protein retention, and 30% increase in survival rates of animals fed an optimised amino acid diet, compared to those fed on a non-specific high protein diet. Similar findings have also been reported in fish (Carter and Bradfield, 1992; Conceição et al., 1997).

Rates of growth and metabolism are also influenced by environmental variables, and in particular temperature because of its effect on biological rate processes (Fauconneau and Arnal, 1985; McCarthy et al., 1999; Robertson et al., 2001a; Robertson et al., 2001b; Whiteley et al., 2001; Whiteley and Faulkner, 2005). Temperature has been shown to have an important effect on fractional rates of protein synthesis in several crustacean species, increasing whole-body k_s values by a Q_{10} of 2-3 (see Whiteley et al., 2001). Changes in fractional rate of protein synthesis can be correlated with certain cellular synthetic properties, such as translational efficiency (k_{RNA}) and protein synthetic capacity (RNA:protein ratios) (Preedy et al., 1985; Pannevis and Houlihan, 1992; McCarthy et al., 1999; Fraser et al., 2002). However, RNA:protein ratios, show an inverse relationship with temperature, being elevated at low temperatures, which is thought to compensate for the effects of low temperatures on synthetic rates processes such as k_{RNA} (data summary, Fraser et al., 2002). Interestingly, the resulting whole-body rates of protein synthesis do not show any metabolic rate compensation at low

temperatures, but continue to decrease from the values measured at higher temperatures following a Q_{10} of 2 (Whiteley et al., 1997).

1.3.1. Effects of Hypoxia on Growth and Metabolism

Environmental oxygen levels have increased dramatically since life began some 3.5 billion years ago. Present day oxygen partial pressure (PO_2) at sea level is approximately 21 kPa, some 7-fold higher compared with levels when complex life began to evolve (Whatley, 1979; Holland, 1994; Bekker et al., 2004). Present day atmospheric oxygen levels may be high, but environmental oxygen is often unstable, and can vary widely from physical and biological interactions in both terrestrial and aquatic habitats. Many animals experience regular normoxic and hypoxic oxygen transitions in their external environment, for example, some intertidal aquatic invertebrates are exposed twice daily to cyclical periods of normoxia at high water, followed by hypoxic exposure during emersion stress at low water.

As a common member of the intertidal fauna, adult decapodan crustaceans survive hypoxia by showing certain behavioural responses and by undergoing a number of physiological adjustments, such as respiratory and circulatory changes. Obviously mobile organisms are able to move away from localised hypoxia (Dandy, 1970; Cochran and Burnett, 1996), but when this is not possible, physiological strategies for hypoxia tolerance must be used. During the initial onset of hypoxia, submerged adult decapods respond by increasing ventilation rates, to increase the flow and volume of water through the branchial chambers, due to elevations in the rate of beating and stroke volume of the scaphognathites (Taylor, 1982; Morris and Callaghan, 1998; Schmitt and Uglow, 1998). This is essentially the same response, only more exaggerated, as that which occurs following pauses in normal ventilation, where tissue / cell oxygen levels can decline in normoxic conditions (Bradford and Taylor, 1982). Hyperventilation in response to progressive hypoxia is a common response for oxygen regulation in animals, and is similarly found in the reptiles and amphibians (Shelton and Boutilier, 1982; Wang et al., 1994; Anderson et al., 2002) teleosts (review,

Hughes, 1973) mammals (Gleed and Mortola, 1991). Ventilatory modifications in decapods exposed to hypoxia also involves an increased incidence of reversals in ventilation direction, which during partial emersion, can draw oxygen into the branchial chambers to oxygenate the branchial water volume (Butler et al, 1978; Taylor, 1982; Wilkens and Young, 1992).

Accompanying the increase in water flow past the gills in hypoxia is an increase in blood flow from the heart. Cardiac output is increased in hypoxia by an increase in cardiac stroke volume, which is sufficient to offset hypoxic induction of bradycardia (McMahon and Wilkens, 1975; Taylor, 1982; Jorgenson et al., 1982; Reiber and McMahon, 1998). Furthermore, blood flow is prioritised and shunted in favour of the sternal artery supplying the gills. Modifications to circulation in this way complements the increased flow and volume of water at the gills, by improving oxygen loading through increased haemolymph supply to the respiratory surface (Airreiss and McMahon, 1994). Blood shunting has also been observed in many other animals in response to hypoxia, and has been well described in amphibian hypometabolism during aestivation (Pinder et al., 1992). In addition, an associated change following cardiac shunting that will be discussed in more detail below, is the corresponding reduction in metabolic rate of poorly perfused tissues (see below).

Improvements in ventilation and circulation during hypoxia in many decapods are combined with, and effectively optimised, by modifications to the oxygen affinity of the respiratory pigment through interactions with acid-base equivalents and certain ions. In progressive hypoxia, an increase in ventilation improves CO_2 excretion across the gills to result in a respiratory alkalosis, which causes a negative Bohr shift and, therefore, helps to sustain oxygenation of the haemocyanin as the haemolymph passes though the gills (Wheatly and Taylor, 1979). In the event of both a prolonged and progressive hypoxia, anaerobic metabolism is activated, which generates a number of metabolic by-products, including lactate, urate, H^+ and CO_2 (Zeis et al., 1992; Taylor and Moore, 1995; Hagerman and Szaniawska 1986; Hagerman and Vismann, 1995). The general

effect of anaerobic metabolism leads to an internal acidosis, and if this is not ameliorated, then a positive Bohr shift will ensue, which will reduce oxygen uptake at the gill. Compensatory responses to the incipient acidosis involve the mobilisation of Ca_2^+ and Mg_2^+ from the carapace to improve the oxygen affinity of the haemocyanin (Taylor and Wheatly, 1981; Klarman and Daniel, 1980; Taylor and Whiteley, 1989). Furthermore, the release of lactate and urate from anaerobic metabolism both result in an increase in oxygen affinity of crustacean haemocyanin, firstly through urate synthesis, and subsequently by lactate production, thereby counteracting a reduction in oxygen uptake following acidosis (Taylor and Whiteley, 1989; Zeis et al., 1992; Morris and Callaghan, 1998).

Interestingly, certain decapod species that regularly experience hypoxia in their natural environment synthesise large multiunit haemocyanins with naturally high affinities for oxygen, thereby, improving tolerance of hypoxia, and increasing the threshold before anaerobic metabolism is employed (Taylor et al., 2000).

Exposure to hypoxia can act as a modulator of not just oxygen affinity of the respiratory pigment, but also the total oxygen carrying capacity in the haemolymph through hypoxia inducible gene expression. For example, improved oxygen carrying capacity has been reported in a number of crustaceans exposed to hypoxia, such as the branchipod crustacean, *Daphnia magna*, and *C. maenas* (Munro Fox, 1948) following corresponding expression of haemoglobin and haemocyanin (Legeay and Massabuau, 1999; Gorr et al., 2004). Although these adjustments to respiration and circulation in hypoxia are centred on the responses shown by adult decapods, recent work has demonstrated a parallel response during development. These include the differential expression of respiratory pigments during development, and also an incidence of bradycardia in response to hypoxia reported in *Procambarus clarkia* and *Nephrops norvegicus*, although modification of ventilation in *P. clarkii* does not occur until juvenile phases (Reiber, 1997; Harper and Reiber, 2004; Eriksson et al., 2006).

When hypoxia is sustained and encountered for long periods of time, the physiological responses by animals can generally be divided into two contrasting groups. First, metabolism in hypoxia or anoxia, as outlined above, can be defended through the modification of respiratory and circulatory systems. Such adjustments are usually associated with the activation of anaerobic energetic pathways (Pasteur effect) and the selective activation of energy efficient pathways that increase ATP yield, either per mole oxygen in hypoxia, or per mole hydrogen in anoxia (Hochachka et al., 1993). Animals that utilise these mechanisms for hypoxia survival are described as oxygen regulators, of which adult malacostracan crustaceans are a good example (see above). Alternatively, hypoxic or anoxic metabolism can undergo a co-ordinated depression, whereby ATP turnover is reduced to a lower steady state level, and anaerobic metabolic pathways are subsequently activated to sustain metabolic depression during prolonged hypoxic exposures (Buck et al., 1993; Hoback et al., 2000; Jackson, 2000; Kolsch, 2001; Mente et al., 2003). Many vertebrates, and also embryonic or small planktonic crustaceans, depress metabolic rate in response to hypoxia, and are classified as oxygen conformers. The fundamental difference between the two strategies is that oxygen regulators maintain cellular energetics at levels similar to normoxic values, whereas oxygen conformers reduce cellular energetics to a low and sustainable level, known as hypometabolism. These two metabolic strategies appear to have evolved uniformly across the animal phyla to counteract the deleterious consequences of oxygen deprivation (ultimately anoxic cell death) when physical avoidance is not possible (Guppy and Withers, 1999; Boutilier and Pierre, 2000; Boutilier, 2001).

1.3.2. **Hypometabolism**

The co-ordinated depression in metabolic rate following hypometabolism occurs not only in response to declining oxygen level, but also against extremes of temperature, desiccation, hypersalinity and starvation (Guppy and Withers, 1999). Characteristically, hypometabolism involves an inhibition of non-essential energy demanding processes, and reallocation of energy to essential energy consuming processes, so that crucially ATP supply and demand remains balanced

(Boutilier 2001). In vertebrates, ATP consumption during normoxia is highest during: protein synthesis > ion-motive ATPases > glucogenesis > ureagenesis (Rolfe and Brown, 1997). As a consequence, many of these processes are rapidly depressed or inhibited during declining oxygen levels. Mitochondrial ATPase, F_1F_0-ATPases, are also inhibited during anoxia, although not all the mechanisms of inhibition are yet known. Binding of an inhibitory subunit (IF_1) has been found in some species, which prevents the enzyme from hydrolysing, or consuming ATP, through proton pumping to alleviate acidosis from the mitochondrial matrix is restricted (St-Pierre et al., 2000).

Animals which show a considerable resistance to hypoxic cell death share a common response within this strategy, e.g: the freshwater turtles, *Chrysemys* sp.; the crucian carp, *Carassius carrasius*; various amphibians, *Rana temporaria* and *Rana pipiens*; the brineshrimp, *Artemia franscicana*; the gastropod, *Helix aspersa*; and *C. maenas.* This common response involves a rapid depression in the rates of protein synthesis (metabolism) during exposure to hypoxia and anoxia (Smith et al., 1996: Hand, 1997; Hofmann and Hand, 1994; West and Boutilier, 1998; Fraser et al., 2001; Packay et al., 2002). The aerobic cost of protein synthesis has been determined in a number of species, and is known, in some cases, to represent more than 50% of basal metabolism, particularly in rapidly growing species. For instance, the fast growing and short lived invertebrate, *Octopus vulgaris*, expends routinely between 35-51% of aerobic metabolism on protein synthesis rates, while the larvae of the African catfish, *Clarias gariepinus,* expend ~43%, and embryos from the sea urchin, *Lytechinus pictus*, expend ~54% (Houlihan et al., 1990; Conceição et al., 1997; Pace and Manahan, 2006). These high values, however, are exceptional, with values ranging from 20-37% of aerobic metabolism being more typical of temperate, adult crustaceans (Houlihan et al., 1989; Whiteley et al., 1996). The high metabolic cost of protein synthesis highlights the potential metabolic saving that can be incurred when protein synthesis rates are suppressed during exposure to environmental hypoxia. A number of other crustaceans also possess such metabolic plasticity, particularly the orders Copepoda and Branchiopoda. Certain species take this response to extremes, e.g. *Diacyclops navus,* by surviving

unfavourable conditions as adult cysts, and *A. franscicana* by surviving as diapausing embryos (Watson and Smallman, 1971; Clegg, 1997; Caceres and Tessier, 2004).

Downregulation of protein synthesis acts through inhibition at the level of translation in hypoxia and anoxia. This has been shown in a range of tolerant species including: the invertebrates, *Littorina littorea and A. franscicana*; the ectothermic invertebrate, *C. carassius,* and the endothermic vertebrate, *Rattus norvegicus* Hofmann and Hand, 1994; Hardewig et al., 1996; Frerichs et al., 1998; Smith et al., 1999; Larade and Storey, 2002). Rates of RNA synthesis are also restructured in response to hypoxia and prioritised in favour of the tissues with normally high rates of protein synthesis, such as the liver.

Collectively, the typical alterations made to protein metabolism by hypoxia tolerant species regulate the metabolic facilitation of rapid and reversible entry into and out of a hypometabolic state. This is a shared and successful survival strategy used by facultative anaerobes, regardless of whether hypometabolism is activated in response to environmental stimuli, or in anticipation of an hypoxic episode (e.g. diapause) (Smith et al., 1996: Hand, 1997; Hofmann and Hand, 1994; West and Boutilier, 1998; Smith et al., 1999; Fraser et al., 2001; Podrabsky and Hand, 2000; Packay et al., 2002). Consequently, cells from facultative anaerobes not only tighten the metabolic 'purse strings and rebalance the ATP chequebook', but importantly electrochemical activity also remains stable, meaning that osmotic homeostasis can be maintained during declining oxygen levels (Boutilier, 2001). The latter being a crucial failure of animals sensitive to hypoxia. Although a great deal of this work has been described in vertebrates, there is accumulating evidence to show that invertebrates show similar metabolic responses to hypoxia and anoxia (Hofmann and Hand, 1994; Hardewig et al., 1996; Larade and Storey, 2002; Packay et al., 2002; Mente et al., 2003).

1.3.3. **Molecular Responses to Hypoxia**

An interesting, and hotly debated component of an animal's defence against low oxygen levels are the mechanisms used to detect the point at which oxygen levels first become limiting. Much of this research has been carried out in vertebrates, where oxygen detection at the organismal level involves carotid bodies in the arteries and in the central nervous system (Mcdonald et al., 1977; Prabhakar, 2000). In crustaceans, chemoreceptors present on the gills carry out the same function, and sense oxygen levels in the ventilation current (Wilkens and Young, 1992; Terwilliger, 1998). Ventilation and cardiovascular responses can be rapidly modulated via the central nervous system, serving to enhance oxygen uptake and delivery, which forms the primary responses to hypoxia as described above.

Oxygen sensing at the cellular level is less clear, with results from a series of interesting experiments that indicate a role for molecular oxygen sensed by a heme protein interaction. For instance, the comparison of erythropoietin (EPO) *in-vitro* expression profiles, following anoxic induction, and following artificial induction using transition metals that simulate physiological hypoxia, provide evidence to support the heme sensor hypothesis (review, Bunn and Poyton, 1996). Simulated hypoxic induction of EPO in human hepatoma cell lines produces a dose responsive reactivity, remarkably similar to the natural expression profile produced as a result of progressive hypoxia (Goldberg et al., 1988; Jiang et al., 1996). Further evidence can be found by studying hypoxia-mediated gene expression in rat hepatocytes (Keitzmann et al., 1993) and also hepatocytes from the western painted turtle, *C. p. bellii* (Land and Hochachka, 1995). Both show metabolic responses mediated by potential changes in heme conformation. In addition, concurrent experiments investigating protein metabolism using *C. p. bellii* have identified oxygen specific proteins which are induced by both physiological and simulated hypoxia, and are associated with changes in hypoxia inducible gene expression (Land and Hochachka, 1995).

Induction of gene expression in response to hypoxia is ubiquitous, and by no means unique to hypoxia or anoxia tolerant organisms. Several genes present in both simple and higher eukaryotic species are expressed during exposure to low

oxygen (Gorr et al., 2006). For instance, activation of anaerobic metabolism in all species sensitive to, and tolerant of, declining oxygen levels involves the expression of a number of genes from the glycolytic pathway (11 in mammals) (review, Hochachka and Lutz, 2001). More recently, the application of DNA microarray technology has allowed the expression of many thousands of gene transcripts to be monitored simultaneously (Gracey and Cossins, 2003). Studies on non-model systems, such as the intertidal fish (*Gillichthys mirabilis*), have highlighted that less than 10% of the differentially expressed genes were down-regulated in the liver during hypoxia (Gracey et al., 2001). In contrast, 60% of the expressed genes were repressed in the skeletal muscle. Most of these genes encoded components of the protein translation machinery, along with genes coding for the most abundant muscle structural proteins. Therefore, the hypoxia response in muscle appears to be part of a common energy saving strategy. Such gene networks have important roles in energy homeostasis, and many have been confirmed as gene targets for the master regulator of hypoxia inducible gene expression, the Hypoxia Inducible factor 1 (HIF-1).

As many as 70 genes expressed in hypoxia or anoxia are mediated by HIF-1 (see below) (Semenza, 2004; Gorr et al., 2006). HIF-1α is a helix loop helix transcription factor, and represents one member of the HIF-1 heterodimer, which is formed by HIF-1α assembling with aryl hydrocarbon receptor nuclear transporter (ARNT, alias HIF-1β). Both are constitutively expressed in normal blood, tissue / cell oxygen levels, and neither are upregulated in response to hypoxia. Hypoxic activity of HIF-1 is related to its DNA binding activity at a hypoxia responsive element (HRE), present in hypoxia inducible genes. HIF-1 is responsible for the activation of a suite of genes important for: cell division and viability; cell death (apoptosis); angiogenesis; and importantly, oxygen and energy homeostasis, either through the enhancement of oxygen delivery, or provision of ATP by alternative metabolic pathways, e.g. anaerobic metabolism (Iyer et al., 1998; Semenza, 2004). Genes activated by HIF-1 include: EPO; haemoglobin; several growth factors (review, Bunn and Poyton, 1996; Ryan et al., 1998); many glycolytic enzymes (Seagroves et al., 2001); and also heat shock proteins (Whitlock et al., 2005). In addition, the rapid reactivity of HIF-1 in

response to changes in oxygen level, and also its ubiquitous nature (see below) are strong evidence of a potential role for HIF-1 in the cellular oxygen sensing mechanism (Semenza, 2004). Furthermore, the accumulation of HIF-1 in hypoxia and simulated hypoxia, as described above with regard to the heme oxygen sensor hypothesis, has also been demonstrated (Wang and Semenza, 1993).

Hypoxic activation of gene expression by HIF-1 relates to changes in DNA binding activity, particularly when oxygen levels decline to PO_2 levels equal to, or below normal tissue / cell physiological oxygen levels i.e. $\leq$ 1 kPa (Semenza, 1999). This is because, unlike in normoxia where HIF-1α is rapidly degraded in < 5 minutes (Huang et al., 1996), in hypoxia, HIF-1α is not targeted for ubiquitin proteasomal breakdown by the von Hippel-Lindau (VHL) tumor suppressor protein. However, these regulatory pathways are very complex and still remain unresolved, although they appear to depend on the many different co-factors that interact with HIF-1. For example, the binding of VHL to HIF-1α is dependant on enzymatic hydroxylation of either a proline or asparagine residue by prolyl domain-containing proteins (PHDs). This takes place in the oxygen sensitive part of the HIF-1α protein, and promotes HIF-1α breakdown in normoxia (Huang et al., 1998; Ivan et al., 2001; Martin et al., 2005).

Due to the universal occurrence of hypoxia, and the requirement of oxygen by animals for respiration, HIF-1, or homologous proteins, have been found in a diverse range of animals. These include a number of vertebrates, such as teleosts e.g. *Oncorhynchus mykiss*, and mammals e.g. *Homo sapiens*, and more recently in invertebrates such as, *Drosophila melanogaster* and *Caenorhabditis elegans* (Bacon et al., 1998; Jiang et al., 2001). Moreover, HIF-1 has recently been found in crustaceans e.g. *Daphnia magna*, and reportedly in the brachyuran *Cancer magister* (Gorr et al., 2004; Head and Terwilliger, 2005). Despite the occurrence of HIF-1 across the Animal Kingdom, most of our understanding comes from studies on mammals, and particularly from physiological experiments characterising the changes in expression of EPO in hypoxia (review, Bunn and Poyton, 1996; Semenza and Wang, 1992). Within the field of mammalian HIF-1

research, recent experiments have switched attention to the role of HIF-1 during embryonic development, and to the role of HIF-1 in cancer research and ischemic cardiovascular disease. This reflects the common role of HIF-1 in angiogenesis, where it plays a pivotal role in vascularisation, both in the formation of the cardiovascular system (Iyer et al., 1997), and in the formation and growth of tumors (Carmeliet et al., 1998). In both cases, HIF-1 is induced by hypoxia resulting from ischemia. The biological implications of HIF-1 and its homologues are far reaching, and yet little is known about its role in the formation of embryonic tissues in crustaceans, especially those that experience hypoxia.

1.3.4. **Cellular Protection from Hypoxia**

A much characterised and ubiquitous cellular response to environmental stress is the activation of stress inducible genes, which code for the synthesis of the heat shock proteins (Hsps). The ubiquitous presence of stress proteins in all organisms is representive of their role in cellular protection during environmental and physiological stress (review, Feder and Hofmann, 1999). A broad range of environmental stressors such as temperature, desiccation, antigens, pollutants, and anoxia can lead to the activation of a constitutively expressed heat shock factor 1 (HSF1). Under stress HSF1 dissociates from hsps into monomer units that reassociate to form an active trimer unit, which subsequently binds to a heat shock responsive target on heat shock protein genes. Here HSF1 is hyper-phosphorylated and gene Hsp transcription is initiated (Craig and Gross, 1991; Heikkila et al., 1997; Tomanek and Somero, 2002). It is unclear what the exact mechanism for such gene expression is; although it is thought that under physiological or environmental stress, normal HSF1 bound Hsps dissociate and bind with high affinity to circulating damaged proteins. This in turn releases HSF1 subunits for trimerisation and binding of the heat shock element (Hse) (Tomanek and Somero, 2002, and cited references).

Families of stress inducible genes are classified by molecular weight, and include the large Hsp family 80-90 kDa, Hsp70 family 65-75 kDa, mid-range Hsps 35-60

kDa, and the small heat shock proteins 15-30 kDa, including ubiquitin 10 kDa (review, Feder and Hofmann, 1999). Each class has been found almost universally amongst prokaryotes and eukaryotes, and all are highly conserved in both form and function, however, the number of genes present in each family is species specific. Continued interest and further examination of the stress protein response has led to the discovery of constitutively expressed Hsps, which function as protein chaperones and prevent structural malformations in normal physiological conditions. These are known as heat shock cognates (Hscs).

Interestingly, continued characterisation of members of the Hsp gene families has shown many to be developmentally regulated, and synthesised at specific phases in development This has been described for a number of invertebrates and vertebrates, including many model organisms e.g. *D. melanogaster*, *A. franciscana*, *Xenopus laevis*, and also various mammals (Bensaude and Morange, 1983; Arrigo and Tanguay, 1991; Ohan and Heikkila, 1995; Gordon et al., 1997; Heikkila et al., 1997; Spees et al., 2003; Geraci et al., 2004). In embryonic *X. laevis*, *D. melanogaster, Danio rerio,* and various mammalian species, Hsp70 and Hsp 90 members are accumulated from maternal sources during maturation, and are functional until the embryonic genome becomes competent during the blastula phase of development (Morange et al., 1984; Arrigo and Tanguay, 1991; Heikkila et al., 1997). The role of these maternal heat shock proteins is unclear, but they may offer the embryo protection against stress during the most vulnerable period of development, or aid initial periods of cell division and differentiation (Basu et al., 2002).

As noted, the importance of Hsps is evidenced by the fact that they are amongst the initial makeup of the embryo proteome. In addition, several other Hsps become inducible later in development, including a number of mid range Hsps, and particularly the small Hsps (Morange et a., 1984 in Wolgemuth and Gruppi 1991; Heikkila et al., 1997; Basu et al., 2002). Of particular interest in diapausing embryos of *A. franciscana* is a small 26 kDa Hsp, identified only in oviparous (diapause) embryos, and shown to undergo nuclear translocation, but not

upregulation, during anoxic stress (Clegg et al., 1995; Clegg et al., 1999). Moreover, Hsp 26 in *A. franciscana* has a maternal origin and is utilised during diapause, conferring tolerance to a range of environmental stressors by preventing apoptosis (Villeneuve et al., 2006). Similar roles of small Hsps have also been identified in *D. melanogaster* and *X. laevis* (Haas et al., 1991; Arigo and Tanguay, 1991; Ohan and Heikkila, 1995; Heikkila et al., 1997).

The Hsp70 family are the most widespread Hsps, found, for instance, in a diverse range of species and throughout the lifecycle (review, Feder and Hofmann, 1999). Expression in embryonic phases of *D. rerio* correlates not only with the onset of developmental events, such as neurogenesis and somitogenesis, but also with environmental stress (Basu et al., 2002; references cited within). Constitutive Hsp70 members are known to function as protein chaperones aiding protein transport, protein folding, and apoptosis (Hofmann et al., 2002; Scott et al., 2003). Stress inducible members such as Hsp 72 function most importantly to target denatured proteins for stabilisation and reconfiguration, preventing irreversible malformation, and the additional energetic requirement of replacement synthesis and degradation. Irreparable, denatured proteins, that can often form cytotoxic molecules, are instead targeted by ubiquitin and tagged for proteolysis.

Hsp70 induction during hypoxia or anoxia has been shown in many species. For example, in intertidal species, exposure to environmentally relevant hypoxic PO_2 levels causes a 2.5-fold increase in Hsp70 levels in the barnacle nauplius, *Balanus amphititre* (Cheng et al., 2003), and a significant increase in the oyster, *Crassostrea gigas* (David et al., 2005). Hsp70 has also been identified as part of the defence strategy used in aestivating and hibernating species, protecting against natural declines in endogenous oxygen through changes in metabolism and circulation (Sills et al., 1998; Scott et al., 2003). Surprisingly, only recent investigations have attempted to characterise cellular protection during embryonic development in marine invertebrates (Geraci et al., 2004; Bonaventura et al., 2006). Yet many are subject to extremes of environmental stress, such as

temperature changes, UV exposure, and variable salinity in planktonic embryonic and larval phases. Also those embryos' that develop in egg masses can be exposed to severe hypoxia (see above).

More recent and less traditional approaches to the study of cellular protection, with particular emphasis on the effects of hypoxia on cell proliferation, have been undertaken. Such experiments have been completed using embryonic *D. melanogaster*, which have shown a combination of Hsp protection, and also cell cycle regulation in response to hypoxic stress (Haas et al., 1991; Douglas et al., 2001). Associated variations in cell cycle duration have also recently been characterised in a number of marine invertebrates (Strathmann et al., 2002). Preliminary evidence suggests that the very vulnerable early embryonic phases, for example during cleavage, show rapid cell divisions, which has been suggested as an adaptation to diminish potential exposure time of this critical period to environmental stress (Staver and Stathmann, 2002). In light of this recent and relatively contemporary research, similar cellular defence strategies could be found to occur during brachyuran ontogeny. Such molecular responses for cell protection could act in concert with physiological strategies to form a global response to hypoxia. This could vary throughout development due to the large changes in growth and differentiation that occur during this period, and the subsequent ontogenetic changes in metabolism and oxygen demand/supply.

In brachyuran crustaceans, studies into the factors influencing growth and metabolism have mainly concentrated on the responses shown by adults. Very little attention has been given to the changes that occur during embryogenesis. Brachyuran embryos are brooded, developing sometimes in their millions, and as a consequence can be packed tightly into the developing egg mass, where they can experience prolonged periods of exposure to hypoxia. This is caused by physical restrictions to water flow and an increase in diffusion distances limiting physical diffusion of oxygen into the egg mass (see above). Only limited information is available to describe the implications of hypoxia on embryonic growth and development, and even less has been made of the cellular response to

hypoxia during ontogeny. Furthermore, recent studies show hypoxia to be important as both a suppressant of growth and development, and interestingly a trigger for vascularisation of the cardiovascular system during embryogenesis, and pathophysiological tumor growth (Carmeliet et al., 1998; Iyer, 1998; Kotch et al., 1999). The characteristics of growth and development during embryogenesis of the edible crab *Cancer pagurus*, and the influence that hypoxia has on this, will be the focus of this thesis.

1.4. Synopsis

The thesis aims to directly characterise the main growth phases in *C. pagurus* embryos by determining whole-embryo fractional rates of protein synthesis and by examining levels of protein accumulation. Such changes will be related to corresponding changes in whole-embryo oxygen uptake rates, in order to estimate metabolic costs during development. To this end, embryonic changes in metabolism during development and in response to physiological hypoxia will also be characterised, accompanied by initial investigations into the underlying physiological and molecular mechanisms.

The specific effects of hypoxia will be examined and related to the conditions naturally present within the egg mass, to assess the extent of maternal care during development and growth. Moreover, the effects of hypoxia on growth and development will be investigated at the level of the whole embryo, and also at the protein and molecular levels. This will involve examination of heat shock protein expression, differences in hypoxia inducible gene expression, and possible changes to BrdU labelled cell proliferation. Furthermore, identification followed by expression levels of a key transcriptional factor (HIF-1α) of hypoxia inducible gene expression will be determined. In addition, the work will be supplemented by further characterising the developmental scheme or profile of the embryonic phases in *C. pagurus*. This will involve both traditional external observations, but more importantly internal characterisation of morphological changes in embryonic development. Direct measurements of embryonic growth and development can then be considered in light of the morphological changes.

Finally, as embryos are likely to be more sensitive to environmental change than adults, such studies will determine whether brachyuran embryos may provide effective biomarkers of environmental hypoxia, possibly as the result of anthropogenic change.

Chapter 2

Characteristics of Growth and Morphology during Embryonic Development of Cancer pagurus

Early phases of embryonic development in *Cancer pagurus* were characterised by the main periods of differentiation, accounting for 70% of the total developmental period. This also included a resting period at the blastula phase, which accounted for 25% of the total development time. Later phases in development, encompassing organogenesis and an intense growth period, were initiated at the eye phase, resulting in the formation of the prezoea embryo.

Similar to adult brachyurans' embryonic growth in *C. pagurus* involved a series of moult cycles, in combination with an uptake of water that supported an increase in size and the attainment of the zoea larval characteristics.

Indices of growth were also used to define growth properties during the embryonic development period in *C. pagurus*. Early development was characterised by relatively small increases to biochemical growth correlates. Later embryonic phases showed large increases of tissue protein, DNA, and RNA content, coinciding with the increase in embryo size, and a continuous decrease in cell size, as cell number increased following the limb formation phase. These results and also Protein:DNA ratios are suggestive of an early period of hypertrophy, between the blastula and limb formation phases, followed by a period of extensive hyperplasia, particularly between the eye and hatching phases in development.

Synthetic indices used in the present study such as RNA:DNA ratio remained relatively constant during embryogenesis, despite variable rates of protein accumulation and rates of development. Interestingly, RNA:DNA ratios were high at the blastula when growth rates were at their lowest.

Finally, maternal control of embryonic development rate in *C. pagurus* appears likely to play an important part in regulating embryonic growth. Ovigerous females remained buried below the substrata, particularly at the blastula and other early developmental phases when growth rates were relatively low. Oxygen availability in the egg mass may, therefore, be central to this behaviour, affecting properties of embryonic growth.

2.1. Introduction

The largest infraorder within the decapod crustaceans is comprised of the most well known and recognisable members, the brachyura, or the true crabs. In addition to the fact that many brachyuran species are commercially fished, their familiarity is also owed to the presence and abundance of crabs amongst the intertidal fauna. Their fairly large size and ease of handling, along with the suitability of some species for maintenance in laboratory aquaria, make them suitable for research purposes. In particular, brachyurans make useful subjects for the study of embryogenesis in marine invertebrates, because all produce lecithotrophic embryos, which are brooded on the abdomen and are easy to remove or to study *in situ*. To date studies on brachyuran development and reproduction have concentrated on: breeding behaviour; copulatory mechanisms; oogenesis; fertilisation; embryo attachment; allometric properties of egg brooding; embryonic development; and the growth and development of pelagic larval phases (Williamson, 1904; Cheung, 1966; Hartnoll, 1968; Hartnoll, 1969; Morgan et al., 1978; Hines, 1982; Anger, 1983; Hines, 1992; Minagawa et al., 1993; Harms et al., 1994; Orensanz et al., 1995; Saigusa et al., 2002; Pinheiro and Hattori, 2003; Costa et al., 2006; Guerrero and Hendrickx, 2006).

Many of the studies on embryonic development are, and still remain, highly descriptive, concentrating on morphological changes during the developmental period (Williamson, 1904; Broekhuysen, 1936; Hartnoll and Paul, 1982; Pinheiro and Hattori, 2003; Costa et al., 2006; Guerrero and Hendrickx, 2006). Whilst this is important, particularly for comparative work, many studies have failed to accurately characterise these morphological changes, due in part to the difficulties encountered in embedding and orientating small brachyuran embryos for sectioning and histological staining (Taylor and Leelapiyanart, 2001). Our current understanding of brachyuran embryogenesis has therefore been best observed from species that produce larger and more practical embryo sizes. This also includes comparative investigations completed using other decapod

members, such as the homarid lobsters (Helluy and Beltz, 1990; Helluy and Beltz, 1991; Harzcsch et al., 1998; Lizardo-daudt and Bond-buckup, 2003) where the diameter of the egg is usually greater than one millimetre and, therefore, at least twice the size of many brachyuran embryos (calculated from egg volume reported in Hines, 1992).

More recently, investigations of brachyuran and decapod embryogenesis have investigated biochemical utilisation of the yolk store, as an index of embryonic metabolism during development (Pandian, 1970; Chu and Koulikawsky, 1994; Subramoniam, 1991; Biesiot and Perry, 1995; Petersen and Anger, 1997; Gonzalez-Baro et al., 2000). This has led to an improved understanding of biochemical changes that are associated with increases in embryonic tissue mass during growth and differentiation and to the changes in rates of development reported previously. However, no studies have correlated indices of embryonic growth to the morphological and biochemical changes described during brachyuran embryogenesis, although a large number of growth related studies have been carried out on brachyuran larval phases and other aquatic organisms, such as larval fish (Buckley, 1979; Anger and Dawirs, 1982; Buckley, 1982; Buckley, 1984; Anger and Hirche, 1990; Anger, 1998). Examination of biochemical growth correlates such as RNA, DNA and protein content during embryonic development, could further increase our understanding of the major growth periods during development and lead to a greater appreciation of the mechanisms responsible for controlling rates of embryonic development in brachyurans, and the specific influence of ovigerous females in manipulating such controls.

As the major periods of growth in adult crabs are confined to specific stages of the moult cycle, the various embryonic moulting cycles reported in crab eggs may also correspond to periods of growth and differentiation, including periods of organogenesis and neurogenesis in the embryos. Furthermore, an appreciation of the factors responsible for controlling embryonic growth and differentiation would aid in the development of effective aquaculture practices for rearing crabs

in captivity, as growth correlates have been shown to be responsive to environmental variables, at least in larval fish (Buckley, 1979; Buckley, 1982; Buckley, 1984; Steinhart and Eckmann, 1992).

There is also evidence that environmental conditions within the egg mass can influence developmental rates in brachyuran embryos (Wheatly, 1981; Naylor et al., 1999; Fernández and Baeza, 2002), and the specific role of declining PO_2 levels in this respect is considered in more detail in Chapter 4. The purpose of the present chapter is to characterise the main growth phases during embryonic development of *Cancer pagurus* to highlight those phases worthy of further investigation into the factors that influence their temporal and developmental progression.

To this end a detailed examination of the embryonic development in *C. pagurus* will be made by describing the main morphological changes as outlined by Helluy and Beltz (1991) and a number of more recent studies (Pinheiro and Hattori, 2003; Guerrero and Hendrickx, 2006). Examination of the developmental phases included traditional biometric measurements on whole-embryos and a histological analysis of sections through the embryos culminating in the formation of a specific scheme for embryogenesis in this species. Secondly, key phases in development were chosen for the determination of cellular growth correlates, such as various nucleic acid ratios, and total protein accumulation by the embryos. Finally, the optimal holding conditions for ovigerous *C. pagurus* and their eggs will be developed to ensure survival of the embryos through to hatching. Brief descriptions of the methodology developed to reduce handling stress for sampling eggs from ovigerous female crabs will also be described.

2.2. Materials and Methods

Adult female edible crabs, *Cancer pagurus,* were obtained from a commercial supplier (The Lobster Pot, Anglesey, 53°18'N, 4°38'W) and from local fishermen (Amlwch, Anglesey, 53°24'N, 4°21'W) during November and December, and transferred to re-circulating marine aquarium at the University of Wales, Bangor. All selected crabs were in good health and most were non-ovigerous. Previous experience has shown that crabs with a loose abdominal apron i.e. the abdominal flap can be easily pulled away from the body to expose the pleopods and gonopores, are generally close to spawning. This feature was used as the main criterion for selecting female crabs so that spawning would occur after the crabs were transferred to the holding tanks in Bangor.

2.2.1. Holding Conditions

A week before the transferral of the crabs, the holding tanks (98L x 46W x 30D inches) were filled with fresh seawater, to which five juvenile *Carcinus maenas* and ten *Palaemon elegans* were added to initiate the biological action of the filtration system. Optimal holding conditions involved application of a new filter system, and maintenance of low temperatures. Seawater from the holding tanks was pumped (Rio 2500) into a filtration tank (60 x 25 x 30 inches) at a flow rate of (350 gallons h^{-1}), equivalent to half the total water volume of the tanks. The filter system was designed in house as a wet/dry trickle filter. As such, the seawater was pumped to the top of the system and onto a plastic tray punctured with holes to restrict water drainage and increase the water transit time through the filter material. As the water drained downwards by gravity it passed through a filter sponge which acted as a mechanical sieve, before draining through the biological filter material and into a reservoir at the bottom of the filter tank. The water was subsequently pumped from the filter reservoir by an external pump and circulated through an external cooling unit before passing back into the holding tank.

On arrival at the University, crabs were tagged around one of the walking legs with plastic bands, and were subsequently identified by the number and colour of these bands. No more than 10 female *C. pagurus* were placed into each holding tank to avoid overcrowding. Holding temperature was maintained at 10°C. The bottom of each holding tank was covered in coarse shell gravel, approximately 30 cm deep, which was obtained from a sand bank off the coast of Anglesey where *C. pagurus* are known to over-winter (A. Tweedale, personal communication). The tanks were decorated with drift wood and large pieces of slate, and the top of each tank was partially covered with wooden boards to reduce the ambient light level. This provided the animals with a natural substratum and plenty of refuges. The animals were left undisturbed during the first 4 weeks of holding to promote spawning activity. The light dark cycle was maintained at 8 h : 16 h, and the crabs were hand fed with either mackerel or mussel once every fortnight prior to system water changes.

2.2.2. Egg Collection and Experimental Regime

Embryos were collected throughout development to include 4 key phases: Blastula; Limb formation (Metanauplius); Eye phase; and Hatching phase (see results). In all cases, eggs were collected by first anesthetising the ovigerous *C. pagurus* by placing a single female onto melting ice for 10-15 minutes. A piece of towel saturated with seawater was placed over the eyes of the animal to reduce stress, and around the embryo mass to prevent direct contact with the ice. Approximately 100mg (fresh weight) of embryos were carefully and quickly removed from the periphery of the embryo mass using sterilised scissors, and placed into a small jar containing sterile seawater at 10°C.

After embryo collection the female crab was returned to the holding aquarium in the exact position from which she was removed. Such a prolonged sampling procedure was necessary to improve access to the egg mass while inflicting the minimum amount of stress to the mother. None of the ovigerous females consumed their embryos or died following this handling method. Throughout development, embryos were removed from the same individual crabs whenever

possible. It is important to note that repeated handling of ovigerous crabs and sampling of the embryos was found to disturb the ovigerous crabs, causing them to discard their eggs. As a result, removal of embryos from the egg mass for identification of the development phase was kept to a minimum, and sampling prioritised for other experiments.

At each phase of development, embryos were washed in sterile U.V. treated seawater to remove any trapped substrata. At first, the phase of development was confirmed from the external characteristics of each batch of embryos. A sub-sample was used for a series of biometric measurements, and a further sub-sample for the determination of growth indices, such as wet:dry weight ratios, nucleic acid concentrations. and estimates of embryonic protein. Finally, a few embryos were prepared for histochemical examination, to determine the internal morphology of the developing tissues and their relationship to the yolk.

2.2.3. **Determination of Developmental Phase**

The embryonic phase of development was determined by examining each batch of washed embryos under a dissecting microscope. Several morphological characteristics were used to identify each phase, such as the total proportion of embryonic tissue present (see below) and the colour of the yolk. For post-limb formation phases, an eye index (μm^2) was measured to follow development (Helluy and Beltz, 1991) and the presence and location of any pigmentation in the embryo was recorded (Broekhuysen, 1936). Biometric measurements were made throughout embryogenesis to include: the diameter of the egg; the area (μm^2) of the pigmentation in the ocular lobes; and the area of yolk within the eggs. The latter was used to determine the approximate proportion (%) of the original yolk store that remained at a given phase in development, and subsequently the proportion of embryonic tissue that was present. This was important for the estimation of total embryonic protein (see below).

2.2.4. **Numbers of Eggs and Wet:Dry Weight Ratios**

Small sub-samples of washed embryos were counted under a binocular microscope (Leica *Zoom 2000*) after removing dead embryos or empty egg cases. Following a count of 100 embryos, the embryos were quickly rinsed in distilled water and gently blotted dry before weighing to the nearest 0.1mg (Oertling NA164). This was repeated a further 5 times on different sub-samples of embryos taken from the same ovigerous mother. The same procedure was repeated on embryos removed from a further five ovigerous crabs at each of the key developmental phases. For dry weight determinations, a sub-sample of washed embryos (approximately 100mg wet weight) were dried to constant weight at 70°C. This procedure was repeated 2 more times on different sub-samples taken from the same ovigerous mother.

2.2.5. **Biometric Determinations**

A small number of washed embryos were viewed under a light microscope (Leica MZ APO). Digital images of the embryos were captured with a mounted camera connected to a computer, using a Hauppage Win TV computer imaging software package. A pulled glass capillary was used to orientate the embryos on the glass slide, to enable the image capture of both dorsal and lateral orientations of the embryos. General observations, such as movements of the embryo (tail flick responses) and the presence or absence of a heart beat, were also noted during imaging. Images were exported into Uthscsa imaging software (version 3), and used for the determination of biometric measurements including the diameter of the egg and the eye, the area of the yolk (μm^2), and the area of ocular pigmentation (μm^2), following calibration against a known standard. The total volume of the egg at each phase in development was calculated according to the formulae of Pinheiro and Hattori (2003) which was $v = 4/3 (\pi r^3)$, where v = volume, r = radius.

Where possible, the embryos had the egg membranes removed for clearer identification of the development phase. Under a microscope (Leica MZ APO) a pulled glass capillary was used to pierce the egg membranes, and to tease the egg membranes away from the embryo. Once the egg membranes were removed a

digital image of the embryo was captured, and the dechorinated embryo was subsequently bathed in toluene blue diluted with seawater. This improved visualisation of embryonic tissue, and highlighted the formation of embryonic limbs and body segments, which was especially useful for comparing developmental differences in morphology between the phases.

2.2.6. **Histochemistry**

Washed embryos were blotted dry to remove residual seawater and placed in Stephaninis' fixative (Stephanini et al., 1967; 2% paraformaldehyde; 15% picric acid; phosphate buffer) and left overnight at room temperature. All embryos were treated identically to allow for tissue damage, such as cell shrinkage, caused during fixation prior to digital analysis. Fixation of the internal tissues was improved by using a pulled glass capillary to pierce the vitelline and embryonic membranes. Embryos were left for a further 1-2 days at 4°C before dehydration in a graded ethanol series (50-100%), 10 minutes treatment in chloroform, and embedding in plastic resin (Technovit 7100, Kulzer) according to the manufacturers' protocol. The embryos were orientated where possible using a light microscope. Serial semi thin sections (4 µm) were cut using a microtome (Reichert-Jung 2050), placed on slides and dried on a heating block. Sections were later stained using haematoxylin-eosin, Schiffs heidenhain haemotoxylin, and toluidine blue. Images were captured using a digital camera (Nikon eclipse 50i).

2.2.7. **Indices of Growth**

1. Embryonic protein content

Approximately 200 mg fresh weight of embryos were ground into a fine powder using a pestle and mortar under liquid nitrogen. Total protein levels in the embryos were determined after precipitation of the embryo homogenate in 2% perchloric acid (PCA) and subsequent alkali digestion in 0.3N sodium hydroxide at 37°C for 60 minutes. A 20µl sub-sample was removed for total protein determination, and measured using a modification of the Lowry technique

(Peterson, 1977). Samples were compared against known standards of bovine serum albumin.

In order to estimate the proportion of embryonic protein per egg, it was necessary to discount the proportion of protein present in the yolk. Initial attempts to isolate the yolk fraction from the embryos proved unsuccessful. This was mainly because separation of the lipid soluble yolk, by either solvent extraction (Bligh and Dwyer, 1959) or density centrifugation (Lubzens et al, 1997), produced inconsistent values for protein from both the yolk and the embryo fractions. Similar difficulties have been observed in other studies on brachyuran eggs (Taylor and Leelapiyanart, 2001). As a compromise, Uthscsa imaging software was used to measure the area occupied by the yolk from 2D digital images of the embryos recorded in dorsal and lateral orientations. Values were expressed as % of the total area. The difference between the total area, and the area occupied by the yolk, was taken to be the area occupied by the embryo. Total protein levels in the embryo were taken to be directly proportional to the % area occupied by the embryo, assuming that protein values per unit weight were similar. At each phase of development, estimations of the area occupied by the yolk were carried out on 10 separate images, representing 10 different eggs. The dimensions of each egg were measured in triplicate.

Values for embryonic protein were used as a means of estimating embryonic growth rate, by calculating mean protein accumulation from one stage to the next. For example, blastula growth rates were taken as the accumulation of protein over the first 6 weeks of development i.e. the duration of the blastula period.

2. RNA and DNA levels

The total RNA content of the eggs was determined in the same tissue homogenates used for total protein determination. Following digestion with 0.3 N NaOH, protein and DNA were precipitated by the addition of ice cold 12% PCA. The acid soluble RNA was removed following centrifugation at 3500rpm, and then pooled following another wash with ice cold 2% PCA. RNA was quantified spectrophometrically (Shimadzu UV260) at wavelengths of 260nm and 232nm according to Ashford and Pain (1986). For subsequent DNA quantification the remaining tissue homogenate was incubated in 10% PCA at 70°C for 25 min, and sub-samples from the resulting supernatant were subjected to the Diphenylamine procedure. Total DNA was measured spectrophometrically (Shimadzu UV260) at 595nm and 700nm using salmon DNA as a standard (Munrow and Fleck, 1966). DNA values were expressed as total DNA content, protein:DNA and RNA:DNA ratios. Total DNA content gives a relative estimate of cell number, protein:DNA describes the average biomass per cell, and RNA:DNA describes the synthetic capacity of the embryonic cells (Cheek et al., 1971; Buckley, 1984; Preedy et al., 1988; Anger & Hirsche, 1990; Anger, 1998). All estimates assume that the DNA content per nucleus remains constant.

2.3. Results

2.3.1. Spawning and Embryogenic Success in Captivity

Between 60-70% of selected female *C. pagurus* spawned large and 'natural' egg masses during each of the second, third and fourth years of consecutive sampling. Spawning most frequently occurred at night and, therefore, the attachment of the eggs to the pleopods was not observed. No females spawned during the first year, and many ovigerous females either consumed their broods or died during this captive sampling period. First year captive crabs also failed to accept food and were unable to tolerate egg collection. This restricted sampling of all key phases and prompted the design of a new filtration system to enhance the water quality of the holding system (see methods). Maintenance of sea water temperatures at 10°C and below was crucial for the long-term survival of the ovigerous crabs. As a result, in each of the subsequent sampling years, approximately 40-50% of ovigerous females successfully completed brooding of their embryos to the hatching phase and released zoea larvae.

2.3.2. Animal Health

Ovigerous crabs sampled during the third sampling year (and one in the fourth) were infected with crustacean shell disease. The symptoms initially appeared as areas of black discoloration on the outside of the carapace, which commonly developed into lesions at articulations between the joints, notably the chelae (figure 2.1). Further investigation by means of dissection revealed the presence of agglutinated haemocytes or nodules that had adhered to the inside surface of the carapace (Vogan et al., 2001). These appear to cause the external discolouration of the cuticle. Fortunately mortality was low in infected ovigerous females and embryonic development proceeded, but other non-ovigerous females were more susceptible to the infection. One notable consequence of this infection in ovigerous females was that embryogenesis was accelerated, and peak hatching occurred approximately 3-4 weeks in advance of hatching periods in other years.

2.3.3. Morphological Changes during Development in Embryonic *C. pagurus*

Nine phases in development have been reported here, but only four of these were chosen as key phases (in highlight) for further experimentation in Chapters 3, 4, and 5 (see figures 2.2 and 2.3). Selection was based on the phases that could easily be identified and those that represented distinct phases of growth and development. Each of these 9 phases are described below. The biometric measurements taken at each of these identified phases of development are summarised in Table 2.1. In addition, figure 2.4 shows the pigmented region of the ocular lobes as a function of development time (weeks), and also with respect to completion of the total developmental period (%).

1. **Blastula phase**: Period 1-6 (up to 8) weeks post spawning (ps).

The blastula embryo appeared completely undeveloped when visualised under a light microscope (figure 2.2, image A). The yolk was comprised of small droplets and occupied almost the entire egg volume, which was (27.17 $\mu m^{3 \times 106}$). The eggs were orange in colour and completely spherical. Figure 2.5, images A and B show histochemical characterisation of the blastula embryo. A perimeter of relatively large cells (5-15µm) in diameter can be seen to envelope the yolk store. Also a polar concentration of ≥100 cells can also be seen, and with evidence of cell migration between the centre and periphery of the yolk. The two vitelline membranes with envelopes 2, and 3, which surrounds the embryo are also visible.

2. Gastrula: Period = 6-9 weeks ps.

The gastrula embryo is the first phase in development that can be easily characterised by external characteristics due to the presence of a clear tissue cap (figure 2.3, image B).

3. Early Limb Formation (Nauplius): Period 9-12 weeks ps.

At the naupliar phase, the early signs of embryonic tissue formation were evident as primordial body regions and appendages (figure 2.3, image C). The yolk droplets were very small and partially fluid, even following fixation in Stephaninis' fixative, which

made dissection extremely difficult. When the egg membranes were removed they came away as a single, relatively thick membrane that had several layers, when compared with the relatively thin egg membrane removed at the blastula.

4. Limb formation (Metanauplius): Period 12-16 weeks ps.

At limb formation the eggs had a clear tissue cap positioned at one pole of the egg, which occupied approximately 20% of the total egg volume (30.34 $\mu m^{3 \times 106}$) (figure 2.2, image B). Following removal of the egg membranes, it was possible to see that the abdomen had grown with respect to the nauplius embryo, and now terminated in an obvious bi-lobed telson fringed with setae. In the limb formation embryo the abdomen was tucked up close against the ventral surface of the embryo, with the telson lying close to the limb buds of the maxillipeds. Tiny cephalic structures, possibly the antennae and antennules, were also apparent. Histochemical staining of embryo sections showed evidence of hyperplasia with respect to the blastula phase embryos, and also very early signs of cell differentiation in the base of the abdomen, which was possibly representative of the developing gut (figure 2.6, image A and B).

5. Eye phase: Period 16-18 weeks ps.

The first evidence of black pigmentation in the eyes (ocular lobes) occured at this phase in development (figure 2.2, image C). The pigmentation marked the lower part of the ocular lobes, but was relatively small (area = 679μm^2) and crescent shaped. The embryo had also increased in size, relative to the limb formation embryo and occupied approximately 30% of the total volume of the egg (33.51$\mu m^{3 \times 106}$). Dissection of the egg membranes revealed evidence of a much larger abdomen with the telson positioned overlaying the mandibles (figure 2.3, image E). Other limbs present at the eye phase included three pairs of maxillipeds on the thorax, and paired antennae and antennules on the cephelothorax. The heart had also begun to form, although the heart beat was not observed (figure 2.7, image D). It is also likely that the embryo had completed a moult cycle with respect to the earlier naupliar phases. Extended fixation caused further separation of the egg membranes at the eye phase, which now included an additional membrane, separate to the thicker membranes observed at the naupliar phases. Histochemical staining of embryo sections still showed only limited evidence of cellular

differentiation and body structures. Where this was apparent differentiation appeared as columns of large columnar cells running part way up the length of the abdomen (figure 2.7, image C, see red arrow), possibly representative of the primordial gut. In addition, the ocular lobes were also relatively large and well developed, and showing initial signs of cellular differentiation (figure 2.7, image B, see red arrow). Average cell size of the developing embryo was notably smaller compared with the blastula phase, with most cells ≤ 8 µm in diameter, although larger cells and elongated cells (10-15 µm) were also present.

6. Eye & Chromatophore phase (Double chromatophoric bridge): Period 18-19 weeks ps.

The embryos were characterised by a number of pigmented features (figure 2.3, image F). The pigmented area in the occular lobes was shaped like a teardrop and had increased in size to 2,339 μm^2. For the first time, other structures in the embryo were pigmented. Two narrow black processes (double bridge) could be seen running down the length of each side of the abdomen and terminating at the telson. These black processes originated from a network of other pigmented processes grouped dorsally above the eyes. The heart had formed and was beating very weakly at ≤ 5 beats min^{-1}. The yolk store remained a deep orange colour, but was reduced in size (62% of the total volume) and the individual droplets had grown larger. This phase in development is also known as the double chromatophoric bridge phase (Pinheiro and Hattori, 2003).

7. Pre-hatching phase 1: Period 19-21 weeks ps.

The pre-hatching period was characterised by a 22% increase in total volume to 47.1 μm^3 $x10^6$. The yolk store was smaller (46% total volume) and had divided into four adjoining lobes (2x small and 2x large) situated centrally in the cephalothorax of the prezoea embryo (figure 2.3, image G). The pigmented area of the occular lobes was 63% larger (6,321 μm^2) than at the eye and chromatophore phase, and was oval in shape and fringed by areas of red pigmentation. The heart appeared as an oval transparent sac structure with scarlet and black chromatophores, and its beat was rhythmic. These formed a series of seven paired parallel red chromatophores lined each side of the dorsal surface of the heart (see figure 2.3, image H pre-hatch 2 phase embryo). The double bridge

chromatophore processes had disappeared and all that remained were lateral pairs of triangular shaped chromatophores, one in each of the 6 abdominal segments. After very careful dissection of unfixed embryos, the developing cephalothorax was surrounded by an exoskeletal structure, possibly relating to the carapace (figure 2.3, image G. The abdomen was almost fully developed and the telson overlapped the optic lobes, resting on the dorsal surface of the embryo by the heart. The telson segment had also become v-shaped, long and sharp, and lined with a set of three small median setae. Regular tail flick contractions of the abdomen were also observed at this phase in development, along with movements which periodically contracted the yolk lobes. Histochemical analysis of sections through the embryo revealed the first evidence of striated muscle fibres, which were present in the abdomen and particularly in the dorsal region of the cephalothorax (figure 2.8, images A, B, C). The embryo had also undergone extensive hyperplasia, when compared to the eye phase embryo.

8. Pre-hatching phase 2: Period 21-22 weeks ps.

The pre-hatching phase 2 embryos were most easily identified by the change of colour and size of the yolk stores (figure 2.3, image H). The yolk has turned ochre in colour and the front two small lobes of yolk had been consumed, leaving only two diamond shaped lobes accounting for approximately 25% of the total egg volume. These were situated behind and below the eyes in close proximity to the gut. The pigmented area of the occular lobes had increased by 40% compared with pre-hatching phase 1 embryos. Heart rate was very rapid and the embryos had become increasingly motile inside the egg membranes.

9. Hatching phase (zoea): Period 22-22.5 weeks ps.

Embryonic development was completed. The zoea larva was fully formed and highly motile within the egg membranes. Figure 2.2 image D shows the zoea larva, which occupied almost the entire volume (55.1 $\mu m^{3 \times 106}$) inside the egg membranes. The remaining yolk stores were often almost entirely exhausted, and not much larger than the pigmented area of the ocular lobes (44,147 μm^2, 16% total volume). The ocular lobes had now become intensely pigmented, kidney shaped and approximately 142 x 82 μm^2 in dimensions. The heart was oval in shape and beated rhythmically $\geq$200 beats min^{-1},

<15°C. The larval rostrum was also formed and lay folded above the heart (figure 2.9, images A and C), and was pigmented with its own chromatophores. Histochemical staining of embryo sections showed the presence of striated muscle formation running the length of the abdomen (figure 2.9, images A and B), and was also notably present in the maxillipeds. Specialised ommatidia were also evident in the eyes, and the gut cavity was now distinct (figure 2.9, images A and B). Average cell size was very small when compared to the other key phases in development, with a cell length of typically just 2 μm.

2.3.4. **Biometric Changes at 4 Key Phases in Development**

1. Embryonic diameter

Table 2.1 shows that the mean values for the diameter of the embryo increased during the developmental period. Changes in diameter were not significant between the blastula and limb formation phases in development (one-way ANOVA, $P = 0.261$), and only a moderate but significant increase had occurred by the eye phase (one-way ANOVA, $P = 0.042$). Mean values were 373 ± 6 μm at the blastula, and 400 ± 9 μm at the eye phase. By hatching phase embryonic diameter had increased to the maximum size recorded during development, representing a highly significant increase with mean values of 472 ± 8 μm (one-way ANOVA, $P < 0.001$).

2. Wet:dry weight ratios

Changes in whole embryo wet weight showed a similar trend during development to changes in embryo diameter (Table 2.1). In summary, there was a small change in wet weight from blastula to eye phase, but a greater change between the eye phase and the hatching phase. Mean values for wet weight were 29 ± 2 μg per embryo at the blastula, and 37.5 ± 3 μg per embryo at the eye phase, representing an increase of approximately 30%. In contrast, hatching phase embryos underwent a 60% increase in wet weight, when compared with the eye phase, with mean values of 60 ± 7 μg. This represented a doubling in total wet weight during the developmental period. There was little change to dry weight during the same period, increasing from 12 ± 2 μg to 15 ± 2 μg per embryo between the blastula and eye phase, after which dry weight remained constant. Wet:dry weight ratios showed little change between the blastula and the eye phase, but increased

markedly by the hatching phase. This coincided with an increase in water uptake during development, with mean values for whole egg water content increasing from 60 ± 1% at the blastula to 75 ± 1.5% at the hatching phase. In addition, water content between the eye phase and pre-hatch 1 phase, and between the pre-hatch 1 phase and the hatching phase, showed increases of 10% and 5% of the total increase.

2.3.5. Growth Indices

Indices of growth were determined at the 4 key phases of development in keeping with the emphasis on these phases in the remaining chapters. Development had a highly significant effect on total embryonic DNA, RNA and protein levels in the embryos of *C. pagurus* (figure 2.10) (Kruskal-Wallis, $P \leq 0.001$), reflecting a large increase in all 3 variables between the eye and hatching phases. At early stages of development (blastula and limb formation) there was no change in the RNA content of the embryos, and relatively small but highly significant changes in DNA and protein e.g. 1.7-fold and 2.5-fold increases, respectively (Mann-Whitney, $P = 0.003$ and $P \leq 0.001$, respectively). However, these changes were not sufficient to alter cellular synthetic capacity (RNA:DNA), which are shown in figure 2.11. Between the eye and hatching phases, DNA content increased by a factor of 10, RNA by a factor of 5, and protein by a factor of almost 7. These changes were reflected by significant changes in the RNA:DNA ratio, as mean values increased from 4.5 ± 0.5 at the eye phase, to 5.7 ±0.06 at the hatching phase (Mann-Whitney, $P = 0.008$). Overall, DNA content showed the largest change during embryogenesis with a 50-fold increase in values from 4.20 ± 0.55 μg embryo^{-1} at the blastula to 220 ± 16 μg embryo^{-1} at hatching (Mann Whitney, $P \leq 0.003$). RNA levels rose by a factor of 12 from 29 ± 8 ng embryo^{-1} to 340 ± 23 ng embryo^{-1}. Finally total mean values for embryonic protein increased 25-fold from 254 ± 12 ng embryo^{-1} at the blastula phase to 6.50 ± 0.59 μg embryo^{-1} at the hatching phase.

Taken as the accumulation of embryonic protein over time, growth rate increased throughout development. After showing relatively little or no change between blastula and limb formation, peak increases were achieved between the eye and hatching phases (figure 2.12). Growth rates were 6 ng protein embryo^{-1} day^{-1} at the blastula, 36 ng protein embryo^{-1} day^{-1} at the eye phase, and 169 ng protein embryo^{-1} day^{-1} at the hatching phase.

Protein:DNA ratios in *C. pagurus* embryos increased significantly between blastula and limb formation phase (Mann-Whitney, $P = 0.021$). Subsequently, protein:DNA levels, which were at their highest during limb formation (91.5 ± 6.6), decreased as development progressed, resulting in values of 67 ± 7.7 at eye phase, and 29.2 ± 2 at hatching phase. Both of these differences were significant (Mann Whitney, $P = 0.039$ and < 0.001, respectively).

2.4. Discussion

2.4.1. Husbandry of Ovigerous Females

Ovigerous female *C. pagurus* caught in pots and landed by fishermen were characterised by depleted egg masses and consumed their eggs within two weeks of captivity. One possible explanation for this behaviour is that the embryos were not fertilised, due to a failure to mate during the breeding season (Lovrich and Vinuesa, 1993). However, the more likely explanation is that ovigerous crabs dropped their eggs as a direct result of the stress experienced during collection and transportation.

Embryo consumption by ovigerous females is a common occurrence in adult decapods that are stressed during early periods of brooding and has been reported in a number of embryological studies to date (Helluy and Beltz, 1991; Garcia-Guerro and Hendricks, 2004; Fernández and Bock, 2000). Females that were sampled to obtain early phase embryos (blastula and limb formation phases) in the present study were most at risk of consuming their egg broods. Ovigerous females brooding embryos at the eye and chromatophore phase, or later phases in development, never consumed their broods even after exposure to repeated handling stress.

Keeping handling stress to a minimum was a crucial aspect during this project, and was necessary to ensure the survival of both the ovigerous female and the embryo brood. The greatest risk facing ovigerous females with broods nearing the hatching phase was the real possibility of exhaustion, following the high energetic costs incurred whilst brooding. For instance, during each year of sampling, a small proportion of females would brood very large healthy embryo masses which experienced much less natural embryo wastage during the brooding period compared with other healthy ovigerous females. However, even though these females were hand fed to maintain their health, they were unable to

complete the final hatching event and subsequently died during the process. It is possible that metabolic reserves decline catastrophically during brooding in certain individuals, since food is not actively sought during the incubation period (Howard, 1982). Metabolic reserves measured during embryo brooding in an Antarctic seastar, *Neosmilaster georgianus*, have been shown to decrease throughout the brooding period (Bosch & Slattery, 1999). Also, rates of oxygen uptake (MO_2) are known to be higher in brachyuran ovigerous females, particularly those brooding embryos at later phases in development, indicating that brooding becomes increasingly demanding to the female crab (Fernández et al., 2000; Taylor and Leelapiyanart, 2001; Baeza and Fernández 2002). In addition, when captive ovigerous females were offered food during early periods of brooding, the animals pushed it away with their chelae. However, food was regularly accepted by ovigerous female crabs brooding later phase embryos, although less voraciously when compared with non-ovigerous females.

In light of the problems associated with the husbandry of ovigerous female *C. pagurus* caught by fishermen, crabs were obtained from a local supplier who bought and sold non-ovigerous crabs collected by divers. These crabs were allowed to spawn in the holding tanks after being transferred to Bangor (see below). Holding conditions were also optimised after the difficulties encountered during year one of the project. This was achieved by improving general water quality, by isolating the holding tanks from the main aquarium, and by installation of a wet / dry trickle filtration system. Also the water was kept at a constant temperature of 10 ± 0.5°C by circulation through an external cooling unit to match the natural temperature range of *C. pagurus*. Previous experience showed that ovigerous crabs kept at higher temperatures (13-15°C), consumed their embryos, and mortality rates were also increased.

Improved water filtration and periodic changes of 1/3rd of the system's seawater were necessary to prevent nitrite (NO_2^-) and ammonia (NH_4^+) accumulation in the holding system, and to maintain nitrate (NO_3^-) levels below 30ppm, which is within the recommended levels for captive marine invertebrates (Camargo et al,

2005). This was important because NO_3^- accumulation in closed recirculating systems is often high and sometimes exposure can be prolonged, since NH_4^+ and NO_2^- ions, which are the initial excretory products, are converted to nitrate NO_3^- especially in oxygenated conditions (Camargo and Ward, 1995; Camargo et al., 2005). Although the toxic effects of NO_3^- is relatively low compared with NH_4^+ and NO_2^-, NO_3^- reduces oxygen transport capacity by transforming the respiratory pigments to non-functional forms (Scott and Crunkilton, 2000; Camargo et al, 2005). Also, NO_3^- toxicity impacts more acutely on early life phases, and could have deleterious effects on the health of embryonic phases as well as long term health effects on the brooding female crabs (review, Camargo et al, 2005).

In summary, the design and implementation of this holding system for *C. pagurus* was crucial for the successful maintenance of adult female crabs and their developing embryos. The survival of the embryos was dependant on the survival of the ovigerous crabs, because it was impossible to keep isolated eggs alive for longer than one month, mainly because the embryos rapidly became infested by bacteria and fungi when separated from the mother. The presence of maternal care would appear crucial for the removal of ectoparasites, particularly nemertean worms, which are a well known factor leading to embryo mortality in Cancrid crabs (Shields and Kuris, 1988; Shields et al., 1991). The long term maintenance of the ovigerous females was necessary as obtaining replacement ovigerous crabs after January was rarely possible.

Finally, the provision of adequate refuges per crab (~20^2 inches), and a natural substratum, were crucial factors for promoting spawning in captivity. Almost all females that spawned in captivity developed their broods up to at least the eye phase in development, and approximately half of these subsequently developed their broods to hatch millions of zoea larvae. Following spawning in the wild, ovigerous *C. pagurus* are known to bury themselves beneath the substratum for approximately two months, and show reduced foraging behaviour during this period (Naylor et al., 1999). The same behaviour was observed in *C. pagurus* maintained in the aquaria in Bangor, suggesting similarities in ovigerous

behaviours to crabs in the wild. Such quiescent behaviour can be difficult to achieve in captivity (Moriyasu and Lanteigne, 1998; Naylor et al., 1999), and is reported to delay the development of the eggs (addressed in Chapters 3 and 4).

2.4.2. Rates of Embryonic Development in *C. pagurus*

Early phases of embryonic development in *C. pagurus* were characterised by the main periods of differentiation. These were followed by organogenesis and growth, (considered here as embryonic tissue formation and protein accumulation), during later phases in development. This apparent biphasic pattern of embryonic development is extensive amongst brachyurans (Broekhuysen, 1936; Moriyasu and Lanteigne, 1998; Taylor and Leelapiyanart., 2001; Pinheiro and Hattori, 2003; Garcia-Guerrero and Hendrickx, 2004; Guerrero and Hendrickx, 2006) and is associated with differences in the rates of development with early phases of development being more prolonged.

The time taken for complete embryonic development in captive *C. pagurus* recorded in the present study was similar to that reported by Naylor et al (1999). Early phases in development lasted approximately 110 days while later developmental phases that followed the initial pigmentation of the ocular lobes were much shorter at approximately 45 days. In the wild, rates of embryonic development are influenced synergistically by the physical characteristics of the surrounding seawater, and in particular, temperature and oxygen, since oxygen demand is positively correlated to temperature within thermal limits (Vernberg and Costlow 1966; Wheatly, 1981; Naylor et al., 1999; Fernández et al., 2000; Baeza and Fernández 2002; Mitchell and Seymour, 2003; Thatje et al., 2003). For example, an elevation in temperature by 13°C, from 11°C to 24°C, has been shown to accelerate embryogenesis by fifty days in the temperate shore crab, *Carcinus maenas* (Wear, 1974). Also, tropical brachyurans naturally have much shorter embryonic periods, owed largely, but not entirely, to the effects of high temperatures (> 20°C) found in the natural environment. The potential influence of differing oxygen levels on embryonic development in *C. pagurus* is discussed in Chapters 3 and 4.

In addition to the well characterised effects of temperature on rates of development in invertebrates, a number of other factors are also known to be important. For example, physiological factors such as egg size (Efford, 1969; Nishino, 1980; Lonsdale and Levinton, 1985; Shields et al., 1991), and endogenous factors such as diapause periods, have also been reported to influence rates of development in a number of species (Wear, 1974; Helluy and Beltz, 1991; Lovrich and Vinuesa, 1993; Moriyasu and Lanteigne, 1998). Other factors including reproductive synchrony of larval release with environmental food supply (Harrison, 1988), and also factors that may have a detrimental effect on the ovigerous female, such as disease, could also play a part.

In the present study, almost all females sampled during the third year suffered from the bacterial infection which causes shell disease in crabs and lobsters (Vogan et al., 2001; Vogan et al., 2002). This infection first became apparent whilst ovigerous crabs were brooding blastula phase embryos, and became progressively worse over time. Contraction of this disease appeared to advance the rate of embryonic development by 3-4 weeks, with respect to hatching periods in other sampling years. The reasons for this are not clear, but it may relate to the unnaturally high levels of ecdysone reported in the haemolymph of infected animals (VanPatten, 2006). Ecdysone levels are normally low during egg brooding, since if molting were to occur part way through the incubation period the embryos would also be shed along with the cuticle. An urge to molt could, therefore, form part of the explanation for why the rates of development were advanced during year 3. The factors regulating this acceleration in growth and development are also unclear, but could be related to maternal oxygen provision to the embryo brood, since temperature, photoperiod and water quality remained unchanged in the present experiment.

The link between maternal ventilation of the embryo mass with rates of development in brachyurans is well documented (Wheatly, 1981, Naylor et al.,

1997; Naylor et al., 1998; Naylor et al., 1999; Fernández et al., 2000, Fernández and Baeza, 2002; Fernández and Brante, 2003) (see Chapter 4). Again, the factors controlling discontinuous ventilation of the egg mass are unknown but could involve environmental cues that entrain brooding behaviours in the ovigerous females. The absence of certain entrainment cues in captivity, such as the lunar cycle, may explain differences in developmental rate. For example, Moriyasu and Lanteigne (1998) reported large differences in rates of embryonic development between captive and wild ovigerous *Chionoecetes opilio*. Indeed, reproductive synchrony is an important feature of decapodan development with larval release governed by a combination of circatidal, lunar, and circadian rhythms (Vries et al., 1991; Morgan and Christy, 1995; Zeng and Naylor, 1997). The absence of periodic cues during the development of *C. pagurus* embryos in captivity raises interesting questions about the factors controlling the duration of embryonic development, and the influence of the health and well being of the adult females.

2.4.3. Embryonic Growth in *C. pagurus*

1. Growth Indices

Growth and development of *C. pagurus* was first examined at the blastula phase, which occurred soon after fertilisation and egg extrusion, when the yolk material was distributed homogenously (centrolecithal) inside the egg, which is common in crustaceans (Muller et al., 2004). Using light microscopy, blastula embryos appeared undifferentiated and evidence of cleavage was not apparent. However, these observations were not supported by the relatively high protein:DNA ratios and the comparatively low DNA content at the blastula, which confirmed the presence of a small number of relatively large cells. Initial periods of cleavage in centrolecithal eggs generally results in a few but large cells. Cleavage is superficial, known as meroblastic cleavage, where the plane of cell division is situated above the yolk (Muller et al., 2004). This whole process ensures cells of common developmental fate are grouped together (Alwes and Scholtz, 2004) as shown in a number of brachyuran embryos (Lovrich and Vinuesa, 1993; Taylor and Leelapiyanart, 2001; Lizardo-daudt and Bond-buckup, 2003; Seneviratna and Taylor, 2006). Although cleavage could not be visualised in live whole embryos

during the present study, similar observations were found following examination of sections through plastic embedded embryos stained with toluidine blue. These sections showed an advanced phase in cleavage with the concentration of cells forming the blastodisc, and also cell migration between this and the centre of the yolk. Similar observations have been made in the freshwater anomuran crab *Aegla platensis* and in the euphausid Meganyctiphanes norvegica (Alwes and Scholtz, 2004; Lizardo-daudt and Bond-buckup, 2003). Further analysis of cleavage at the blastula phase will be addressed in Chapter 5.

Following the blastula phase in development, the growth indices closely followed the progressive changes in embryo size and morphology. Development was marked by significant increases in tissue protein, DNA, and RNA content as the embryos increased in size, and a continuous decrease in cell size as cell number increased following the limb formation phase. Overall the results suggest that there is an early period of hypertrophy between the blastula and limb formation phases, followed by a period of extensive hyperplasia, particularly between the eye and hatching phases, indicating an increase in embryonic tissue formation. Such periods of intense growth occur in adult crustaceans during the moult cycle at the pre- and postmoult phases (Mayrand et al., 2000; N. M. Whiteley, unpublished observations). A similar pattern of growth has also been reported in the Japanese larval flounder *Paralichthys olivaceus* (Gwak and Tanaka, 2002; Gwak, 2003). Histochemical sectioning of embryonic *C. pagurus* sections, at all of the key phases in development, confirmed the large increase in cell number during development. In addition, cells were often seen in mitosis at the limb formation phase.

Another useful indicator of growth during development is the RNA:DNA ratio or the cellular capacity for protein synthesis, which is a common measurement in the study of growth in larval fish (review, Buckley et al., 1999). The values for RNA:DNA reported here were within the range of values reported for larval fish and rock scallop *Crassadoma gigantea* (Whyte et al., 1990; Buckley et al., 1999; Gwak and tanaka, 2002; Gwak, 2003). In the present study, the RNA:DNA ratio

remained relatively constant during embryogenesis, despite variable rates of protein accumulation and variable rates of development. The lack of any developmental changes in RNA:DNA ratios contrasts to the response reported in the rock scallop *C. gigantea*, and to the response in post hatching phase larval fish, where the RNA:DNA ratios can show large changes during development (Buckley, 1979; Buckley, 1982; Buckley, 1984; Whyte et al., 1990). The reasons for this are not clear; the main possibility is that the presence of the endogenous yolk store can cause problems with the use of RNA:DNA as a sensitive measure of growth, as reported in larval fish studies (Buckley et al., 1999).

Interestingly, in the present study and despite the low protein accumulation and morphological development during the blastula phase, the RNA:DNA was relatively high. Whole embryo RNA levels were also high, and similar to those found at the limb formation phase, even though limb formation embryos had undergone considerable growth and differentiation by this phase (see below). High RNA:DNA ratios have also been reported in *C. gigantea* at the blastula, and are indicated to be elevated in sea urchin blastula embryos (Epel, 1967; Whyte et al., 1990; Rees et al., 1995). However, a high RNA content at the blastula in *C. pagurus* may simply be an artefact of vitellogenesis, as oppose to an indication of elevated synthetic capacity. For example, Adiyodi and Subramonium (1983) have found that maternal RNA is packaged into maturing oocytes during oogenesis in Emerita sp. (addressed in chapter 3). Although ribosomal RNA forms the bulk of total tissue RNA, maternal mRNA transcripts may also contribute to the high vitellogenic RNA content at the blastula, e.g. transcripts for heat shock proteins (Hsps) (addressed in chapter 3; Clegg et al., 1994; Clegg et al., 1995; Heikkila et al., 1997; Clegg et al., 1999). In light of this, analysis of the yolk constituents would provide useful information as an extension to this work.

2. Protein accumulation

Microscopical observations, such as the size of the yolk store and histochemical identification of cell division and differentiation, showed no differences between

blastula embryos sampled close to extrusion and those sampled several weeks after spawning. Embryos remained as blastulae for up to 6 weeks making this the most prolonged phase of development. Not surprisingly growth rates (protein accumulation) of the blastula embryos were relatively low. Extended time periods, along with slow embryonic growth and lack of differentiation at the blastula phase, has been reported in *C. pagurus* and in several other decapod species (Wear, 1974; Lovrich and Vinuesa, 1993; Moriyasu and Lanteigne 1998; Naylor et al., 1999; Taylor and Leelapiyanart, 2001). These authors suggest that the extended period as blastulae is related to a period of quiescence or diapause. It is possible that the observed plateau in growth and development at the blastula in *C. pagurus* could be related to maternal control of rates of embryonic development. This is based on the observation that ovigerous *C. pagurus* crabs carrying blastula phase embryos were continuously buried beneath the substrata, and therefore potentially deprived of oxygen. This suggestion is investigated in Chapter 4.

Rates of development between the blastula and limb formation in *C. pagurus* was also relatively slow and accounted for more than half of the total developmental period. Slow rates of development were accompanied by a low rate of protein accumulation by the embryo, which was not dissimilar to that recorded during the blastulae period. Similarly, other temperate brachyuran species are characterised by relatively prolonged metanaupliar phases (Shields et al., 1991; Taylor and Leelapiyanart, 2001). A low rate of protein accumulation could explain why early phases in development are relatively prolonged. This could also be a direct consequence of low protein synthetic activity, and also the PO_2 levels present in the egg mass, factors which will be addressed in Chapters 3 and 4.

Advancement to the eye phase triggered an intense period of growth and differentiation, particularly to the post mandibular region in embryonic *C. pagurus*. Rates of development increased considerably, and embryogenesis was completed by as little as six weeks later, accounting for 30% of the total developmental time period (see figures 2.4 and 2.12). The eye phase appears to

mark a transition in rates of development for a number of other brachyuran species, as indicated by increases in the rates of biochemical reactions and by overall increases in metabolic rate (Subramoniam, 1991; Lovrich and Vinuesa, 1993; Petersen and Anger, 1997; Taylor and Leelapiyanart, 2001). There are, however, some exceptions with *Chionoecetes opilio* embryos characterised by a quiescent or diapause period at the eye phase (Moriyasu and Lanteigne, 1998). In the present study an increased rate of protein accumulation at the eye phase, probably due to hyperplasia, appears to be part of the main reason for an increase in developmental rate, although maternal brooding behaviours could also be important.

At the hatching phase the embryos were considered to be zoea. By this phase, the yolk stores had decreased in size and were surrounded by large transparent lipid globules. These could offer further metabolic substrates for growth, as lipids represent a major energy reserve for the embryo and larvae during development (Lovrich and Ouellet, 1994; Anger, 1998). In contrast, esterase activity at hatching phase in *Emerita asiatica* is low, and indicative of a low lipid utilisation (Subramoniam, 1991). Lipid stores at the hatching phase could instead function to improve buoyancy during the pelagic zoeal phases, as found in marine zooplankton (Visser and Jonasdottir, 1999; Campbell and Dower, 2003).

Attainment of the hatching phase in *C. pagurus* marked the completion of a rapid and compressed period of growth and development. Accelerated rates of development represent the period of organogenesis that occurs close to hatching in brachyurans (Naylor et al., 1999; Taylor and Leelapiyanart, 2001; Pinheiro and Hattori, 2003 Guerrero and Hendrickx, 2006). In *C. pagurus*, increased growth rates at the hatching phase are supported by an increase in cell number, and cellular synthetic capacity, which is similar to the relationship observed in larval fish (Buckley, 1979; Buckley, 1982; Buckley, 1984; Steinhart and Eckmann, 1992). Consequently, hatching phase embryos underwent a large accumulation of protein, which was 4-fold greater than the total protein accumulated between extrusion and the eye phase. Also this protein was accumulated during a much

shorter time interval, representing just 30% of the total developmental period. Rapid growth rates in larval and juvenile fish also occur during high protein accumulation, or retention efficiency (Conceicao et al., 1997; Carter et al., 1998). These changes to rates of development in *C. pagurus* appear to reflect changes in protein metabolism, which will be addressed in the following chapter.

2.4.4. **Biometric Changes and Water Content**

The diameter of the embryo increased steadily between the blastula and eye phase, followed by a large increase between the eye and the hatching phase. Changes in diameter generally coincided with the absorption of water, which caused an increase in embryonic volume for the total developmental period of approximately 100%. Many marine invertebrates show a large increase in the rate of water absorption during development (Pandian, 1970; Sheader, 1996; Petersen and Anger, 1997), in contrast to terrestrial invertebrates, which show very little water exchange to avoid desiccation (Gilchrist, 1978; Gibbs et al., 1997; Rivers et al., 2002).

The relationship between water uptake during development in *C. pagurus* and changes in wet weight was not clear. This may relate to alterations in the mass of the yolk store during development and the synthesis of new egg membranes during moulting (see below) (Taylor and Leelapiyanart, 2001). It is important, however, to note that since water content varied little between early phases, these changes were potentially more an indication of an increase in tissue mass. Similar changes in dry weight were observed during embryogenesis in *H. araneus* (Petersen and Anger, 1997). At later phases, changes in wet weight were mainly related to changes in water content, as embryonic dry weight remained constant after the eye phase, for reasons described below.

The main periods of water uptake in embryonic *C. pagurus* corresponded to organogenesis and hatching. This pattern of water absorption appears to be common in crustaceans, and has been identified in a number of decapods

including: *H. gammarus*; *H. araneus*; *Hemigrapsus crenulatus*; and *C. maenas* (Pandian, 1970; Petersen and Anger, 1997; Chung and Webster, 2004; Seneviratna and Taylor, 2006). Maximum water content at the hatching phase could serve a range of purposes during development. For example, elevations in water content are known to increase buoyancy by decreasing specific weight, in preparation for pelagic life phases (Pandian, 1970; Petersen and Anger, 1997). Also, increased water content in the egg may improve thermoconforming functions, by increasing the specific heat capacity of the whole larva, enabling body temperature to equilibrate more rapidly to environmental temperature, which could be important during pelagic life phases (Pandian, 1970). Finally, in addition to moulting and osmotic hatching, recent evidence also suggests that water uptake is important for osmoregulation during embryonic development (Seneviratna and Taylor, 2006).

2.4.5. **Embryonic Moult Cycles**

Crude changes in embryo morphology visualised on whole embryos were enhanced by dissecting the eggs to reveal more specialised changes, such as those associated with embryonic moult cycles. In particular, an increase in embryonic tissue and morphological differentiation during the limb formation, or metanauplius embryo, was associated with a moult cycle. Similar observations were made at the eye, protozoea and hatching phases (see below) as described in other crustacean taxa (Goudeau, 1976; Goudeau and Lach, 1983; Helluy and Beltz, 1991). Therefore the timing of growth and differentiation in crustacean embryogenesis appears to be highly conserved.

Observations of the protozoea embryo revealed a number of interesting features, such as the first appearance of a zoeal carapace, which was folded and looked oversized. The 'extended' carapace may function to permit further growth of the protozoea and prezoea embryo, and or could also act as a flotation device to improve buoyancy for pelagic zoeal phases. By the protozoea and pre-zoea phases the embryos were noticeably motile in the egg membranes, and heart beat was also observed for the first time.

The heart is the first physiological organ, and the cardiovascular system the first system, to become functional during development (Harper and Reiber, 2004). The formation of the heart was first observed in late limb formation and eye phase embryos, although this was pre-heart beat. In embryonic *C. pagurus,* the heart beats at the protozoea phase were weak and arrhythmic to begin with, but were followed by an increased rate of beating to $\geq$ 200 beats min^{-1}, with visible haemolymph circulation at the prezoea phase. Embryonic cardiac competence in decapods could form an important adaptation to hypoxia during development. Circulation of the haemolymph and haemocyanin in this way would improve oxygen loading and tissue delivery, increasing tolerance to hypoxia similar to mechanisms that have been well described in adult decapods (review, McMahon, 2001; Harper and Reiber, 2004) (addressed in Chapter 4). Tail flicking was also observed at the protozoea and prezoea developmental phases, which was supported by the first appearance of striated muscle fibres in the body, but pre-dominantly in the abdomen of prezoea phase embryos, indicating that the embryos had a functioning muscular system by this stage.

In summary, *C. pagurus* spawn a single large egg mass during the winter, which is brooded for approximately six months, culminating with the synchronised release of zoea larvae with the summer plankton bloom. Determination of variables that can be used to assess growth, and the observations on the main morphological changes, reveal that the growth and development of *C. pagurus* embryos is not continuous, but switches between relative quiescent and active periods of growth and differentiation. More specific and direct measurements of growth in *C. pagurus* embryos, determined as changes in protein synthesis rates at the key phases in development are given in the next Chapter, while the effects of environmental oxygen levels on growth and metabolism in *C. pagurus* eggs is covered in Chapter 4.

Figure 2.1. Male and female examples of *Cancer pagurus* with crustacean shell disease. (a) Arrows show lesions formed on the underside of the carapace and on the chelae. (b) Arrow showing the early symptoms of the disease, which appear as localised blackening of the cuticle.


A
1 inch


b
1 inch

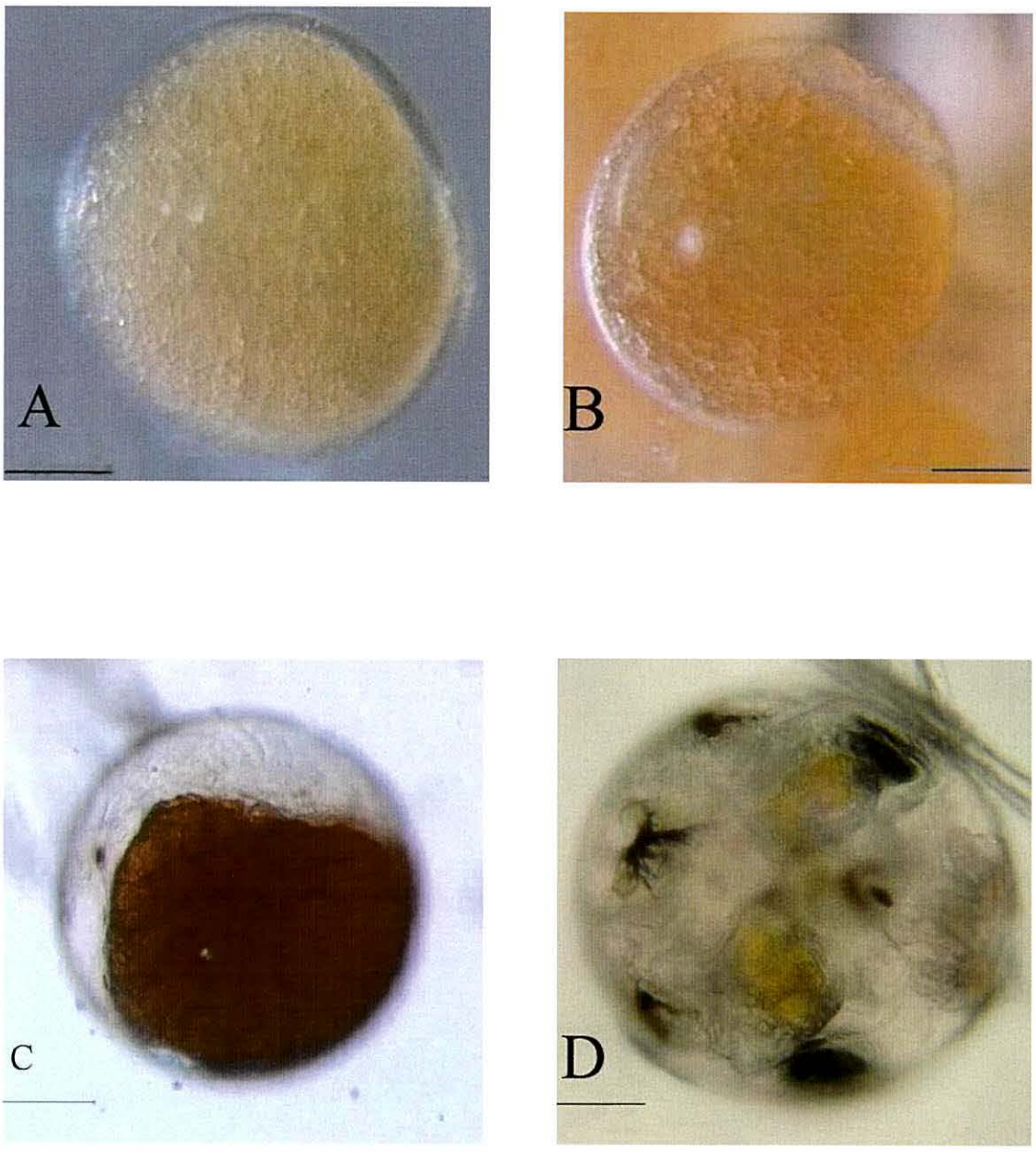
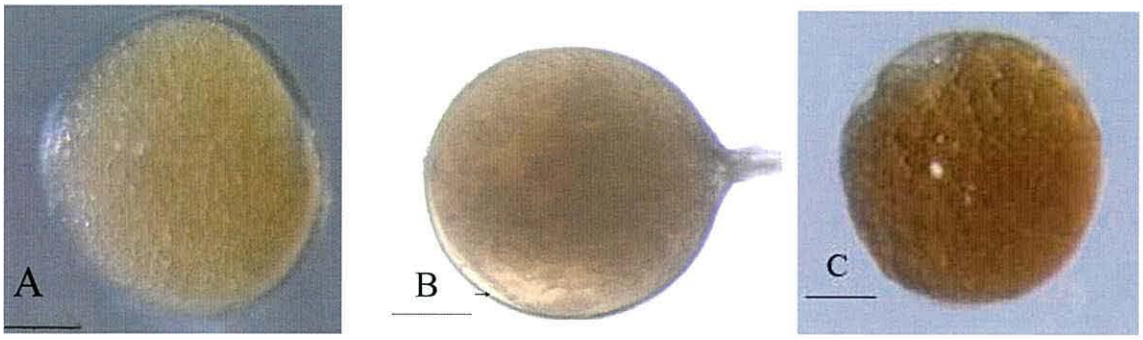
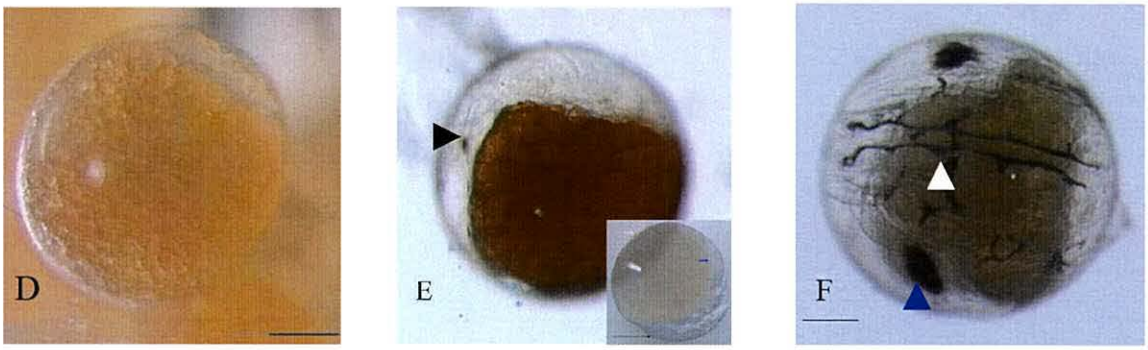
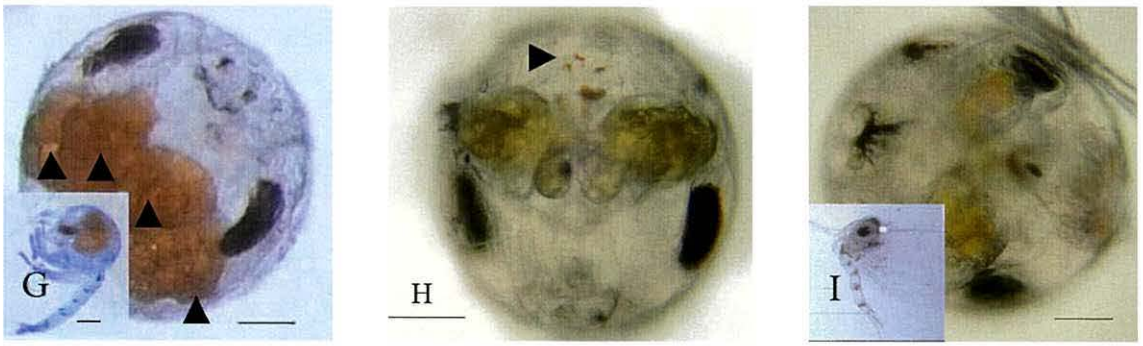


Figure 2.2. Key phases in embryonic development of *Cancer pagurus*. (A) Blastula. (B) Limb formation. Transparent area is the developing embryo. (C) Eye phase. The embryo has large ocular lobes that show partial pigmentation. (D) Hatching. The first phase zoea larva is fully formed. Most of the yolk has been consumed and has also changed colour. The zoea embryo is highly motile inside the egg membranes, and the heart beats rapidly.

Figure 2.3. All phases in the embryonic development of *Cancer pagurus*. (A) Blastula. (B) Gastrula phase, arrowhead shows the germinal disc. (C) Nauplius. (D) Limb formation, transparent area is the dorsal side of the developing embryo. (E) Eye phase, black arrowhead shows the pigmentation in the eye. Also, note the developing abdomen, and relatively large ocular lobe (blue arrow) present in the dissected embryo. (F) Eye and chromatophore phase. White arrowhead showing the black pigmented chromatophore processes (double bridge) in the abdomen, blue arrowhead showing the large pigmented ocular lobes. (G) Pre-hatch 1, note the 4 remaining yolk lobes (arrowheads), and absence of chromatophore processes. Also, note the features of the dissected embryo bathed with toluidine blue. (H) Pre-hatch 2, the embryo has large and intensely pigmented ocular lobes, and the yolk has changed colour from orange to ochre. Black arrowhead shows the scarlet pigmentation surrounding the heart. (I) Hatching phase, with hatched zoea in indent.


A
B
C


D
E
F


G
H
I

Morphological phase (n)	Proportion of development time (%)	Diameter (µm)	Volume ($\mu m^3 \times 10^6$)	Fresh weight µg per egg	Dry weight µg per egg	Pigmented area of occular lobe μm^2	Yolk (% volume)	Yolk colour
blastula (6)	25	373 ± 6	27.2 ± 1.2	29 ± 2	12 ± 2	N/A	95	orange
Gastrula (3)	14	N/A	N/A	N/A	N/A	N/A	92	orange
Nauplius (5)	14	382 ± 11	30.3 ± 1.4	N/A	N/A	N/A	88	orange
Limb formation (6)	17	387 ± 9	30.3 ± 1.4	36.5 ± 3	15 ± 3	N/A	77	orange
Eye (7)	9	400 ± 8	33.5 ± 1.3	37.5 ± 3	15 ± 2	735 ± 28	70	orange
Eye & Chromatophore (5)	5	413 ± 9	36.9 ± 1.3	N/A	N/A	2339 ± 191	62	orange
Pre-hatch 1 (5)	9	448 ± 12	47.0 ± 1.4	47.6 ± 5	14 ± 2	6321 ± 606	46	orange
Pre-hatch 2 (5)	5	459 ± 11	51.3 ± 1.4	N/A	N/A	10548 ± 567	25	Ochre
Hatch (6)	2	472 ± 8	55.1 ± 1.5	60 ± 7	15 ± 1.5	11413 ± 499	16	Ochre

Table 2.1. Biometric measurements at nine characterised phases of embryonic development in *Cancer pagurus*, and wet:dry weight ratio measurements at all four key phases in development.

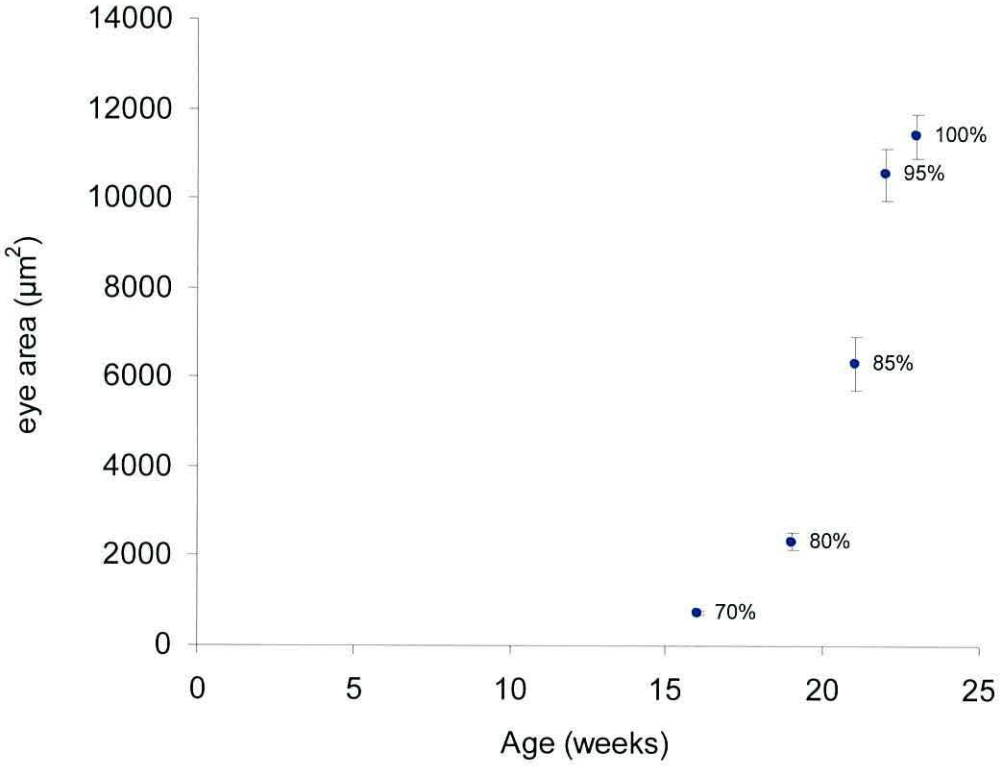


Figure 2.4. Eye index during % completion of embryonic development of *Cancer pagurus* maintained at 10°C. The area (µm²) of the pigmented region in the ocular lobes was measured using digitally captured images of each appropriate developmental phase using Uthscsa imaging software. Values are means ± SE, generated by making measurements on 6 different embryos on a single image, which was repeated on a further 5 images for each appropriate developmental phase.

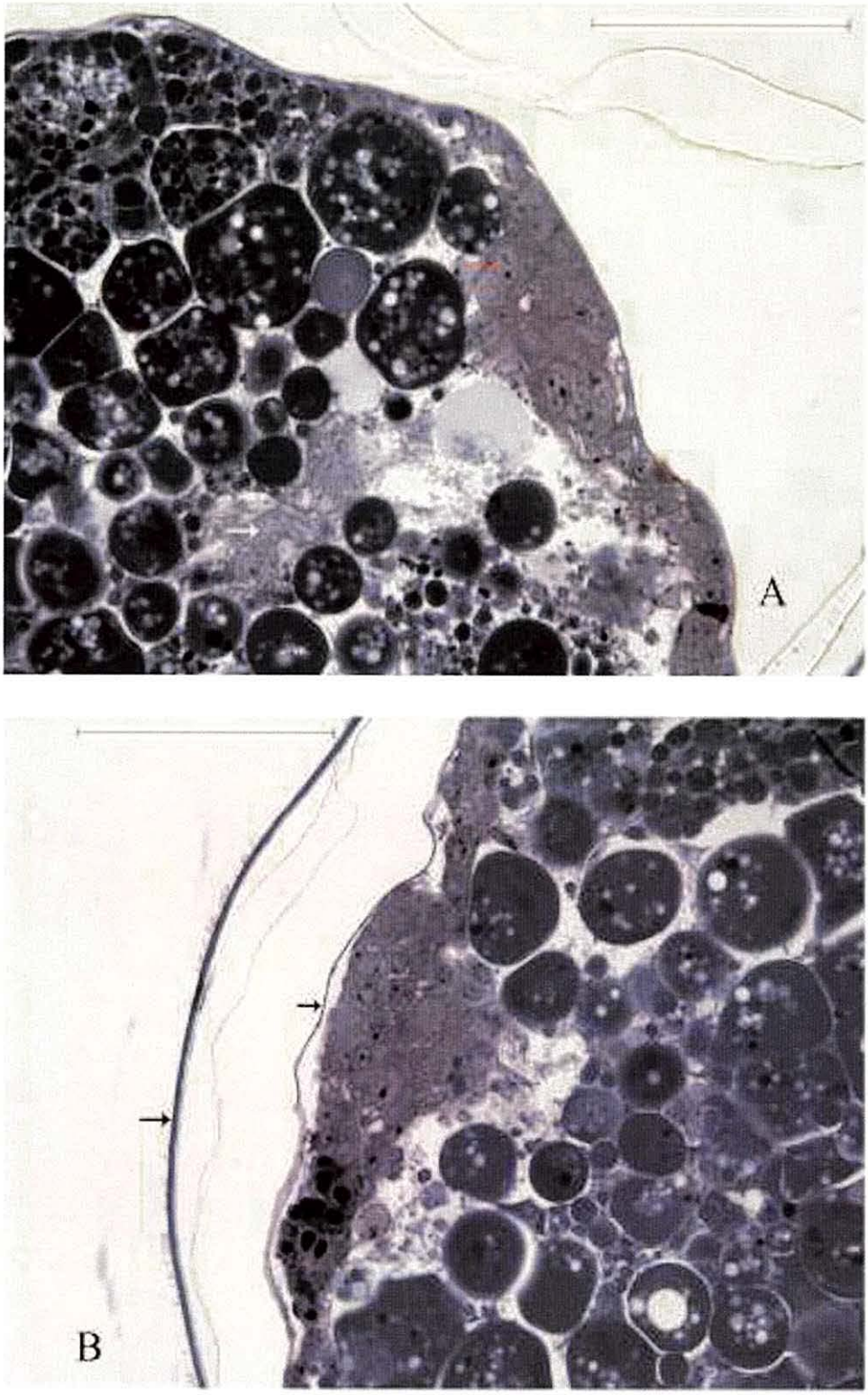


Figure 2.5. Blastula phase in embryonic development of *Cancer pagurus*, longitudinal sections stained with toluidine blue; scale bars 100µm. (a) Red arrow shows early cells present in the blastodisc; white arrow shows cellular migration between the blastodisc and centre of the egg. (b) Black arrows show the egg membranes, the large arrow points to the vitelline membranes, and the small arrow shows the first embryo membrane.

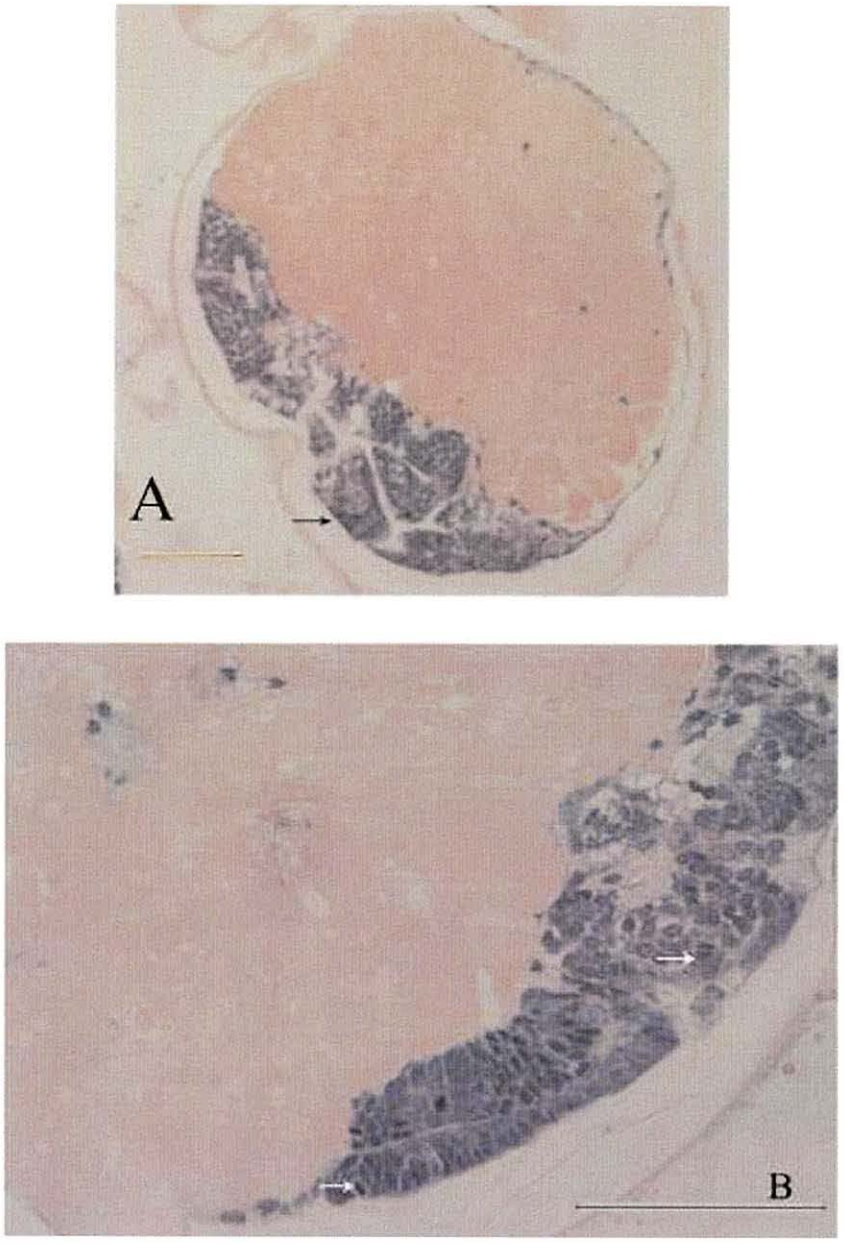


Figure 2.6. Limb formation phase in embryonic development of *Cancer pagurus*. Longitudinal sections stained with hemotoxylin and eosin; scale bars 100μm. (a) Arrow shows the growing abdomen. (b) Arrows show cells in mitosis.

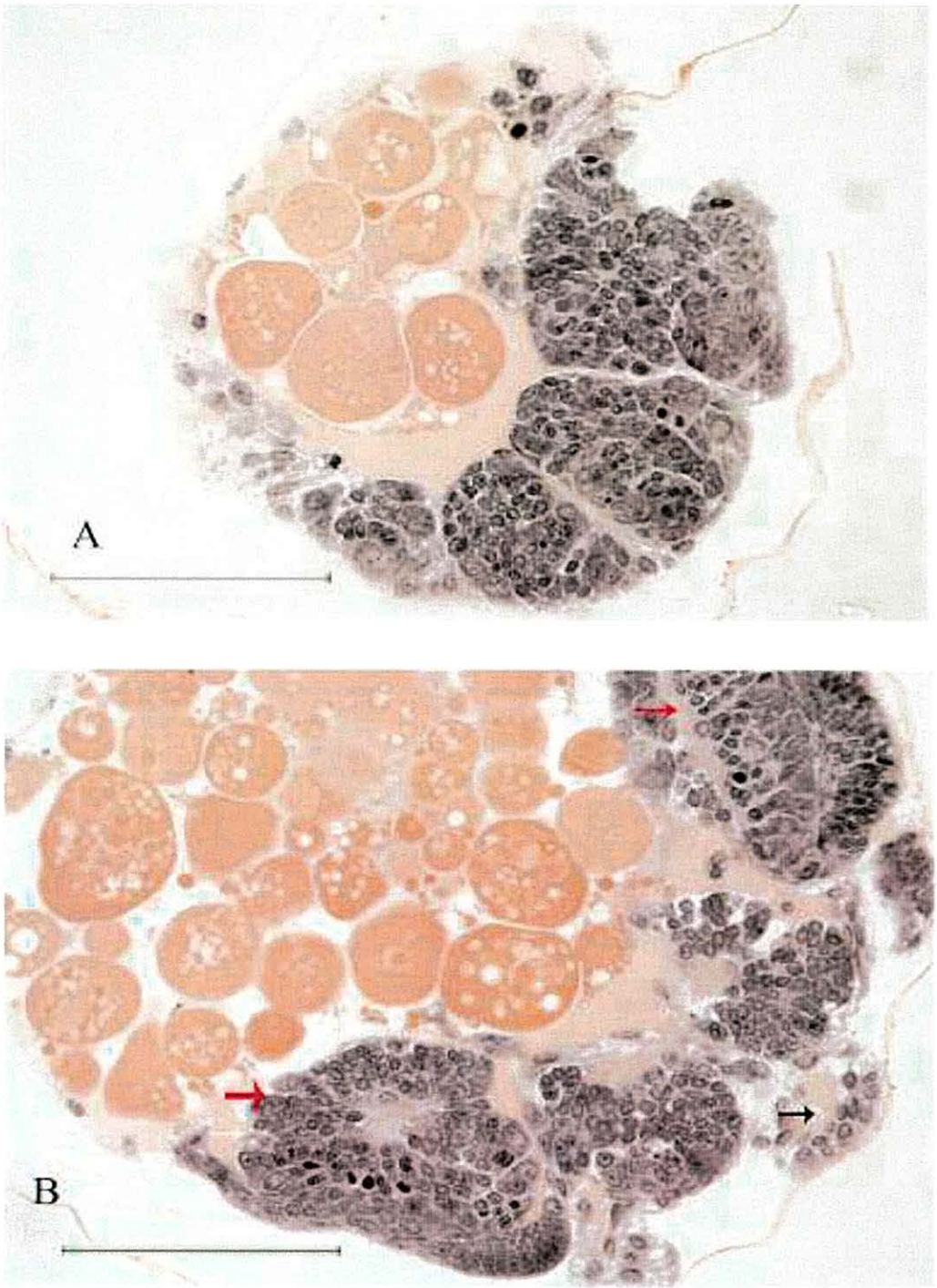
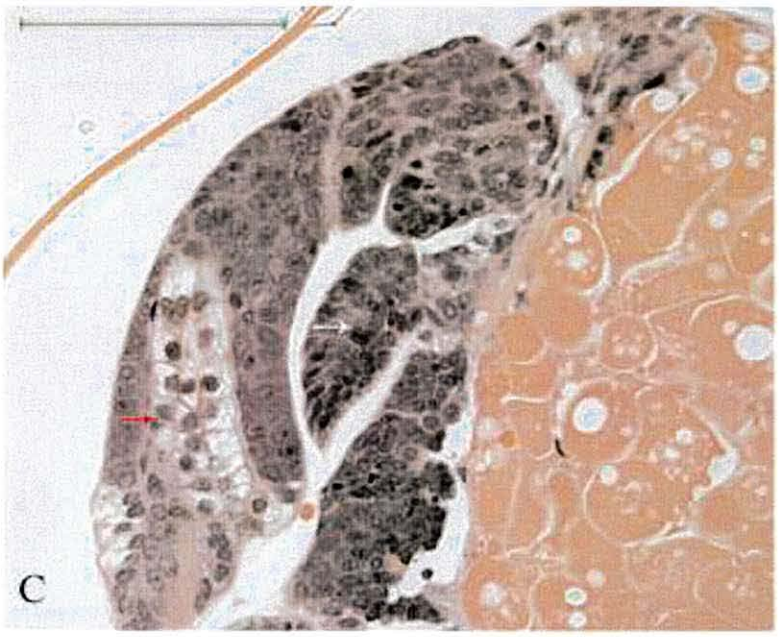


Figure 2.7. Eye phase in embryonic development of *Cancer pagurus*, sections stained with hemotoxylin and eosin; scale bars 100µm. (a) Oblique section shows large tissue formation in the whole embryo, and also the large increase in cell number. (b) Cross section with red arrows showing the large ocular lobes, the black arrow shows the telson.



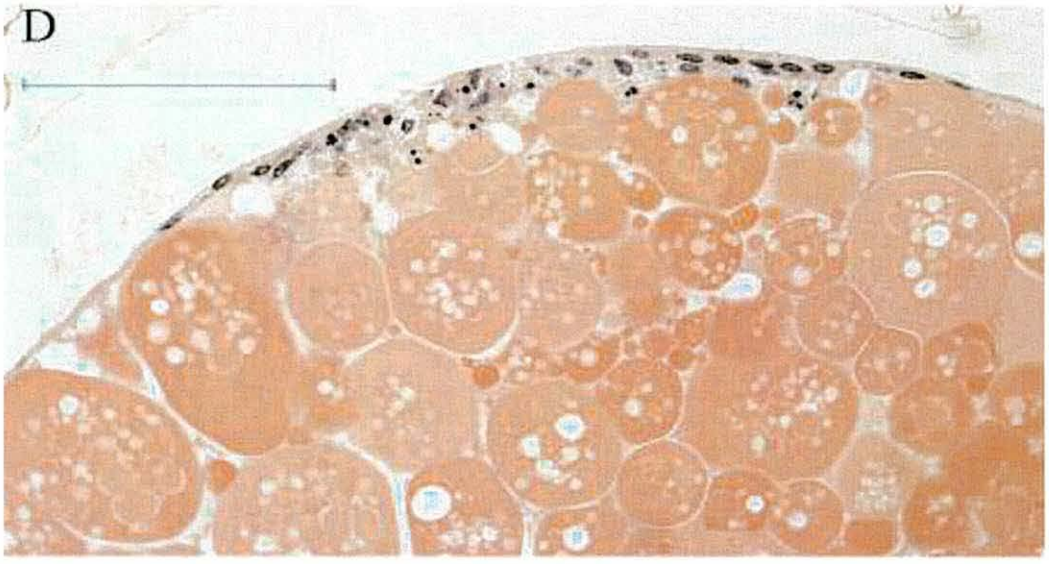


Figure 2.7 continued. (c) Longitudinal section with red arrow shows a 100µm developing cell column, which could be the early cells of the gut at the base of the abdomen. White arrow shows early formation of a maxilliped. (d) Longitudinal section with white arrow pointing to a developing cell layer which could be the heart.

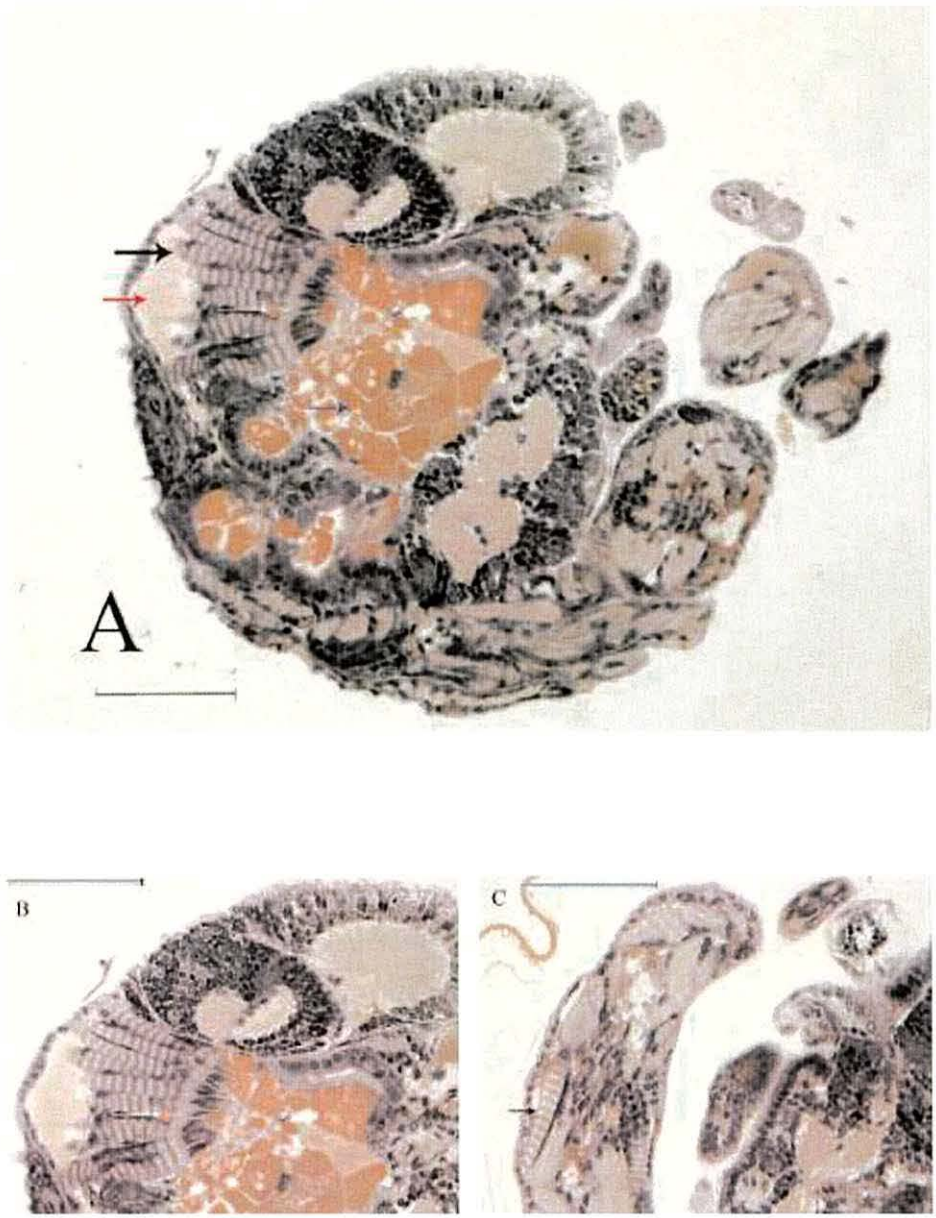


Figure 2.8. Pre-hatch 1 phase in embryonic development of *Cancer pagurus*, sections stained with hemotoxylin and eosin; scale bars 100µm. (a) Longitudinal section showing whole animal, note the extensive cell differentiation and huge increase in cell number. Black arrow shows striated muscle concentrated at the dorsal surface of the animal above the gut. Red arrow shows what could be the heart. Both features also shown in image B. Blue arrow shows the remaining yolk located centrally and predominantly in the gut. (C) Longitudinal section through the abdomen and maxillipeds. Black arrow showing striated muscle in the abdomen.

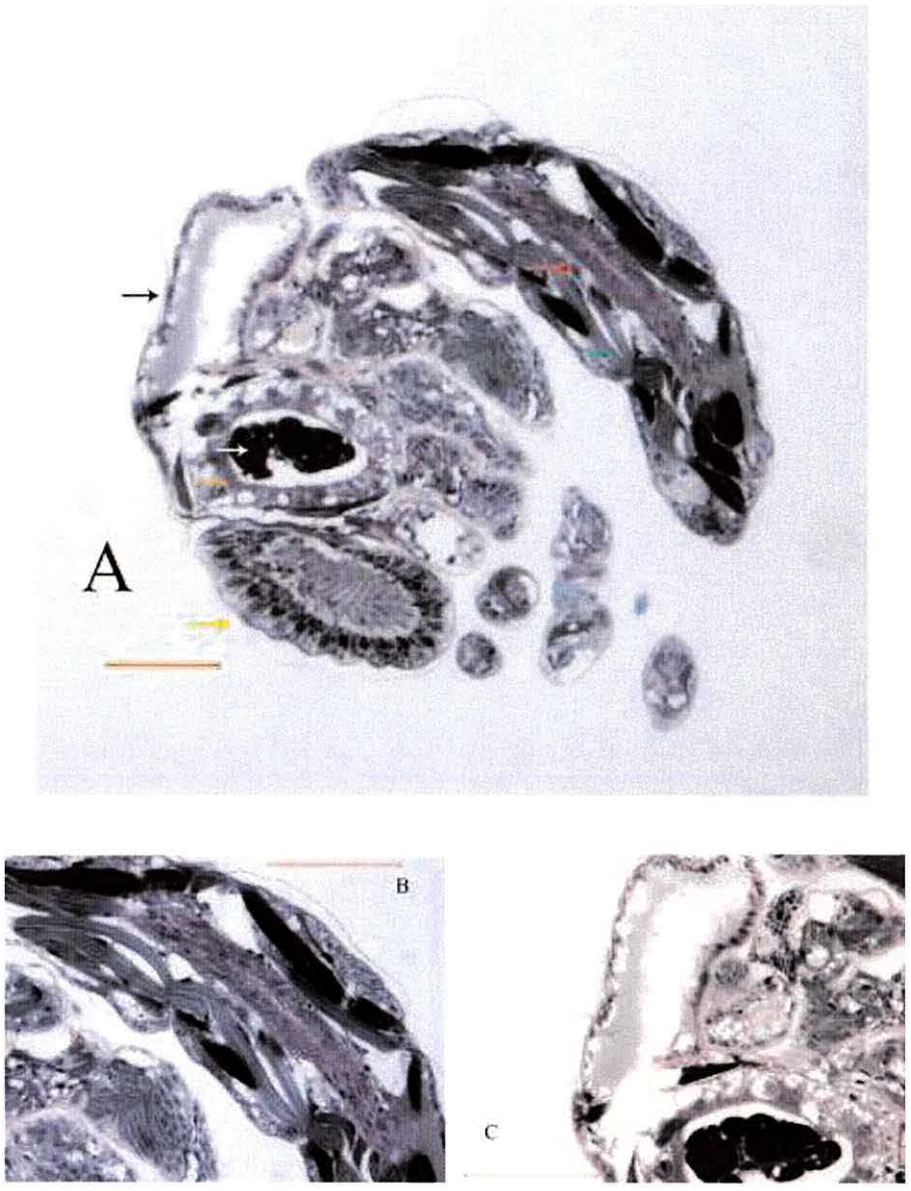
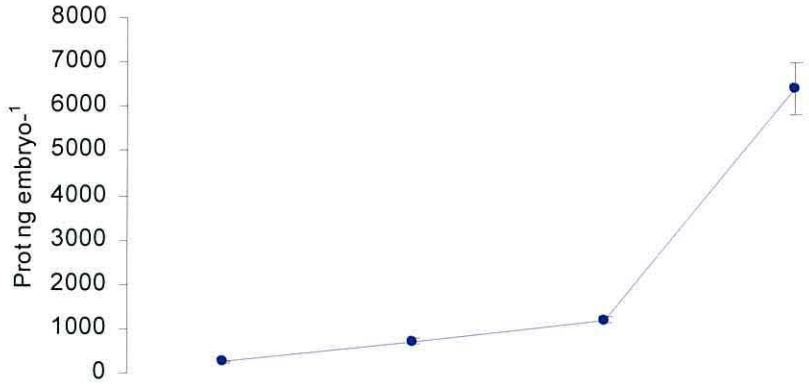
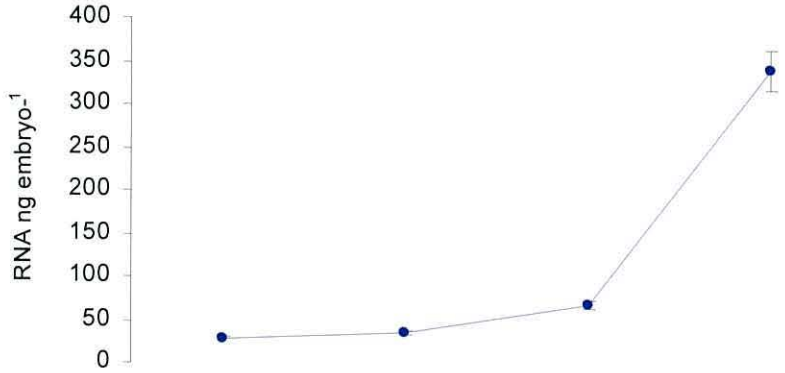


Figure 2.9. Hatching phase in embryonic development of *Cancer pagurus*, sections stained with Schiffs heidenhain hemotoxylin; scale bars 100µm. (a) Longitudinal section with yellow arrow showing the ocular lobe; orange arrow showing the gut; white arrow showing the remaining yolk encapsulated by the gut; black arrow showings the rostrum; green arrow showing the main muscle groups in the abdomen; red arrow showing the gut tissue in the centre of the abdomen. (b) Enlarged view of the abdomen. (c) Enlarged view of the rostrum and gut cells encapsulating the yolk.

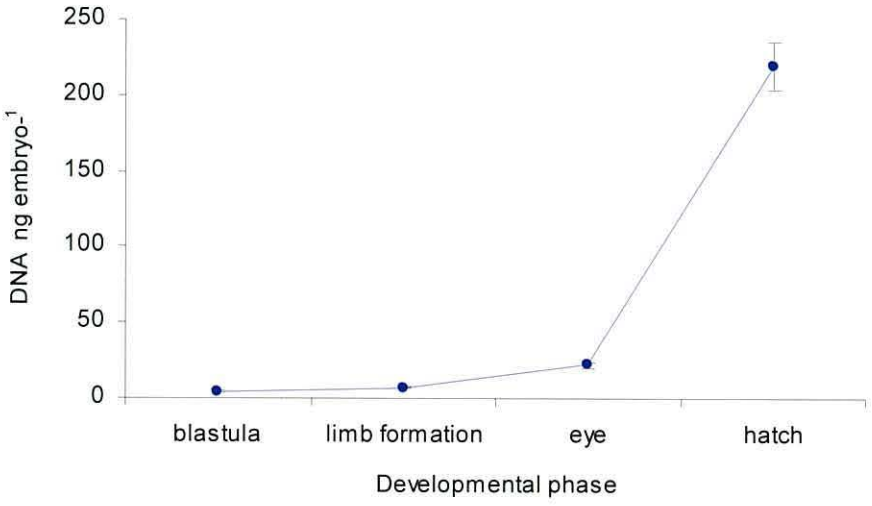
Figure 2.10. Developmental changes in total embryonic RNA, DNA, and protein at each of the four key phase of embryogenesis (Blastula, Limb formation, Eye, Hatching) in *Cancer pagurus*. Values are means ± SE (n = 16, except at the blastula where n= 10, and at the eye phase, n = 30).


8000
7000
6000
5000
4000
3000
2000
1000
0
Prot ng embryo-1

a

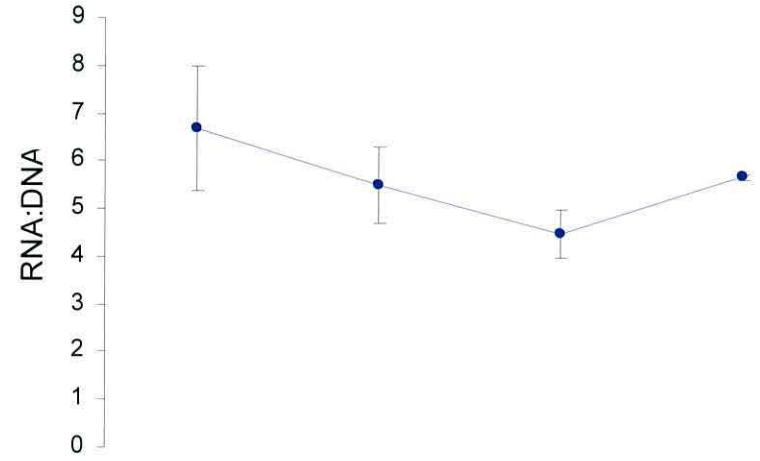

400
350
300
250
200
150
100
50
0
RNA ng embryo-1

b

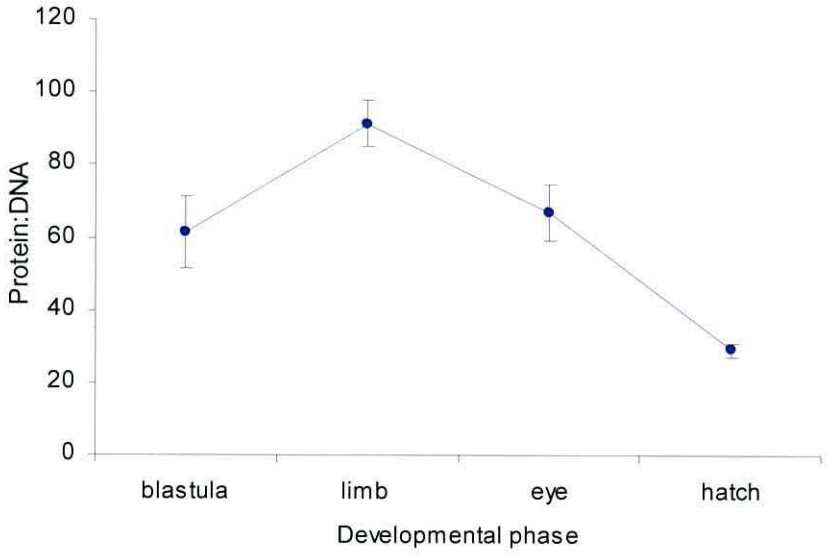

250
200
150
100
50
0
DNA ng embryo-1
blastula
limb formation
eye
hatch
Developmental phase

c

Figure 2.11. Developmental changes in RNA:DNA and protein:DNA ratios at each of the four key phase of embryogenesis (Blastula, Limb formation, Eye, Hatching) in *Cancer pagurus*. Values are means ± SE (n = 16, except at the blastula where n= 10, and at the eye phase, n = 30).


RNA:DNA
9
8
7
6
5
4
3
2
1
0

a


Protein:DNA
120
100
80
60
40
20
0
blastula
limb
eye
hatch
Developmental phase

b

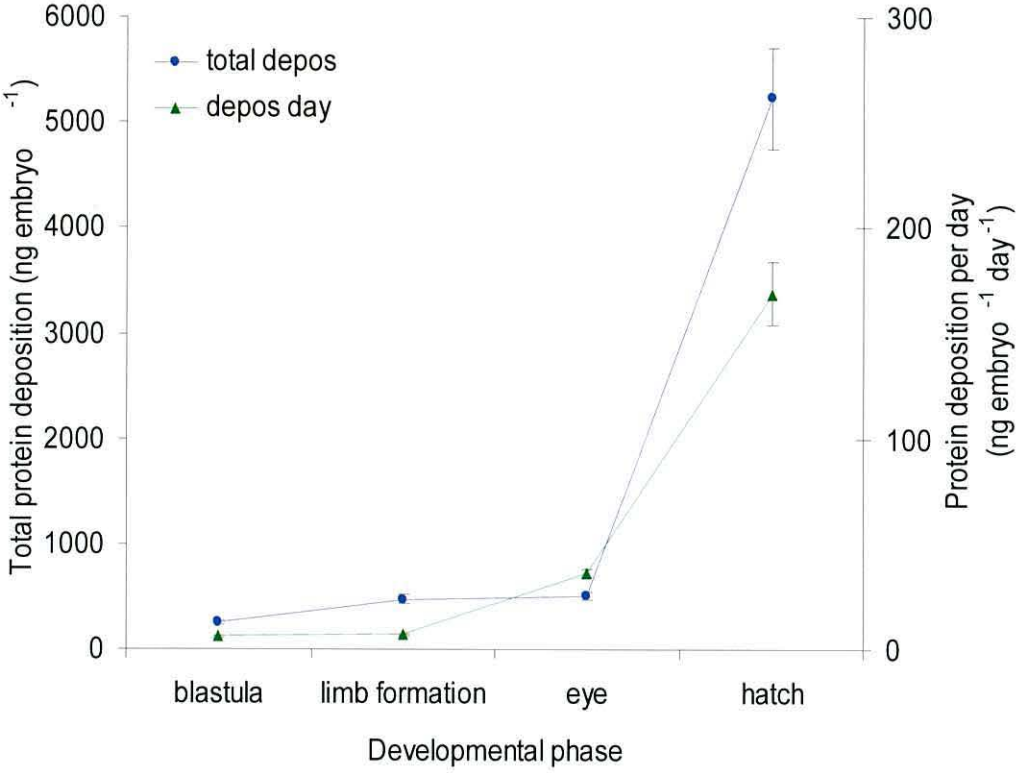


Figure 2.12. Changes in protein deposition per day, and total protein deposition at each of the four key phases in development (Blastula, Limb formation, Eye, Hatching) of *Cancer pagurus*, at 10°C. Values are means (n = 16, except at the blastula where n = 10, and at the eye phase, n = 30).

Chapter 3

Ontogeny of Protein Synthesis and Metabolic Rate in *Cancer pagurus*

Metabolic rate increased by 10-fold during embryonic development in *Cancer pagurus*, and was powerfully related to the increase recorded in embryo size. The greatest increase occurred between the eye and hatching phases, where whole embryo $\dot{M}O_2$ increased from 214 ± 17 pmol embryo^{-1} h^{-1} to 832 ± 56 pmol embryo^{-1} h^{-1}. This large change coincided with organogenesis, maximum levels of protein synthesis, and the major growth period of the embryo.

Rates of protein synthesis during embryonic development were investigated by using a flooding dose of tritiated phenylalanine, following successful validation of the technique with brachyuran embryos. Fractional rates of synthesis (k_s) increased significantly and by 8-fold between the Blastula and Limb formation phases, with mean values increasing from 1.45 ± .57 % day^{-1} to 11.3 ± 3 % day^{-1}. Despite this increase the rate of protein accumulation did not change, and therefore protein synthesis reflected protein turnover. Maximum k_s values were recorded at the eye phase, with mean values of 24.4 ± 5.1 % day^{-1}, the highest values reported in the literature for crustaceans at low temperatures. This coincided with organogenesis and subsequently a rapid and large accumulation of protein. By hatching phase, the mean k_s had fallen to 6.3 ± 2 % day^{-1}, reflecting the completion of embryonic growth and the formation of the zoea larva. Unlike changes in $\dot{M}O_2$ changes in k_s were not positively correlated with size, but instead were directly related to changes in RNA activity, which also exhibited a phase specific relationship during development.

The metabolic costs of protein synthesis were also calculated during development by correlating values of whole embryo $\dot{M}O_2$ and absolute values of protein synthesis, for which reasons are discussed for the poor correlation between the two variables.

3.1. Introduction

The true crabs (Brachyura) have made successful transitions from the marine environment into freshwater and terrestrial habitats, and as a result show a variety of different life history strategies (Wear, 1967; Bliss, 1968). Despite the range of environments inhabited by brachyurans, and the tolerance of some species to varying degrees of terrestrialness, they all produce lecithotrophic eggs, which are brooded on the abdomen, and often in a large egg mass (Hines, 1982; Hines, 1991). Modifications, however, occur in the size of the eggs, the brood size, and the duration of embryogenesis, as well as differences in fecundity (Wear, 1974; Hines, 1982; Hines, 1991; Shields et al, 1991; Taylor and Leelapiyanart, 2001). As a consequence, developmental times for brachyurans can be extremely variable with certain species hatching in little more than two weeks (Garcia-Guerrero and Hendrickx 2004), whilst embryogenesis in Lithodids, which represent an extreme example, can last up to two years (Lovrich and Vinuesa, 1993; Petersen and Anger, 1997). Generally species that produce smaller eggs have larger brood sizes, but produce embryos that grow and develop rapidly, relative to species with large eggs. For example, comparative data compiled using crabs from the family Cancridae show this relationship (Shields et al., 1991), as do many other, but not all, crustacean groups (Patel and Crisp, 1960; Wear, 1974; Hines, 1982; Lonsdale and Levinton, 1985).

Development times are also influenced by physical factors in the environment. For example, the effects of temperature on rates of development have been well characterised for a range of species (Wear, 1974; Vidal, 1980; Hoegh-Guldberg and Pearse, 1995; Moriyasu and Lanteigne, 1998). Several studies, however, have also shown that embryonic development in a number of crustacean groups can be strongly affected by endogenous factors, such as periods of diapause, which are indirectly linked with environmental conditions (Lovrich and Vinuesa, 1993; Helluy and Beltz, 1991; Hairston, 1996; Gyllstrom and Hansson, 2003; Hand, 1998). It is fairly common for brachyuran embryos to be arrested at a

given phase in development (Lovrich and Vinuesa, 1993; Petersen and Anger 1997; Moriyasu and Lanteigne, 1998; Taylor and Leelapiyanart, 2001). As a consequence, brachyuran species that exhibit periods of diapause can have relatively prolonged brooding or incubation periods, and show more pronounced phase specific changes in rates of growth and development (Lovrich and Vinuesa, 1993; Moriyasu and Lanteigne, 1998). The onset of diapause or quiescence during development can easily be observed through the absence of progressive morphological changes to the embryos, and by changes in metabolic rate (see below).

Recent studies have investigated changes in metabolic rates in brachyurans during embryonic development. As expected, the general trend is an increase in whole-embryo rates of oxygen uptake ($\dot{M}O_2$) with development, as shown in: the edible crab, *Cancer pagurus*; the spider crab, *Hyas araneus*; and two grapsid crabs, *Heterozious rotundifrons* and *Cyclograpsus lavauxi* (Petersen and Anger, 1997; Naylor et al., 1999; Taylor and Leelapiyanart, 2001). Increases in $\dot{M}O_2$ during development have been correlated with growth periods, since rapid periods of development are reflected by elevations in oxygen consumption.

Allometric relationships, such as mass specific changes to metabolic rate, using data compiled from many studies have also been shown (Taylor and Leelapiyanart, 2001). This relationship, however, is not clear during embryogenesis, because of difficulties associated with accurate estimates of tissue mass. A number of recent studies also show evidence of a reduction of metabolic rate at diapause phases in development, although rates of lipid metabolism in *H. araneus* were maintained during the diapause period (Petersen and Anger 1997; Taylor and Leelapiyanart, 2001). Similar depressions in metabolic rate during diapause phases of development have been extensively characterised in another crustacean, the brineshrimp, *Artemia franciscana*. During such periods both metabolic rate, and the rates of protein synthesis, have been found to undergo dramatic reductions (Hofmann and Hand, 1994; Clegg,

1997). A similar reduction in rates of protein synthesis following diapause has been found during embryogenesis of the killifish, *Austrofundulus limnaeus* (Podrabsky and Hand, 2000).

While previous studies have characterised changes in metabolic rate and changes in biochemical composition during development, fewer studies have investigated the corresponding changes in protein synthesis rates, which are closely related to growth (Rees et al 1995, Fujiwara and Yasumasu 1997, Pace and Manahan, 2006). Rates of protein synthesis in fish larvae can be rapid during the larval phases in development and is thought to be associated with a reduction in degradation rates, resulting in increased protein retention (Conceição et al., 1997; Carter et al., 1998). In addition, there is increasing evidence to show that protein synthesis rates are closely associated with rates of oxygen uptake (Houlihan et al., 1989; Pannevis and Houlihan, 1992; Houlihan et al., 1993; Whiteley et al., 1996), and yet no information is available to show if the variables are related during ontogeny, when high $\dot{M}O_2$ levels correlate to rapid periods of develop in brachyurans (Naylor et al., 1999; Taylor and Leelapiyanart, 2001). As protein synthesis rates are sensitive to declining oxygen levels, and as hypoxia is a common feature of brachyuran egg masses (see Chapter 4), it is interesting to establish whether $\dot{M}O_2$ and k_s are related during embryonic development, and if this relationship changes during the early periods of differentiation and the main periods of growth and organogenesis. Moreover, both of these metabolic variables are responsive to environmental variables and, therefore, could provide a subtle indication of some of the potential physiological effects of anthropogenic and or environmental change on embryo survival, development and growth (Considered in Chapter 4, Wheatly, 1981; Fauconneau and Arnal, 1985; Naylor et al., 1999; McCarthy et al., 1999; Whiteley et al., 2001; Baeza and Fernández, 2002; Mitchell and Seymour, 2003; Whiteley and Faulkner, 2005).

The reproductive characteristics of *C. pagurus* make it the species of choice for studies of this kind, since the embryos are spawned in early winter followed by

an over-wintering period when the ovigerous females brood their eggs whilst buried in the substratum. Development continues through to early summer when the eggs hatch as pelagic zoea larvae. The four key phases in development, identified in Chapter 2, will be used for these experiments. The four phases encompass both the relative quiescent phases and the active periods of embryogenesis. Measurements include both oxygen uptake rates and fractional rates of protein synthesis in embryos removed from the female crabs and exposed to normoxia. The purpose will be to establish developmental changes in both of these variables and their relationship to prolonged periods of incubation. Chapter 4 will concentrate on the specific effects of hypoxia on embryonic rates of metabolism and protein synthesis, to establish the role of molecular oxygen in the control of embryogenesis in *C. pagurus*.

3.2. Methods

3.2.1. Animal Collection and Egg Sampling

Female *C. pagurus* were collected and maintained in aquaria at the University of Wales, Bangor as outlined in Chapter 2. Embryos were sampled as described previously. Rates of oxygen uptake and protein synthesis were studied at 4 key phases of embryonic development in *C. pagurus:* blastula; limb formation; eye phase; and hatching phase. All 4 phases are morphologically distinct and were identified by light microscopy under low magnification by the series of characteristics described in Chapter 2.

3.2.2. Rates of Oxygen Uptake

$\dot{M}O_2$ were determined in a micro-cell repirometer maintained at 10˚C (RC-300 Respirometer, Strathkelvin Instruments). Changes in oxygen partial pressure (PO_2) were measured using a polarographic oxygen electrode connected to an oxygen meter (1302 electrode, and Oxygen Meter 781, Strathkelvin Instruments). For each set of readings, less than 40 mg of embryos were placed into the respirometer cell with 1ml of sterile, aerated seawater. After sealing the respirometer with the oxygen electrode, the sea water was mixed with a magnetic stirrer to prevent oxygen stratification, and PO_2 levels were recorded continuously for five minutes. At the end of the measurement period the seawater was replaced with a fresh sample of aerated, sterile seawater, and the process repeated a further three times on the same sample of embryos. Measurements were subsequently taken on two further batches of embryos from the same mother. As a consequence, PO_2 readings for a single $\dot{M}O_2$ estimate were taken in triplicate from three batches of embryos. PO_2 readings were also taken from the respirometer without the embryos prior to each set of measurements to act as controls. At the end of each run it was not possible to check the viability of the embryos, except at the hatching phase, where the presence of a beating heart was used.

3.2.3. Rates of Protein Synthesis

1. Validation of the flooding dose methodology

Whole-embryo fractional rates of protein synthesis were determined by adding a flooding dose of labelled phenylalanine to the incubation seawater surrounding the embryos in vivo. A similar approach has been used to successfully determine protein synthesis rates in larval fish and larval sea urchins, as well as a hepatic cell line (Land et al., 1993; Smith and Houlihan, 1995; Conceição et al., 1997; Pace and Manahan, 2006).

As this was the first time that the flooding dose technique had been used to detect protein synthesis rates in crab embryos, it was first necessary to validate the technique, and establish whether the labelled amino acid was able to pass through the various egg membranes and flood the amino acid free pools. It was also necessary to establish whether the labelled amino acid was being incorporated into new embryonic protein. Subsequently a preliminary time course experiment was carried out on the eye and chromatophore phase embryos of another marine brachyuran, *Necora puber*. The ready availability of *N. puber* made it an ideal substitute for *C. pagurus*, whose availability was limited. The permeability of its egg membranes and the amino acid uptake rates were taken to be similar to those of *C. pagurus* embryos, working on the assumption that egg permeability is affected by habitat type, with marine eggs more permeable than freshwater and terrestrial eggs (Epel, 1967; Shilling and Manahan, 1990; Loeffler and Lovtrup, 1970; Susanto and Charmantier, 2001; Woods and Hill, 2004). Rates of protein synthesis were estimated in approximately 200mg of embryos after 30 and 60 min incubation in UV treated seawater containing 150 mmol l^{-1} phenylalanine (Phe) and L-[2-6 ^{3}H] Phe (Amersham, specific activity 122 Ci $mmol^{-1}$) at 50 μCi ml^{-1}. The theoretical specific activity of the labelled incubation media was 740 $dpm.nmol^{-1}$ phe. The incubated embryos were held at 10°C in a temperature controlled water jacket, and the incubation medium was continuously, but gently aerated and stirred.

Using the incubation conditions and the optimal time interval determined above, approximately 200mg of *C. pagurus* embryos were used to determine protein

synthesis rates at each of the 4 key phases of development. After the period of incubation, the embryos were rapidly washed four times in 2ml of UV filtered seawater to remove any residual incubation medium, drained and then frozen in liquid nitrogen for storage at -80°C.

2. Analysis of samples

The specific activity and concentration of Phe in the free pool and protein bound fractions were determined by the method of Garlick et al (1980) using the modifications described in Whiteley et al (1996). In addition, further modifications were developed in the present study to enhance the enzymatic conversion of Phe to β-phenylethalamine (β-PEA) with use of tyrosine decarboxylase (see below). In summary, each sample of eggs were ground to a fine powder in liquid nitrogen, and the proteins and nucleic acids precipitated in 2% ice cold perchloric acid (PCA). The resulting homogenates were centrifuged at 3500 rpm at 4°C for 15 minutes. The supernatants, representing the intracellular free pools, were decanted and stored at -20°C for subsequent enzymatic conversion of Phe to β-PEA. Following 2 further washes in cold 2% PCA, the pellet was digested in 0.3N NaOH at 37°C for 1 hour. Two subsamples of 20 µl were removed from the alkali digestion for total protein (embryo and yolk) determination (see Chapter 2). Protein and DNA were precipitated by the addition of ice cold 12% PCA, and acid soluble RNA quantified as described in Chapter 2.

The remaining protein pellet was hydrolysed in 6N HCl in sealed screw cap tubes for 18-24h at 110°C. After hydrolysis, the lids were removed from each sample tube and the acid levels left to evaporate at 80°C. Following evaporation, tissue amino acid samples were resuspended in 1.5 mol l^{-1} citrate buffer (pH 6.3), instead of 0.5 mol l^{-1} (Garlick, 1980), as recommended by Victor Preedy (personal communication). Flourometric determination of β-PEA present in both free pool and protein-bound fractions were established following enzymatic conversion of Phe by tyrosine decarboxylase. Tyrosine decarboxylase with co-factor pyrodoxal phosphate suspended in 1.5 mol l^{-1} citrate buffer, were added to

the protein hydrolysates at 1.4 units ml^{-1} and 0.5 mg ml^{-1}, respectively, and to the free pool supernatants at 0.7 units ml^{-1} and 0.5 mg ml^{-1}, respectively. Samples were sonicated (Dawe Sonicleaner) 4 times at 5 seconds each (the development of this crucial step is described below). Samples were subsequently incubated overnight at 52°C. The conversion efficiency of tyrosine decarboxylase was estimated by simultaneously converting Phe standards of known concentrations (0-150 nmol ml^{-1}). Following solvent extraction (see below), β-PEA recovery was measured fluorometrically (Wallac VICTOR^2 1420 Multilabel Counter) against known standards at an exitation of 355 nm and an emission of 460 nm (Suzuki & Yagi, 1976). The specific activities of the free pool and protein-bound fractions were determined by scintillation counting in an Optiphase 'Hisafe' liquid scintillant using a Wallac WinSpectral 1414 scintillation counter. ^3H counting efficiency was 37%. The specific activity of Phe in each sample was expressed as $dpm.nmol^{-1}$.

3.2.4. Development of Sonocatalysis for Phe Conversion

Initially samples were converted to β-PEA using the enzyme phenylalanine decarboxylase as described by Whiteley et al (1996). Conversion efficiency of Phe to β-PEA with this enzyme ranged between 40-70% (S. J. McCleary, unpublished observations). However, part way through the present project phenylalanine decarboxylase became unavailable from commercial sources. An alternative enzyme, tyrosine decarboxylase, the original enzyme used by Garlick et al (1980), was subsequently used to complete the analysis of the remaining samples, which included some second year and all third year samples. Enzymatic conversion efficiency of Phe to β-PEA using tyrosine decarboxylase was highly variable, because conversion to β-PEA occurred due to the impurities of phenylalanine decarboxylase present in the batches of tyrosine decarboxylase. As a result the mean conversion efficiency was very low between just 1-3%, although very occasionally conversion was much higher and similar to Phe decarboxylase. The various attempts to improve conversion efficiencies by optimising enzyme and substrate concentrations were met with limited success. Modifications to the protocol were also directed at β-PEA recovery following

Phe conversion. This was achieved by extending the solvent extraction times from 10 sec to 300 seconds per sample (Prof V. Preedy, personal communication). Although the modifications to the extraction protocol improved β-PEA recovery, further optimization was needed and this lead to the development of a sonication step.

3. Ultrasonication

Sonication has been used in industry for the best part of a century. Alfred Loomis first discovered the properties of ultrasound when transmitted through a liquid in 1927, following earlier military experiments into marine propulsion systems at the close of the 19th century. Sonication combines the properties of high power and high frequency sound waves in a liquid. It is characterised by a phenomena known as cavitation. Cavitations are created by passing soundwaves with high energy (15 KHZ-1 GHZ) and high acceleration (80,000g) through a liquid, to form tiny bubbles, merely microns in diameter that rapidly grow and collapse. These bubbles are transient, lasting microseconds, before violently collapsing. The nature of this reaction transforms acoustical energy into kinetic energy, as bubbles collapse and cause compression of liquid molecules. This transformation creates intense localised heat and pressure, known as 'hotspots', which have temperatures in excess of 5000ºC and pressures in excess of 1000 atmospheres (Suslick, 1989). The cyclical property of repeated expansion and compression creates mass mixing, improved energy transfer, and extreme heat in liquids bathed with ultrasound. Such properties make sonication an extremely useful tool for both industrial, and biochemical applications. Subsequently, there are only a small number of references that currently report the use of sonication for the enhancement of biological reactions, such as those that have applied it to remediation studies (references within, Suslick 1998).

Preliminary experiments on the application of sonication during enzymatic conversion of Phe to β-PEA, by the impurities associated with tyrosine decarboxylase resulted in an appreciable increase in Phe conversion efficiency, resulting in values very similar to the conversion achieved with Phe

decarboxylase. The incorporation of this technique was, therefore, a crucial modification to the success of the flooding dose protocol.

3.2.5. Calculations

Rates of oxygen uptake were calculated from the decline in PO_2 over a known period of time, the solubility coefficient of O_2 in seawater at 10°C (Harvey, 1955), and the volume of water in the respirometer. All values were corrected to STPD and expressed as pmol O_2. embryo^{-1}. h^{-1}.

Fractional rates of protein synthesis (k_s, % protein synthesised day^{-1}) were calculated according to Garlick et al (1980):

k_s = Sb/Sa x 24/t x 100

where Sb is the specific activity of protein bound Phe (dpm.nmol^{-1}), Sa is the specific activity of free pool Phe (dpm.nmol^{-1}), and t is the incubation time in hours (h).

For the calculation of absolute rates of protein synthesis (A_s), it was necessary to account for the proportion of total protein present in the embryo by excluding the proportion present in the yolk (discussed in Chapter 2). Briefly, the yolk fraction, expressed as a percentage of the total area (μm^2) of the egg (2D), was estimated at each of the four key phases of development. Pre-recorded images were used for area measurements using Uthscsa imaging software version 3 (see Chapter 2). Total protein levels in the embryo and the yolk were subsequently calculated from their individual area percentages of the whole egg, and then transformed into their respective proportions of the total protein value to obtain a mean value for embryonic protein at each key phase in development. These calculations were based on the assumption that the total protein was present in equal proportions in the yolk and the embryo throughout the developmental period. Estimated values

for embryo protein were then used to calculate absolute rates of protein synthesis, and translational efficiency of the RNA, or the RNA activity (k_{RNA}) according to the equations:

A_s (ng protein embryo^{-1} day^{-1}) = k_s /100 x embryonic protein (ng protein embryo^{-1})

where k_s is the fractional rate of protein synthesis.

RNA activity (k_{RNA}) Preedy et al. (1988):

k_{RNA} (µg protein. µg RNA^{-1}. day^{-1}) = 10 x k_s /RNA:protein

Protein synthetic capacity (RNA:protein ratio) is the capacity for protein synthesis expressed in µg RNA.mg embryonic protein^{-1}.

3.2.6. Statistical Analysis

All values are given as means ± SE. Prior to statistical analysis all data were tested for normality of data distribution using the Kolmogorov-Smirnov test and homogeneity of variances using the Levene test. Where appropriate data was log transformed, and subsequently the effect of development on the dependent variables was tested by one-way ANOVA followed, when significant, by the LSD *post-hoc* test. Non-parametric data was analysed by a Kruskal-Wallis ANOVA. Significant differences were analysed by a Mann-Whitney test. The results were also subject to regression analysis completed with ANOVA. Statistical analyses were performed using SPSS software (SPSS version 12; SPSS Inc., Chicago, Il, USA). The level of significance used in all tests was $P < 0.05$.

3.3. Results

3.3.1. Rates of Oxygen Consumption

The Whole embryo values of oxygen uptake rates are shown in Figures 3.1 a+b. Rates of whole-embryo oxygen uptake ($\dot{M}O_2$) increased significantly during development (one-way ANOVA, $P < 0.001$). At the blastula phase, $\dot{M}O_2$ was significantly lower than at all other phases of development, with mean values of 85 ± 10 pmol embryo^{-1} h^{-1} (one-way ANOVA, $P < 0.001$). $\dot{M}O_2$ levels were approximately 2-fold greater by the limb formation phase with mean values of 182 ± 22 pmol embryo^{-1} h^{-1}, which remained unchanged at the eye phase at 214 ± 17 pmol embryo^{-1} h^{-1}. At the hatching phase $\dot{M}O_2$ had increased by approximately 10 fold, with respect to the values recorded at the blastula, to reach values of 832 ± 56 pmol embryo^{-1} h^{-1}. This mean value was significantly greater than the mean obtained at all of the previous phases of development (one-way ANOVA, $P < 0.001$). Mass specific rates of $\dot{M}O_2$ showed a similar relationship to whole-embryo $\dot{M}O_2$ values during development (Table 3.1b). Values were lowest at the blastula phase (2.93 ± .28 pmol O_2. mg^{-1}. h^{-1}), and showed almost a 5-fold increase over the developmental period, achieving maximum values at the hatching at 13.86 ± .94 pmol O_2. mg^{-1}. h^{-1}.

*Note all statistical analysis performed on log transformed values.

Figure 3.2 shows the direct relationship between mean whole embryo $\dot{M}O_2$ and mean whole embryo DNA content during development. The relationship between the two variables was highly significant (ANOVA, $P = 0.006$).

3.3.2. Validation of the Flooding Dose Methodology

Experimental Time-Course

The mean specific activities of the amino acid free pools and the protein-bound fraction in embryonic *N. puber*, is given along with the resulting k_s values in Figure 3.3. Mean specific activities in the free pools of the eye and chromatophore phase embryos were 577 ± 151 dpm.nmol^{-1} at 30 minutes, and 561 ± 179 dpm.nmol^{-1}after 60 minutes incubation. Although these values were slightly lower than the theoretical specific activity of the bathing media, there was no significant difference between specific activities after 30 and 60 minutes labelling, suggesting that incorporation was stable (one-way ANOVA, P = 0.945). Protein-bound specific activities continued to increase from 0.91 ± 0.08 dpm.nmol^{-1} at 30 minutes to 1.71 ± .28 dpm.nmol^{-1} at 60 minutes, which represented a significant change (one-way ANOVA, $P < 0.024$). The resulting k_s values were similar at 30 and 60 min incubation, giving mean values of of 12.23 ± 4.9 % day^{-1} and 11.62 ± 4.7 % day^{-1}, respectively (one-way ANOVA, P = 0.930). An incubation time of 60 minutes was subsequently selected as the optimal time period for subsequent estimations of protein synthesis rates in crab embryos using the flooding dose technique.

Specific activities in the free pools of embryonic *C. pagurus* at the 4 key phases in development are shown in Figures 3.4. Values represent those taken after labeling for 60 minutes at 10°C. Mean specific activities of the free pools were elevated at all 4 phases. Specific activities were 1431 ± 381 dpm.nmol^{-1} at the blastula and 1176 ± 188 dpm.nmol^{-1} at the limb formation phase. Specific activities declined at both the eye and hatching phases to give means of 603 ± 61 and 790 ± 83 dpm.nmol^{-1}, respectively. Means values were only significantly lower at the eye phase, when compared with the blastula and also when compared to the limb formation phase (Mann Whitney, P = 0.024 and P = 0.015, respectively).

Specific activities of the protein bound fraction after 60 minutes labeling at 10°C, and for each of the 4 key phases in development in *C. pagurus* are shown in Figure 3.4. Incorporation rates were at their lowest at the blastula phase with mean values of 0.70 ± 0.16 dpm.nmol^{-1}. A highly significant increase in protein-bound specific activity occurred at the limb formation phase giving mean values of 4.84 ± 0.64 dpm.nmol^{-1} (one-way ANOVA, $P = 0.008$), which was similar to the value recorded at the eye phase (5.72 ± 0.80 dpm.nmol^{-1}). Protein-bound specific activity decreased significantly between the eye and hatching phase, as mean values returned to values similar to those recorded at the blastula, at 2.14 ± 0.92 dpm.nmol^{-1} (one-way ANOVA, $P = 0.006$).

3.3.3. Rates of Protein Synthesis and Associated Variables

Developmental changes in fractional rates of protein synthesis (k_s) at 10°C are shown in Figure 3.5. Development had a highly significant effect on k_s in embryonic *C. pagurus* (one-way ANOVA, $P < 0.001$). Fractional rates of synthesis increased significantly ($P < 0.001$) during early phases of development, with mean values increasing almost 8-fold, from the mean value recorded at the blastula of 1.45 ± .57 % day^{-1}, to 11.3 ± 3 % day^{-1} at the limb formation phase. The maximum k_s values recorded during development occurred at the eye phase, where mean values reached 24.4 ± 5.1 % day^{-1}, representing a 17-fold increase from blastula values. By hatching phase, the mean k_s had fallen to 6.3 ± 2 % day^{-1}, which was four times lower than the values measured at the eye phase, but similar to the mean value recorded at the limb formation phase ($P = 0.075$).

*Note all statistical analysis performed on log transformed values.

Absolute rates of protein synthesis (A_s) during development are shown in Figure 3.7. A_s showed a large increase during development reaching maximum values at the eye phase and remaining unchanged through to hatching phase, where values were 306 ± 69 ng protein embryo^{-1} day^{-1}. This marked a highly significant (one-

way ANOVA, $P < 0.001$) 78-fold increase from the mean values recorded at the blastula phase (3.8 ± 1.48 ng protein embryo^{-1} day^{-1}).

*Note all statistical analysis performed on log transformed values.

The relationship between mean whole embryo A_s levels as a function of mean whole embryo DNA levels (standardised) during the developmental time frame is shown in Figure 3.8. Changes in standardised A_s showed a similar trend to the changes in k_s during development, with values undergoing a relatively large increase between the blastula and eye phase, followed by a decline of a similar order at the hatching phase.

Figure 3.5 shows the developmental changes in RNA activity (k_{RNA}), which represents protein synthesis rate per unit of RNA, and RNA:protein ratio, which gives an indication of the capacity for protein synthesis by estimating tissue ribosomal content (Preedy et al., 1988). Development at 10°C had a significant effect on k_{RNA} in *C. pagurus* embryos, but had no effect on the RNA:protein ratios. k_{RNA} increased by approximately 40% between limb formation and the eye phase, with mean values increasing from 2.90 ± 0.72 µg protein.µg RNA^{-1}.day^{-1} to 4.43 ± 1 µg protein.µg RNA^{-1}.day^{-1}, respectively. Although this increase was not significant (one-way ANOVA, $P = 0.241$), the mean value for k_{RNA} at the eye phase was the highest achieved during the developmental period. At the hatching phase, log k_{RNA} showed a significant decrease (one-way ANOVA, $P = 0.002$) marking a 75% reduction from the raw values recorded at the eye phase (1.14 ± . 36 µg protein.µg RNA^{-1}.day^{-1}). The relationship between k_{RNA} and k_s during development is shown in Figure 3.6. The two variables showed a marked and significant linear relationship during development (one-way ANOVA $P = 0.03$). In contrast, RNA:protein ratios remained relatively constant during development, being 45.6 ± 5.3 at limb formation, 63.2 ± 8.7 µg RNA.mg protein^{-1} at the eye phase and 51.5 ± 2.4 ug RNA.mg protein^{-1} at the hatching phase (Kruskal-Wallis, $P = 0.364$).

3.3.4. Metabolic Costs of Protein Synthesis

The relationship between whole embryo rates of metabolism (pmol embryo^{-1} h^{-1}) and absolute rates of protein synthesis (A_s, ng protein embryo^{-1} hr^{-1}) is shown in Figure 3.9. This Figure can be used to estimate the energetic costs associated with protein synthesis during development of *C. pagurus*, based on the relationship between $\dot{M}O_2$ and A_s using the data collected from the separate experiments. Figure 3.9 shows that the mean whole embryo rates of $\dot{M}O_2$ and mean A_s are not closely related in *C. pagurus*. Consequently, the estimation of a mean metabolic cost for protein synthesis in embryonic *C. pagurus* using this relationship is likely to be unrealistic. Instead, the relationship between mean $\dot{M}O_2$ and A_s values at each phase was examined by converting $\dot{M}O_2$ values per embryo to mmol ATP.g protein^{-1} synthesised, using the assumption that 1 mmol O_2 was equivalent to 6 mmol ATP (Reeds et al., 1985).

Using this approach the theoretical cost of protein synthesis was shown to be significantly different during development (KRUSKAL-WALLIS, $P \leq 0.01$). Mean costs were at their highest at the limb formation and hatching phases, with mean values of 383 ± 83 and 469 ± 73 mmol ATP. g protein^{-1} synthesised, respectively. The eye phase was characterised by the lowest metabolic cost with mean values of 131 ± 22 mmol ATP g protein^{-1} synthesised, despite there being no change in A_s with respect to the hatching phase. These values, however, should be treated with caution as there was little correlation between $\dot{M}O_2$ and A_s, and resting rates of $\dot{M}O_2$ were difficult to record in hatching phase embryos.

In addition to the metabolic costing analysis examined for protein synthesis during development, background energetic cost, analysed as the $\dot{M}O_2$ determined at zero protein synthesis was also tested. This analysis demonstrates differences in the background energetics during embryonic development, in terms of the present study, as changes occur in embryo size, complexity and activity during development. Background energetic cost in *C. pagurus* embryos

remained stable at early phases until the hatching phase, where they were significantly higher (ANCOVA, $P \leq 0.001$).

3.4. Discussion

Typically, egg incubation times in *C. pagurus* are relatively prolonged when compared with other temperate brachyurans inhabiting British coastal regions, such as *Carcinus maenas* and *N. puber* (S. J. McCleary, Unpublished observations; Wear, 1974). In the wild, ovigerous female *C. pagurus* are known to spend an over-wintering period buried beneath the substratum (Williamson, 1900; Naylor et al., 1999). During this time period the embryos remain at the blastula phase in development. Recent data suggests that a period of diapause or quiescence during early phases in development is important for prolonging the incubation period in this species (Wear, 1974; Naylor et al., 1999). Data from the present study supports this view due to the combination of low metabolic rates, low synthetic activities, and low rates of protein synthesis, both fractional and absolute in the normoxic blastula embryos of *C. pagurus.* There are a number of other brachyurans and decapods that are known to exhibit resting phases during embryogenesis including: a New Zealand grapsid crab, *H. rotundifrons*; the snow crab, *Chionoecetes opilio*; the spider crab, *Hyas araneus*; the American lobster, *Homarus americanus*; and the southern king crab, *Paralomis granulosa* (Helluy and Beltz, 1991; Lovrich and Vinuesa, 1993; Petersen and Anger, 1997; Moriyasu and Lanteigne, 1998; Taylor and Leelapiyanart 2001; Sibert et al., 2004). In each of these species, the incubation period is relatively prolonged i.e. > 6 months, and is associated in some species with a reduction in metabolic rate during embryogenesis (Petersen and Anger, 1997; Taylor and Leelapiyanart 2001).

Commonly, diapause periods during development occurs at the formation of the blastodisc following early cleavage, also with a second resting phase reported at the eye phase in the boreal spider crabs. Resting phases during embryogenesis

and the resulting increases in incubation time are thought to occur in order to synchronise larval phases with seasonal changes in biotic and abiotic factors (Wear, 1974; Shields et al, 1991; Petersen and Anger, 1997; Moriyasu and Lanteigne, 1998).

3.4.1. Rates of Oxygen Uptake during Development

Whole-embryo $\dot{M}O_2$ levels at the blastula were the lowest recorded during development. A similar response has previously been observed in *C. pagurus* and in other brachyuran species (Naylor et al., 1999; Taylor and Leelapiyanart, 2001; Baeza and Fernández, 2002). Similarly, the mean value for mass specific $\dot{M}O_2$ was lower than at all other embryonic phases, however, it was considerably higher when compared with two species of grapsid crab, but remarkably similar to diapause phases in *H. araneus* (Petersen and Anger, 1997). It is important, however, to consider that the relatively low metabolic rate reported here, may not only be a reflection of a resting phase in blastula embryos. Alternatively, it is possible that the relationship between size and metabolic rate is responsible for the low metabolic rates measured at the blastula phase. This explanation was tested by examining the relationship between whole-embryo $\dot{M}O_2$ and total DNA content as a measure of cellularity or embryo size (see below).

As embryonic development in *C. pagurus* progressed beyond the blastula phase, the embryos increased in size as shown by the considerable increase in the proportion of embryonic tissue, wet weight, and the DNA content of the embryos (see Chapter 2). Whole-embryo $\dot{M}O_2$ values during the developmental period increased 10-fold, with the greatest increase occurring between the eye and hatching phases, which coincided with organogenesis, the major growth period described in Chapter 2, and also with increased embryo motility e.g. tail flicking. As both embryo size and $\dot{M}O_2$ increased during embryonic development, the scaling of $\dot{M}O_2$ was investigated by plotting $\dot{M}O_2$ against total DNA content, because the latter value is least likely to be affected by the yolk. Figure 3.2 shows that there was a close relationship between whole-embryo $\dot{M}O_2$ and DNA

content, suggesting that the changes in $\dot{M}O_2$ during development were caused by an increase in embryo size. A similar response was observed in previous studies using *C. pagurus* embryos, and in the closely related species, *Cancer setosus*, although the total increase in $\dot{M}O_2$ was less than the increase measured during embryonic development of two grapsid crabs (Naylor et al., 1999; Taylor and Leelapiyanart, 2001; Baeza and Fernández, 2002).

It is important, however, to emphasise that direct comparisons of $\dot{M}O_2$ data between studies are difficult due to differences in the measurement techniques and the experimental regime used. Also, complications arise in the accurate determination of embryonic phases, which can make phase specific comparisons in $\dot{M}O_2$ levels unreliable. The latter is related to the relatively small size of brachyuran embryos, which make them difficult to handle and, in particular, prepare for general histology and examination using light microscopy. When histological examination is possible, and the vitelline membranes have been removed, morphological features can be observed that remain hidden when the embryos are examined whole (see Chapter 2, Figure 2.2). This can lead to difficulties in accurately determining developmental phase. For example, the eye phase embryos described in this study following the removal of the vitelline membranes, appear to be at the same phase in development as gastrulation phase grapsid embryos, described and classified from external appearances using light microscopy by Taylor and Leelapiyanart (2001). Therefore, the embryos appear to be at gastrulation but are in fact at a more advanced phase in development, having differentiated cephalic and thoracic appendages being at the eye formation phase. Dissection of both gastrulation and eye phase embryos in *C. pagurus* revealed considerable morphological differences (see Chapter 2), concluding that a clearer definition of the various developmental phases in brachyuran embryos can only be made by removing the embryos from the egg membranes. In addition, the use of vital stains such as toluidine blue after membrane removal also helps to enhance the resolution of embryonic features, such as the presence of limb buds.

3.4.2. Fractional Rates of Protein Synthesis during Development

The fractional rates of protein synthesis (k_s) recorded during embryonic development in *C. pagurus* were typically much higher than values reported in the literature for adult crustacean tissues (Whiteley et al., 1996; Robertson et al., 2001 a,b; Whiteley and Faulkner, 2005). However, both tissue specific variations in k_s, e.g. adult *C. maenus*, 0.3-17 % day^{-1} (El Haj and Houlihan 1987, Houlihan et al, 1989; Mente et al., 2003) and whole animal k_s values, e.g. larval fish < 1-70 % day^{-1}, can vary tremendously, being dependant on a number of factors including: species; body size; temperature; dormancy, and nutritional status (Carter and Bradfield, 1992; Houlihan et al., 1993; Conceição et al., 1997).

Unlike $\dot{M}O_2$, embryonic k_s in *C. pagurus* was not positively correlated with size. Instead, k_s showed a phase specific relationship during embryonic development, with rates increasing to their highest levels (24.4 ± 5.1 % day^{-1}) at the eye phase, before falling prior to hatching. This is the highest whole animal k_s value recorded in crustaceans at 10°C, when compared to existing data for adult crustaceans. However, when the effects of body size are taken into account and the data scaled to an animal of 1g wet weight, the embryonic k_s values compare closely to the values measured in the temperate isopod, *Idotea rescata*, at 14°C (Whiteley et al., 1996). Unfortunately, no data is available in the literature for k_s values in crustacean embryos or larvae. Despite this, the values reported in the present study are within the range of those reported in the lecithotrophic larva of the gastropod, *Haliotis rufescens*, and the embryonic and larval phases of *Lytechinus pictus* (Varvra and Manahan, 1999; Pace and Manahan, 2006). Furthermore, these values are similar to k_s values recorded during preliminary experiments using brachyuran embryos taken from *N. puber* and *C. maenas* (Whiteley, unpublished observations). In general, k_s values are relatively high during embryonic and larval phases in development, when compared with adult

life phases, but values for adult *C. pagurus* are currently not available to make this comparison with the embryonic values collected in this study.

The marked phase specific changes observed in mean k_s values recorded in embryonic *C. pagurus*, could be a reflection of ontogenetic changes during development. For example, at limb formation whereby the developing embryo underwent a period of differentiation with the appearance of various limb buds and the abdomen, k_s was elevated compared with the blastula phase. Furthermore, at the eye phase, which was characterised by an increase in embryo proportion, protein accumulation, and the onset of organogenesis, k_s was markedly elevated when compared with all of the key phases in development.

Part of the changes to k_s during embryonic development in *C. pagurus* may also be influenced by the proportion of highly synthetic tissues, such as the gills or gut, as found in larval fish (Houlihan et al., 1986; Houlihan et al., 1988). Tissue specific measurements of k_s in adult *C. maenas* also show the high values in the gut (Houlihan et al., 1989; Mente et al., 2003). In terms of the present study, morphological observations of the gut coincided with both the limb formation but mainly the eye phase in development, where k_s was relatively high (see Chapter 2). In addition, recent studies into the chemical changes in embryonic metabolism of *Emerita asiatica* and *Hyas araneus*, also suggest a phase specific pattern in metabolic rate and metabolic consumption of the biochemical yolk store during brachyuran embryogenesis (Subramoniam, 1991, Petersen and Anger, 1997). Both of these studies also show a period of low metabolic rate, or yolk consumption, during early developmental phases, followed by increases during the eye phase and again during organogenesis. Similarly, Naylor et al (1999) reported a large increase in metabolic rate during organogenesis in *C. pagurus*.

Finally, it is also important to consider the influence of embryonic moult cycles on embryonic k_s. Work on adult crustaceans has shown that k_s values in muscle

tissue are related to the moult cycle, with a 6-fold increase in k_s during the pre-moult period in adult *H. americanus*, and also during the post-moult period in adult *C. maenas* (El Haj and Houlihan, 1987; El Haj et al., 1996). This has been shown to reflect both increased hyperplasia and hypertrophy in the muscle fibers (Mayrand, 2000; N. M. Whiteley Unpublished observations). In *C. pagurus* new egg membranes were observed at the limb formation and eye phases, indicating the occurrence of an embryonic moult cycle, which coincides both with an increase in k_s values, but also with periods of hyperplasia (see Chapter 2).

3.4.3. RNA:Protein Ratios and RNA Activities

Changes in k_s recorded in the present study occurred at constant protein synthetic capacity, but reflected changes in ribosomal activity (k_{RNA}). A similar relationship between k_s and k_{RNA} has also been shown in *H. americanus* during pre-moult (Houlihan et al., 1989; El Haj et al., 1996), following a meal in juvenile shrimp, *Litopenaeus vannamei,* and adult *C. maenas* (Houlihan et al., 1989; Mente et al., 2003) and also during rapid growth in larval herring (Houlihan et al., 1995). This relationship appears to be relatively common amongst ectotherms, and has been shown to regulate rapid changes in protein synthesis rates for example, in response to temperature and oxygen increase (Whiteley et al., 2001; Mente et al., 2003; Storch et al., 2003). A possible reason for the constant synthetic capacity in *C. pagurus*, may again be due to the influence of the energy store present in the form of the lecithotrophic yolk mass, as reported in Chapter 2 with respect to RNA:DNA ratio. RNA:protein is also, therefore, less likely to change until nutritional condition is more variable, for example during the zoea larval phases where food resources are not reliable and must be actively sought. Similarly, protein synthetic capacity and rates of protein synthesis are highly correlated in post hatching phases in development of larval fish, but not during lecithotrophic phases in development (review Buckley et al., 1999).

In the present study it was difficult to link rates of protein synthesis to rates of protein turnover during embryonic development, since rates of protein

degradation were unknown. However, attempts were made to estimate protein degradation during development, and subsequently protein retention efficiency by comparing mean values for embryonic protein accumulation with mean *A*s. At the blastula phase absolute rates of protein synthesis were not sufficient to account for the total amount of protein accumulated at the end of this period in development, assuming that protein accumulation occurred at a constant rate. Absolute rates of protein synthesis at the blastula do not, therefore, account for the observed growth, suggesting that rates of protein degradation were also lower, reducing protein turnover, and providing further evidence for a reduction in growth during this phase in development. In sea urchin and *Xenopus laevis* embryos there is an initial brief burst in the rates of protein synthesis followed by a decline during early cleavage (Gross et al., 1964; Epel 1967; Grandin and Charbonneau 1989; Rees et al., 1995). A similar situation may exist in *C. pagurus* blastulas. This suggestion is supported by the relatively large amounts of ribosomal RNA present at the blastula (see Chapter 2), since the elevated RNA levels may be required to activate an initial pulse of protein synthesis in the zygote and, therefore, help to trigger cell differentiation.

In general, the rate of protein accumulation, or growth, in embryonic *C. pagurus* appeared to be correlated with protein retention efficiency, which was highest at the hatching phase. This is in agreement with results reported in larval fish, and the short lived cephalopod, *Octopus vulgaris*, which also grows rapidly as a result of high protein retention (Houlihan et al., 1990; Conceição et al., 1997; Carter et al., 1998). It is noteworthy that the highest fractional rates of protein synthesis in *C. pagurus* embryos were not characterised by the highest protein retention efficiency. The reasons for this are not clear, but protein retention efficiency could be related to the types of tissue present in the embryo. For instance, tissue specific protein retention efficiencies have been shown in both adult vertebrates and invertebrates, and retention efficiencies in highly synthetic tissues e.g. gill tissue, are found to be relatively low (Haschemeyer and Smith, 1979; Fauconneau and Arnal, 1985; Houlihan et al., 1990). Protein growth rate in embryonic *C. pagurus* was also interpreted using *A*s values standardised against whole embryo DNA, thus for cell number. To this end, rates of growth increased

from the blastula and reached its peak at the eye phase, where it was very similar to limb formation, before declining at the hatching phase. So it would appear that growth in embryonic *C. pagurus* is related to whole embryo k_s and protein retention.

Interestingly, the mean values for whole embryo *A*s remained elevated at the hatching phase, despite the drop in k_s values, probably because of the large increase in the number of cells present in the embryo, and the elevation in cellular synthetic capacity reported in Chapter 2. However, standardised *A*s values at the hatching phase were relatively low and similar to blastula values, where growth rates were negligible, and rates of protein synthesis gave a good indication of protein turnover rates. The reduction in absolute rates per cell in hatching embryos could result in a reduction in metabolic demand per cell prior to hatching. A reduced, or stable metabolic rate, has been shown in certain fish and frog embryos at the point of hatching competence. This is believed to conserve energy in preparation for the hatching event where metabolic rates are known to increase (Bradford and Seymour 1985; Darken et al., 1998). A similar response may operate in brachyuran embryos, although this was not evident with respect to the whole-embryo $\dot{M}O_2$ values reported here, which continued to increase, but as a direct result of the hatching process itself. Examination of $\dot{M}O_2$ between Pre-hatch 2 and the hatching phases may be a useful addition to this study, to determine if metabolic rate shows a similar response to that found in fish and frogs. Nevertheless, a decrease in k_s at the hatching phase was not unexpected, since most embryonic growth and differentiation had already been completed earlier in development. It is also likely that the increasing activity of the emerging zoea increased energy expenditure, reducing the amount available for k_s.

3.4.4. Theoretical Costs of Protein Synthesis during Development

By relating $\dot{M}O_2$ and absolute rates of protein synthesis (*A*s) an indirect estimate of theoretical metabolic cost for protein synthesis can be obtained. Such an approach produces estimates that are highly variable, with costs in a wide range of species ranging from 50 mmol ATP.g protein^{-1} to several thousand mmol ATP.g protein^{-1} synthesised (Houlihan et al., 1990; Pannevis and Houlihan., 1992; Land et al., 1993). This is because measurements of $\dot{M}O_2$ represent a summation of many different metabolically demanding processes, which are not separated by this approach. Data from the present study confirms this, since the hatching phase embryos had significantly higher background energetic costs at zero protein synthesis when compared with earlier phases in development. This reflecting the embryos increased size, motility particularly during hatching, and physiological complexity at the later zoea phases in development. In general, compiled data for estimated values of the metabolic costs of protein synthesis demonstrate an inverse relationship between metabolic costs and *A*s (Pannevis and Houlihan., 1992). This is a reflection of the fixed cost and variable cost components of protein synthesis, with RNA synthesis comprising the fixed cost, and protein synthesis the variable cost (Pannevis and Houlihan., 1992; Whiteley et al., 1996; Smith et al., 1999). As rates of protein synthesis increase, the fixed cost, contributes proportionally less of the total metabolic cost of protein synthesis. However, in the only other study to examine embryonic and larval costs of protein synthesis in a temperate species, Pace and Manahan (2006) reported a fixed cost relationship, regardless of protein synthesis rates in the sea urchin, *Lytechinus pictus*.

In the present study, whole embryo $\dot{M}O_2$ showed a poor correlation with whole embryo *A*s, with $\dot{M}O_2$ increasing throughout development, and *A*s increasing between the blastula and the eye phase, but remaining stable at the hatching phase. There could be a number of reasons causing the discrepancy between $\dot{M}O_2$ and *A*s values in embryonic *C. pagurus*. Firstly the $\dot{M}O_2$ values recorded at the hatching phase are unlikely to represent resting values, as the embryos

were hatching and making the transition to free-swimming larvae within the respirometers. Also, this effect was potentially exacerbated at the hatching phase by the relative reduction in protein synthetic activity. The metabolic costs of protein synthesis for hatching phase embryos would therefore incorporate these effects, resulting in an overestimate of the true metabolic cost of protein synthesis. Second, ontogenetic changes could lead to redistribution in energy expenditure during embryonic development. For example, the competence of certain organ systems, such as the cardiovascular system, could influence oxygen uptake and circulation, and alter the balance between oxygen supply and demand (Spicer, 1994; Reiber, 1997; Harper and Reiber, 2004). Finally, changes in energy expenditure relating to osmoregulation could also result in a redistribution of energy allocation during development. For example, in sea urchin embryos and larvae, Na^+/K^+-ATPase appears to show variable costs at different phases in development, and there is recent evidence of ontogenetic changes in osmoregulation in crab embryos (Seneviratna and Taylor, 2006).

For these reasons, estimates for the metabolic cost of protein synthesis reported here should be treated with caution. However, it is tempting to suggest that metabolic costs in *C. pagurus* were also fixed over a highly variable range of protein synthesis levels, i.e. for the limb formation and hatching phases. Although at the eye phase, when the embryos were rapidly developing and the whole-embryo As was similar to the hatching phase, the costs were greatly reduced. Theoretical costs of protein synthesis calculated here in embryonic *C. pagurus* were similar to values reported for other invertebrates such as various crustacean species and sea urchin embryos, and also developing vertebrates e.g. larval teleosts (Houlihan et al., 1992; Whiteley et al., 1996; Whiteley and Faulkner, 2005; Pace and Manahan, 2006). The effects of hypoxia on As and $\dot{M}O_2$ levels will be discussed in Chapter 4. Hypoxia could potentially restrict hatching movements and give a clearer indication of the relationship between As and $\dot{M}O_2$.

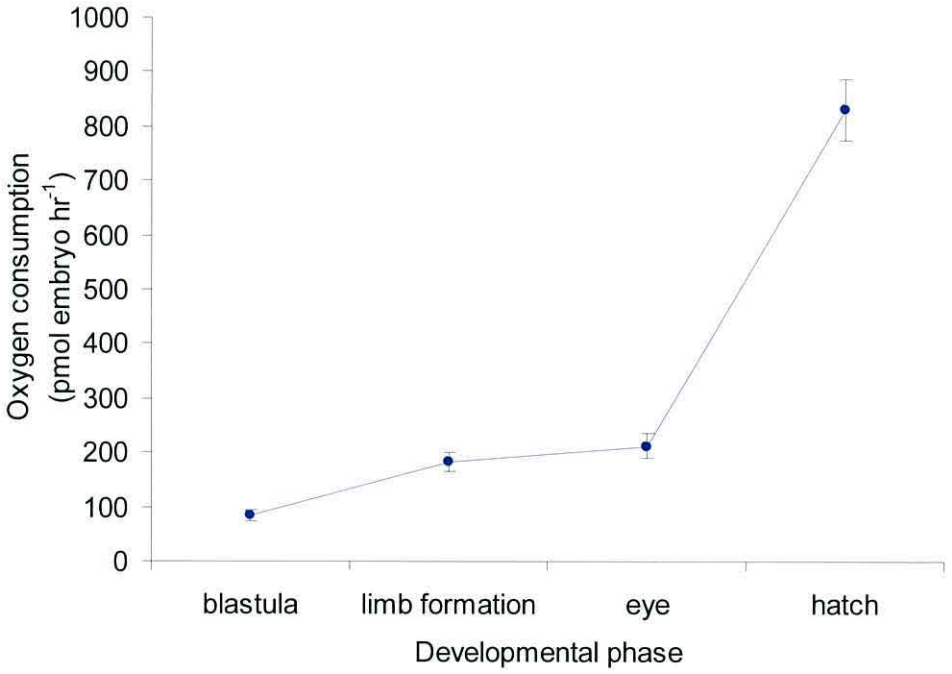
3.4.5. General Conclusion

In summary, this study shows that embryonic $\dot{M}O_2$ is highly correlated to cell number and thus whole embryo size during development, with slight deviations occurring only at the blastula and hatching phases. The greatest increases in mean values of whole embryo $\dot{M}O_2$ values occurred following organogenesis and hatching, as shown in a number of other brachyurans during embryogenesis. Mass specific rates of $\dot{M}O_2$ changed in a similar fashion to the values measured in other brachyurans, with early phases having $\dot{M}O_2$ values lower than those predicted by tissue mass (Taylor and Leelapiyanart, 2001).

Following difficulties encountered with the accurate determination of the embryo tissue mass e.g. separation of the embryo from the yolk, and the mass of the egg membranes, discussed previously by Taylor and Leelapiyanart (2001), a scheme was developed to estimate embryonic protein when corrected for the protein present in the yolk. These estimations were made using area measurements recorded on digitally captured images of the embryos and yolk in two different orientations. Corrected values of embryo protein content were subsequently used to calculate absolute rates of protein synthesis and growth rates. It is possible that blastula embryos of *C. pagurus* are metabolically quiescent, as indicated by the relatively low $\dot{M}O_2$ values and low rates of protein metabolism, although this could also be a reflection of allometric relationships with whole-embryo $\dot{M}O_2$ showing a close relationship to embryo size. In contrast to embryonic $\dot{M}O_2$, k_s values were not correlated with size, but peaked at the eye phase before declining at the hatching phase.

The relatively high rates of protein synthesis at the eye phase corresponded to the rapid increase in rates of development observed in Chapter 2. In addition, the elevation to k_s at the eye phase preceded and thus initiated, the major period of growth observed during embryonic development in *C. pagurus*. Estimates of the

metabolic cost of protein synthesis, showed a phase specific relationship during development, with the lowest costs at the eye phase, and the highest costs at both the limb formation and hatching phases. The reduction in k_s at hatching, despite the maintenance of elevated total As, suggests that cellular synthetic rates are reduced, although this doesn't have an overall effect on whole-embryo $\dot{M}O_2$, although this could possibly relate to the increased activity of the hatching embryo.



a

Development	Whole embryo (MO_2) pmol embryo^{-1} h^{-1}	Mass specific pmol embryo^{-1} h^{-1}
Blastula	85 ± 10	2.93 ± .3
Limb formation	182 ± 22	4.98 ± .4
Eye	214 ± 17	5.68 ± .4
Hatch	825 ± 38	13.86 ± .9

b

Figure 3.1. (a) Effect of development on whole embryo rates of oxygen uptake ($\dot{M}O_2$) in *Cancer pagurus* at 10ºC. Values are means ± SE (n = 6 except for the blastula, where n = 5). (b) Tablulated values of whole embryo rates of oxygen uptake in the developing embryos of *Cancer pagurus* at 10ºC.

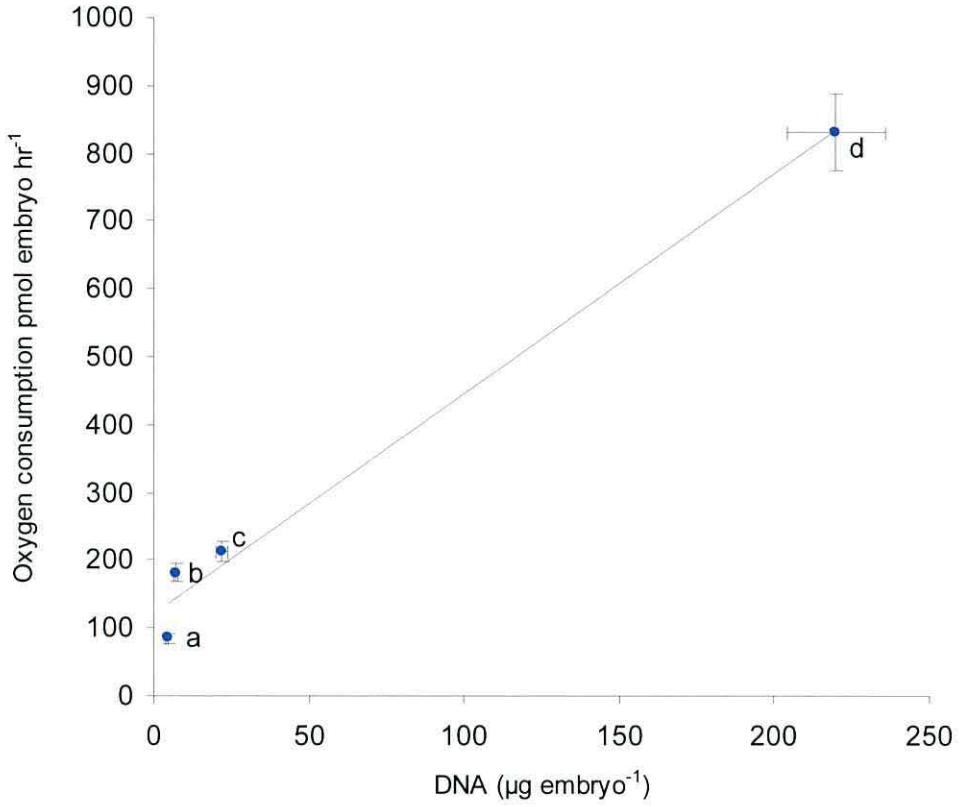
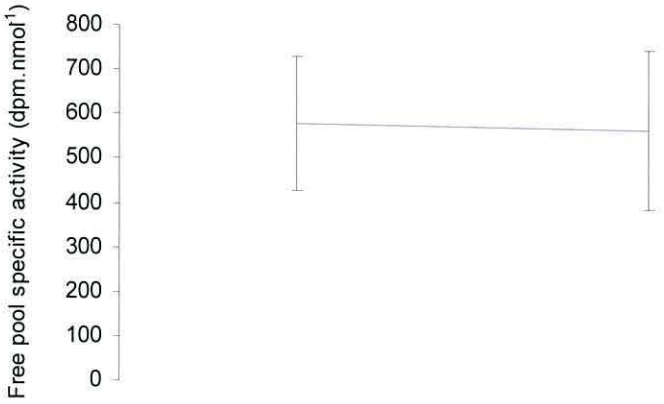
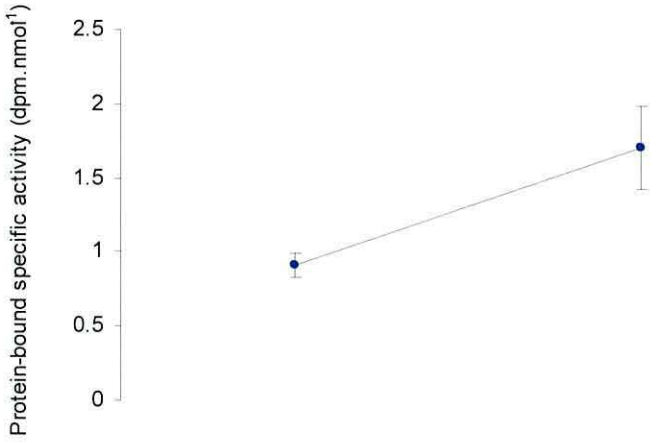


Figure 3.2. Mean whole embryo rates of oxygen uptake ($\dot{M}O_2$) as a function of mean whole embryo DNA in *Cancer pagurus* at 10°C. The broken line is the relationship between $\dot{M}O_2$ and whole embryo DNA for all key phases in development (a = Blastula, b = Limb formation, c = Eye, d = Hatching), and is described by the linear regression equation y = 3.2303x + 123.35, r^2 = .988; *P* = .006.

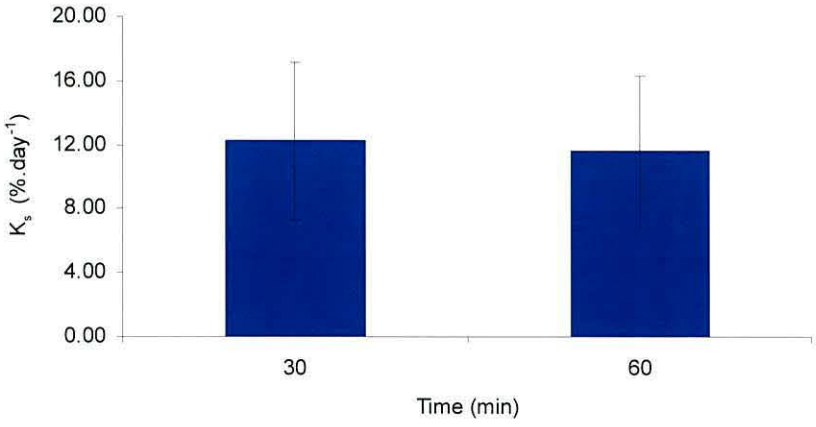
Figure 3.3. (a) Specific activities of the free-pool and (b) protein bound phenylalanine in embryonic *Necora puber* 'eye and chromatophore' phase after incubation in (^{3}H) phenylalanine at 10ºC, along with resultant changes in (c) whole animal fractional rates of protein synthesis (*K*s). Values are means ± SE (n = 5).


Free pool specific activity (dpm.nmol-1)
800
700
600
500
400
300
200
100
0

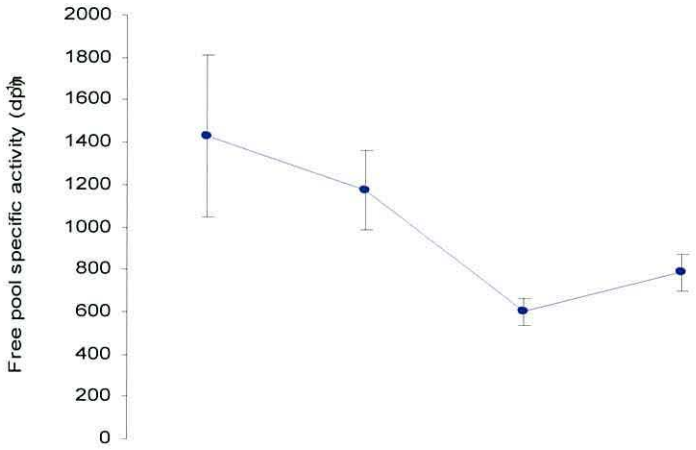
a


Protein-bound specific activity (dpm.nmol-1)
2.5
2
1.5
1
0.5
0

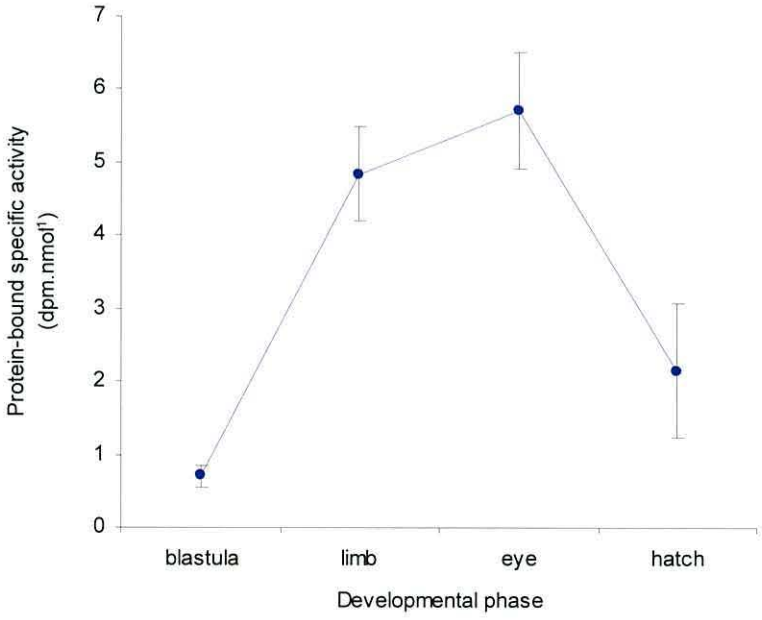
b


Ks (%.day-1)
20.00
16.00
12.00
8.00
4.00
0.00
30
60
Time (min)

c



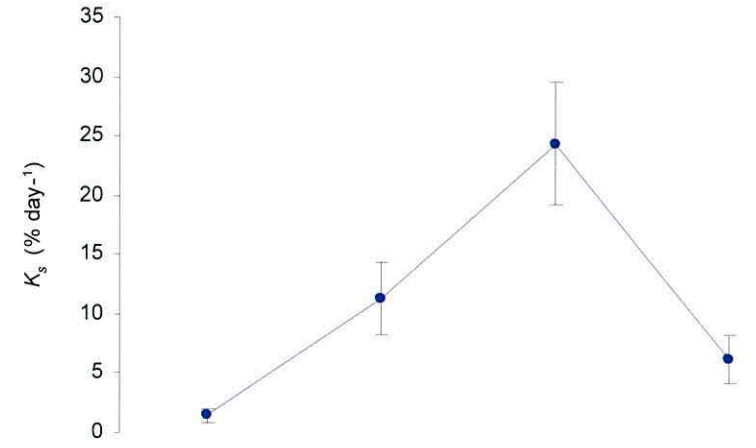
a



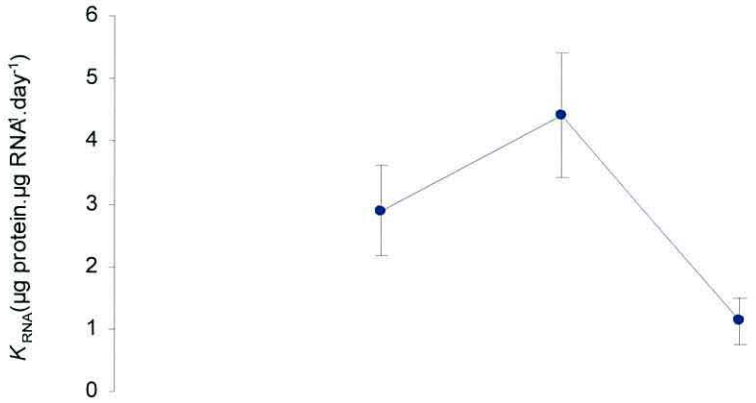
b

Figure 3.4 Specific activities of the free-pool and protein bound phenylalanine at all key phases in embryonic development (Blastula, Limb formation, Eye, Hatching) in *Cancer pagurus* after incubation in (^{3}H) phenylalanine at 10°C. Values are means ± SE (n = 6 except for the blastula, where n = 4).

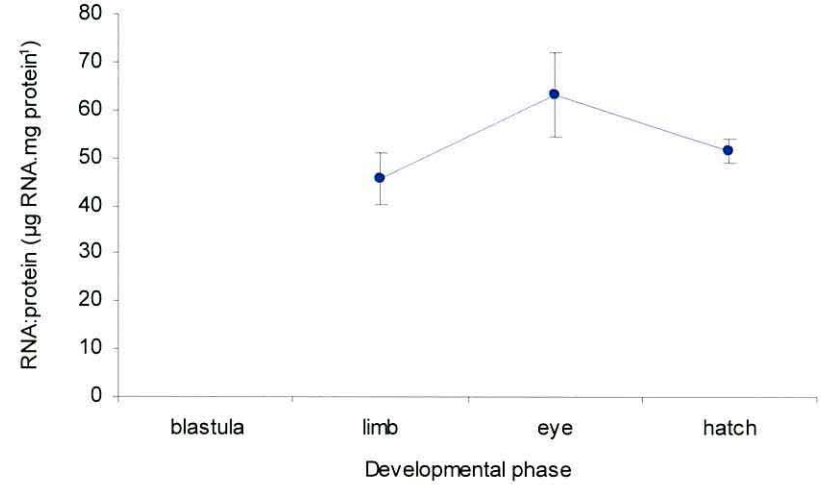
Figure 3.5. Changes in whole embryo fractional rates of protein synthesis (K_s, % day^{-1}) (a), RNA activity (k_{RNA}) (b), and RNA:protein ratios (c), during development in *Cancer pagurus* at 10°C. Values are means ± SE (n = 6 except for the blastula, where n = 4).


35
30
25
20
15
10
5
0
K_s (% day-1)

a


6
5
4
3
2
1
0
K_{RNA}(µg protein.µg RNA^{1}.day^{-1})

b


80
70
60
50
40
30
20
10
0
RNA:protein (µg RNA.mg protein^{1})
blastula
limb
eye
hatch
Developmental phase

c

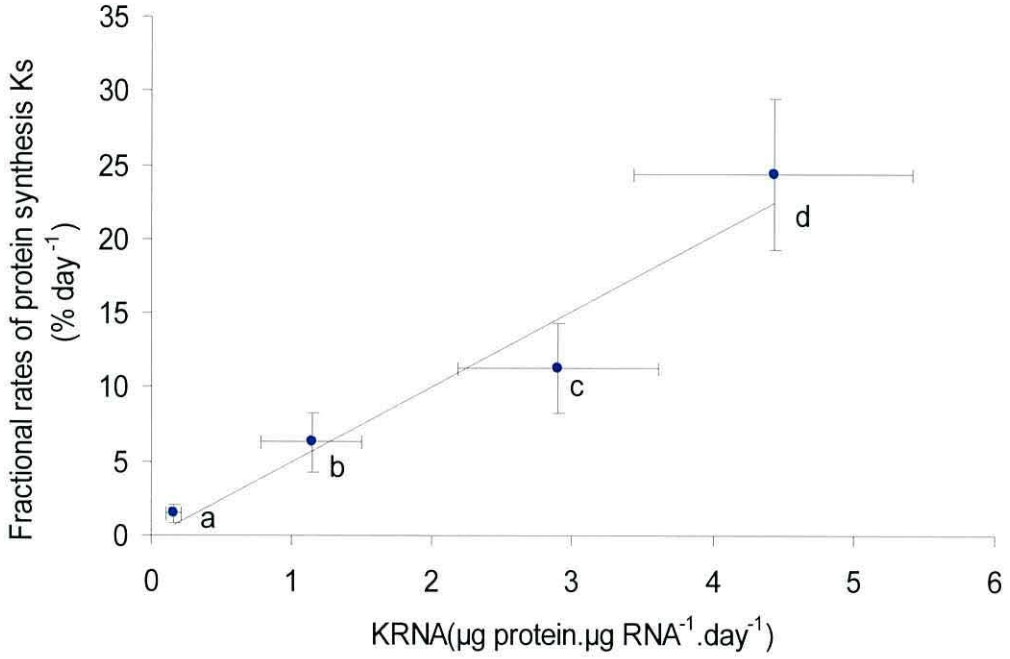


Figure 3.6. The relationship between mean RNA activity (k_{RNA}) and mean fractional rates of protein synthesis (K_s, % day^{-1}) during development (a = Blastula, b = Limb formation, c = Eye, d = Hatching) in *C. pagurus* at 10°C. The broken line is the regression of k_{RNA} and K_s, and is described by the linear regression equation y = 5.1x – 0.09, r^2 = .95; P = .028. Data at the blastula substituted in by using values recorded in 2 kPa hypoxia (see Chapter 4).

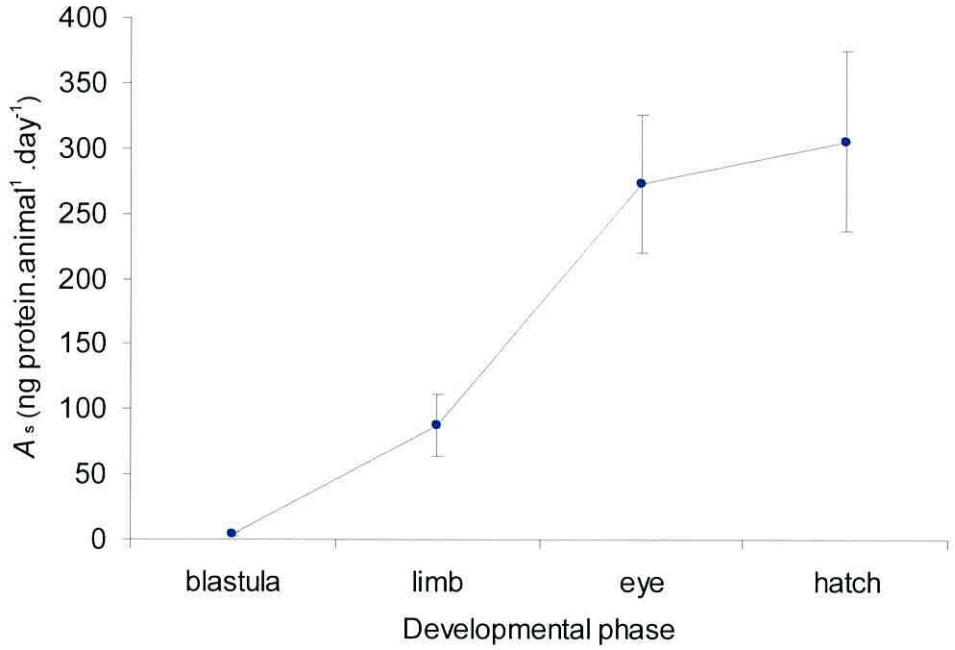


Figure 3.7. Changes in whole embryo absolute rates of protein synthesis (A_s) during development in *C. pagurus* at 10ºC. Values are means ± SE (n = 6). Data at the blastula substituted in by using values recorded in 2 kPa hypoxia (see Chapter 4).

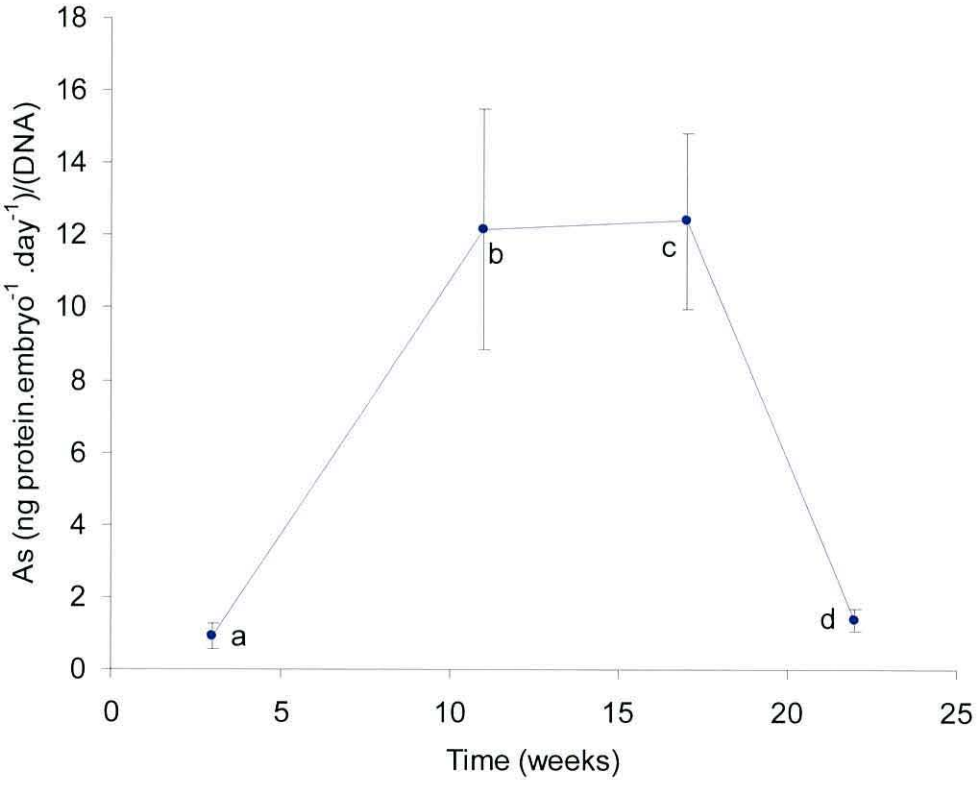


Figure 3.8. Mean whole embryo absolute rates of protein synthesis (A_s) as a function of mean whole embryo DNA at all key phases (a = Blastula, b = Limb formation, c = Eye, d = Hatching) in development of *C. pagurus* at 10ºC.

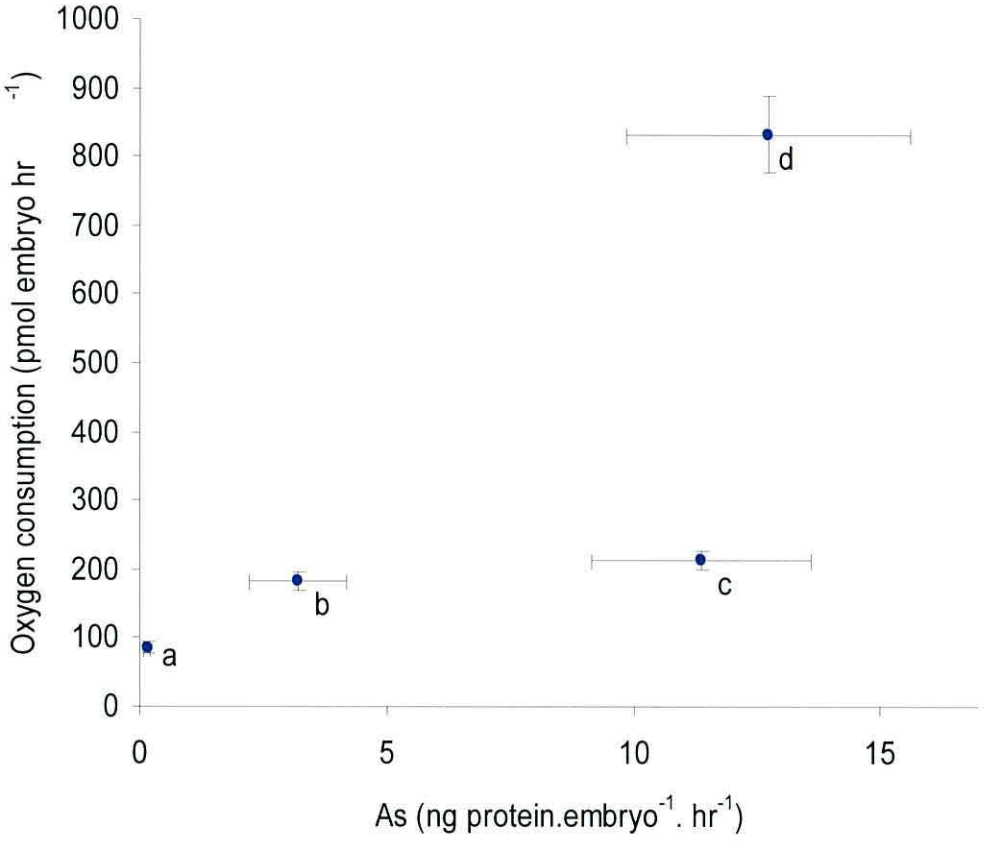


Figure 3.9. The relationship between mean absolute rates of protein synthesis (A_s) and mean whole embryo rates of oxygen uptake ($\dot{M}O_2$) during embryonic development (a = Blastula, b = Limb formation, c = Eye, d = Hatching) in *C. pagurus* at 10°C.

Chapter 4

Influence of Hypoxia on Rates of Metabolism and Protein Synthesis in embryonic *Cancer pagurus*

Ovigerous *C. pagurus* incubating blastula embryos remained continuously buried beneath the substratum, consequently restricting maternal oxygenation and, therefore, convective oxygen supply to the developing embryos. Subsequent phases in development experienced a continued increase in the level of oxygen present in the developing egg mass, which coincided with an increase in the frequency of maternal abdominal flapping behavior.

Incubation of blastula phase embryos in hypoxic PO_2 found naturally in the egg mass had little short-term effects on metabolic rate, and no effect on the rates of protein synthesis and RNA activity (k_{RNA}). These findings are indicative of a quiescent or diapause period at the blastula, which may be correlated to the natural brooding hypoxia induced through maternal behavior. An increased level of oxygen present in the egg mass at subsequent phases in development related to an increased embryonic metabolic sensitivity to hypoxia. Both the eye and hatching phases were found to be strict oxygen conformers, which was also supported by the absence of a significant increase in embryonic anaerobic lactate production in response to hypoxia.

Generally, k_s levels were reduced to a common value of 5% day^{-1} following exposure to a five fold reduction in oxygen level, although these reductions were only significant at the limb formation phase and the eye phase. Rates of protein synthesis and k_{RNA} at the eye phase showed a 5-fold and a 4-fold respective decrease in response to a progressive hypoxic series. Reasons are discussed for the incidence and the effects of hypoxia during embryonic development in *C. pagurus*.

Finally, the metabolic costs of protein synthesis in hypoxia were also investigated in light of the difficulties experienced in Chapter 3. This was found to comprise a fixed cost of 149 ± 4 mmol ATP. g $protein^{-1}$.

4.1. Introduction

Many marine invertebrates deposit their embryos in large egg masses of different shapes and sizes, related, in part, to improving oxygen provision to the developing embryos (Strathmann and Chaffee, 1984; Lee and Strathmann; Strathmann and Hess, 1999). In brachyuran crustaceans, oxygen provision by the ovigerous female appears to be vital during the brooding period to facilitate embryonic development and hatching as pelagic larvae. Furthermore, recent studies suggest that the main reproductive effort associated with egg brooding in brachyurans is comprised of maternal oxygen provision to the developing embryos (Fernández et al., 2000; Taylor and Leelapiyanart, 2001; Baeza and Fernández, 2002; Fernández and Brante, 2003). This is especially important in certain Cancrid crabs such as *Cancer pagurus*, which can spawn in excess of 3 million eggs, twice the normal allometric relationship found between brood size and female body size in brachyurans (Hines, 1982; Hines, 1991). As a result of this large brood size, the developing embryos of *C. pagurus* are tightly packed together in the brooding egg mass, which can be several centimetres wide and deep.

According to Fick's First Law of Diffusion, natural passive oxygen supply to biological structures is potentially restricted to 1mm. In terms of brachyuran egg masses even this is likely to be an overestimate, because of the demand and competition for oxygen between developing siblings, and also the poor convective flow through the interstitial spaces between the developing embryos of certain species (Fick, 1855; Krogh, 1941; Lee and Strathmann, 1998; Fernández et al., 2000). For example, continuous measurements of PO_2 levels using microoptode fibres, sampling of egg mass water PO_2 using a syringe and cannula tubing, and MRI imaging of water flow in the developing egg masses of *C. pagurus*, have revealed relatively poor circulation of water through the egg mass compared with other brachyuran species. This results in stagnation of water

flow and probably the formation of large boundary layers further restricting oxygen diffusion into the egg mass (Naylor et al., 1999; Fernández et al., 2000).

The effects of hypoxia during embryonic development has a range of implications, for example, in several species of mollusc whose embryos often rely on oxygen diffusion from the environment; exposure to hypoxia can cause impedance of growth and shell formation (Strathmann and Strathmann 1995; Cancino et al. 2003), and in certain species, hypoxia is recognised as one of the major causes of embryonic mortality (Eriksson and Wickland, 2001; Steer et al. 2002). Many marine species rely on passive oxygen supply to their developing embryos. Those species that produce egg masses, as a consequence can have strongly asynchronous patterns of development, with embryos from the centre of the egg mass showing delayed hatching compared with those positioned at the periphery of the egg mass (Chaffee and Strathman, 1984; Marois and Croll, 1991; Booth, 1995; Starthman and Strathman 1995; Steer et al. 2002). Even during egg brooding, where the embryos benefit from convective oxygen supply, developing embryos can experience hypoxia. In brachyurans, the oxygen levels in the developing egg mass can also become severely hypoxic, and diminish by more than 85% only millimetres beneath the peripheral embryos. For example, Naylor et al (1999) reported a fairly constant PO_2 level in excess of 14.6 kPa at the periphery of the developing egg mass in *C. pagurus*. However, PO_2 levels at the centre of the egg mass were only 4.5 kPa, and in the same species but examined in a different study they were 2.1 kPa, and similar to hypoxic egg masses reported for gastropod molluscs (Booth, 1995; Fernández et al., 2000). Similar findings have also been reported in other Cancrid crabs (Fernández et al., 2000; Fernández et al., 2003).

Brooding hypoxias in brachyuran egg masses can be associated with egg packing arrangements and the frequency of oxygen provision behaviours by the ovigerous female, such as abdominal flapping, ventilatory reversals and pauses (Wheatly, 1981; Fernández et al., 2000; Baeza and Fernández, 2002; Fernández and Brante, 2003). The situation is compounded in *C. pagurus*, as following spawning

ovigerous females bury themselves entirely beneath the substratum, thereby restricting convective oxygen supply to the developing embryos. They can remain buried in this position when undisturbed for up to two months, even in laboratory aquaria (Williamson, 1900; Naylor et al., 1999).

When faced with a prolonged exposure to hypoxia, animals are essentially known to use one of two well established lines of physiological defence (Hochachka and Lutz, 2001). Metabolic rate is defended by switching from aerobic to anaerobic respiration, which is part of a response also involving improved oxygen transport through hyperventilation and changes to oxygen affinity of the respiratory pigment (Hoback et al., 2000; Kolsch et al., 2001). Alternatively, the well established and most successful response shown in for example, embryonic *Artemia franciscana*, and the anoxia tolerant turtle, *Trachemys scripta elegans*, is a depression of aerobic metabolism (Hochachka, 1986; Clegg, 1993; Hand, 1997; Lutz and Storey, 1997; Guppy and Withers, 1999). A crucial aspect of the latter is a large reduction to rates of protein synthesis to reduce ATP demand during energy limited conditions.

The strategy used by crab embryos exposed to declining oxygen levels is unclear, although embryos of *C. pagurus* and *Cancer setosus* appear to show an increase in oxygen sensitivity as development progresses (Naylor et al., 1999; Baeza and Fernández, 2002). Also, while it appears that ovigerous females ventilate the developing embryos according to demand during development (Wheatly, 1981; Naylor et al. 1999, Fernández et al. 2000; Fernández et al. 2003), periods of hypoxia may be experienced at all phases of development. For Cancrid embryos, this can best be interpreted as an artefact of the cyclical nature in which oxygen is provided to the embryos, and the burying behaviour of the ovigerous female below the substrata. This is most pronounced at early developmental phases, particularly during the blastula phase, where oxygen levels at the centre of the egg mass can vary between anoxia and normoxia (Naylor et al., 1999; Fernández et al., 2000; Baeza and Fernández, 2002; Fernández and Brante, 2003).

While the effects of hypoxia on growth and metabolism have been investigated on a range of invertebrates (Land et al., 1993; Chu and Ovsianico-Koulikowsky, 1994; Strathmann and Strathmann 1995; Clegg, 1996; Hand, 1997; Naylor et al., 1999; Staples et al., 2000; Baeza and Fernández., 2002; Steer et al. 2002; Cancino et al. 2003; Mente et al., 2003;) and vertebrates (Portner et al., 1991; Zhou et al., 2001; Fraser et al., 2001), only a few of these studies have determined rates of oxygen uptake ($\dot{M}O_2$) along with fractional rates of protein synthesis (k_s, % day^{-1}) (Houlihan et al., 1989; Land et al., 1993; Smith and Houlihan, 1995; Mente et al., 2003; Pace and Manahan, 2006). Even fewer studies have been made on the effects of hypoxia on protein metabolism during ontogeny (Clegg, 1996; Hand, 1998), and equally as limited are investigations covering the effects of hypoxia during brachyuran development (Wheatly, 1981; Naylor et al., 1999; Baeza and Fernández, 2002). Hypoxic responses are of particular interest in brachyurans, as early periods of embryonic development involve morphological differentiation and relatively low rates of protein synthesis, followed by periods of rapid growth and elevated rates of protein synthesis at later phases in development (see Chapter 3). Naylor et al (1999) also established that oxygen sensitivity increases during development in embryonic *C. pagurus*, suggesting that higher rates of protein synthesis and growth are associated with a greater reliance on oxygen supply to the egg mass and, therefore, higher rates of oxygen demand.

This study will further investigate the relationship between rates of oxygen uptake and protein synthesis in embryonic *C. pagurus*, to determine if changes in growth rates during development influence sensitivity to hypoxia, and to investigate whether rates of protein synthesis are regulated as part of a global response to hypoxia. The involvement of anaerobic metabolism will also be examined by the determination of whole-embryo lactate concentrations to establish whether metabolic rate is defended during hypoxia.

The reproductive behaviour of *C. pagurus* make this an interesting choice of species to base this study, due to the large size of its egg mass, and its reproductive brooding behaviours, meaning that hypoxia is a regular occurrence, especially at early phases of development. A greater understanding of the metabolic responses of *C. pagurus* embryos and their growth-related consequences, such as the possible control of hatching, will not only increase our understanding of ontogenic changes, but will provide valuable information on the relative sensitivities of brachyuran embryos to other forms of environmental hypoxia, including those events caused by anthropogenic impacts. The occurrence of hypoxic episodes in the natural environment, can be natural e.g. tidal regime (Nilsson and Nilsson, 2004), or anthropogenic, which is an increasing problem, especially in estuarine areas which are poorly mixed, or where stratification of the water column prevents mixing of the sea water with atmospheric oxygen (Diaz and Rosenberg, 1995; Bricker, 1997). In addition, the current rise in global marine CO_2 levels has lead to an increase in the eutrophication of aquatic environments (Cheng et al., 2003), and hence an associated decrease in aquatic PO_2.

4.2. Methods

4.2.1. Animal Collection and Egg Sampling

Female *C. pagurus* were collected and maintained in aquaria at the University of Wales, Bangor as outlined in Chapter 2. Eggs were sampled as described previously.

Rates of oxygen uptake, rates of protein synthesis, and determination of partial pressure of oxygen present in the developing egg mass, were studied at the 4 key phases of *C. pagurus* embryonic development outlined in Chapter 2 and studied in Chapter 3.

-

4.2.2. Rates of Oxygen Uptake MO_2

Experiments determining the rate of oxygen uptake ($\dot{M}O_2$) were carried out using the same experimental setup as outlined in Chapter 3. In short, approximately 40mg of embryos were placed into 1ml of sterile aerated seawater, and continuously mixed to prevent oxygen stratification during measurements. Oxygen partial pressure (PO_2) was recorded continuously, and allowed to fall through a range of oxygen levels that occur naturally in the egg mass. Measurements were recorded for at least five minutes at PO_2 levels of 18, 12, 8 and 2 kPa. After the completion of each measurement series, the seawater was removed and replaced with a fresh sample of sterile aerated seawater, and the process repeated a further 2 times on the same sample of embryos. Two further batches of embryos taken from the same mother were treated in the same way, resulting in 3 separate readings run in triplicate. Control measurements were taken before each set of measurements using the same setup containing only sterile aerated seawater. The viability of the embryos were determined where possible, e.g. at later phases by assessing heart rate. Standardised rates of oxygen uptake were calculated, and measurements were expressed as pmol $embryo^{-1}$ hr^{-1}.

4.2.3. Oxygen Partial Pressure in the Egg Mass

Experiments determining the level of oxygen present in the developing egg mass were completed by withdrawing 500µl of seawater from the centre of the egg mass using a needle and syringe. A small amount of this seawater was passed by a polarographic oxygen electrode (Radiometer ES047) connected to a blood gas meter (Radiometer acid base analyser PHM71) maintained at 10°C. The process was completed on another five ovigerous female crabs at approximately the same phase in development.

4.2.4. Rates of Protein Synthesis

Whole-embryo fractional rates of protein synthesis in hypoxia were determined in crab embryos in vivo, by adding a flooding dose of labelled phenylalanine to the surrounding seawater (150 mmol l^{-1} phenylalanine (Phe) and L-[2-6 ^{3}H] Phe (Amersham, specific activity 122 Ci $mmol^{-1}$) at 50 µCi ml^{-1}. Samples were incubated for 60 minutes at 10°C and the incubation medium was continuously, but gently aerated and stirred. Severe hypoxia was maintained by supplying a gas mixture of air and nitrogen via a gas mixing pump (see Chapter 5) to maintain the PO_2 of the incubation medium at the selected partial pressure of 2 kPa. Fractional rates of protein synthesis were measured at each of the 4 key phases in *C. pagurus* development. Samples were processed as described in Chapter 3.

1. Analysis of samples

The specific activity and concentration of Phenylalanine (Phe) in the free pool and bound protein fractions were determined enzymatically using the technique of Garlick et al (1980), following modifications described in Whiteley et al (1996) and those outlined in Chapter 3. Total protein was measured against known standards of bovine serum albumin using a modification of Lowry technique (Peterson, 1977). RNA was determined using a spectrophotometer (Shimadzu UV260) according to Ashford & Pain (1986), and total DNA also measured spectrophometrically (Shimadzu UV260) at 595 nm and 700 nm with salmon DNA as a standard (Munrow and Fleck, 1969).

4.2.5. Lactate Accumulation

Experiments determining whole embryo lactate accumulation during incubation in normoxia, and exposure to hypoxia (2 kPa) were mainly completed using the eye and chromatophore phase embryos of *Necora puber*, due to its ready availability. Preliminary experiments were also trialled on the eye phase embryos of *C. pagurus*. Approximately 3 g (wet weight) of crab embryos were sampled as previously described, and left to acclimate at 10°C in gently aerated seawater for 30 minutes. In *N. puber* ~200 – 300 mg of embryos were instantly snap frozen following removal from the egg mass. These were used to act as control samples for embryonic lactate present in the developing egg mass at this phase in development. The embryos were subdivided into two treatment groups according to oxygen partial pressure (PO_2) and placed into glass chambers (6 x 4 cm) filled with temperature acclimated sterile seawater. After sealing the incubation chambers, a small needle connected to either a gas mixing pump (Wösthoff SA27/3-F, Bochum, Germany) for treatment with hypoxia, or an air pump (Whisper 400) for incubation in normoxia, was inserted through the lid, to bubble the appropriate gas mixture through the seawater. This also served to gently mix the eggs to ensure an even distribution of the prevailing PO_2 level. Both incubation chambers were then placed into a water bath (Grant Y22) and submerged almost entirely to maintain constant temperature. Each treatment group was incubated for 4 hours, after which the eggs were blotted and snap frozen in liquid nitrogen.

1. Analysis of Samples

Each sample of eggs were ground to a fine powder in liquid nitrogen and proteins precipitated in 8% cold perchloric acid (PCA). The resultant deprotenised sample supernatants were centrifuged for 3500 rpm at 4°C for 15 minutes, and carried through for enzymatic determination of tissue lactate accumulation (Sigma, Lactate Dehydrogenase, cat: 826-6). All steps were performed on ice and according to the manufacturer's instructions. Briefly, two sub-samples of 100 µl were added to enzyme preparation solution (Nicotininamide adenine dinucleotide, Glycine buffer, deionised water, Lactate dehydrogenase), vortexed and incubated at 37°C for 30 minutes. Tissue lactate was determined

spectrophometrically (Shimadzu UV260) at 340 nm, using lactate acid as a standard.

4.2.6. Calculations

Outlined in Chapter 3.

4.2.7. Statistical analysis

All values are given as means ± SEM. Prior to statistical analysis all data were tested for normality of data distribution using the Kolmogorov-Smirnov test, and homogeneity of variances using the Levene test. The effect of hypoxia at each phase in development was tested following log transformations of the data by one-way ANOVA and when significant, by the LSD *post-hoc* test. Non-parametric data was analysed by a Kruskal-Wallis ANOVA, with significant differences analysed using the Mann-Whitney test. The results were also subject to regression analysis completed with ANOVA. Statistical analyses were performed using SPSS software (SPSS version 12; SPSS Inc., Chicago, Il, USA). The level of significance used in all tests was $P < 0.05$.

4.3. Results

4.3.1. Rates of Oxygen Consumption

The influence of progressive hypoxia on the rates of oxygen uptake $\dot{M}O_2$ in *C. pagurus* embryos at each of the 4 key phases of development is shown together in Figure 4.1. Since whole embryo $\dot{M}O_2$ in normoxia was shown to increase with size and with development (see Chapter 3), oxygen levels in the respirometer at the hatching phase decreased very rapidly, and as a consequence these experiments were completed in half the time taken for at the blastula phase.

Figure 4.2a shows that progressive hypoxia had very little effect on $\dot{M}O_2$ at the blastula phase, even following a five fold reduction in oxygen level to 2 kPa (one-way ANOVA, $P = 0.227$). This was confirmed following a regression analysis, which also showed that there was no relationship between $\dot{M}O_2$ and progressive hypoxia (Regression, $P = 0.722$, slope; $r^2 = 0.08$). Despite these, *post hoc* analysis revealed that significant differences in $\dot{M}O_2$ were present between PO_2 of 12 and 2 kPa ($P = 0.045$), with values for $\dot{M}O_2$ decreasing by 36%, from 119 ± 11 to 75 ± 9 pmol embryo^{-1} h^{-1}.

At limb formation whole embryo $\dot{M}O_2$ decreased significantly during progressive hypoxia, with mean values ranging from 182 ± 13 pmol embryo^{-1} h^{-1} at normoxia, to 99.7 ± 5.5 pmol embryo^{-1} h^{-1} at a PO_2 of 2 kPa (Figure 4.2b). The regression value for the relationship between $\dot{M}O_2$ and progressive hypoxia was highly significant (Regression, $P = 0.08$; $r^2 = .84$). Although, mean values of $\dot{M}O_2$ were approximately identical at normoxia and a PO_2 level of 12 kPa, and showed only a relatively small decrease (15%) between normoxia and a PO_2 of 8 kPa. The critical partial pressure of oxygen (Pc), defined in this study as the PO_2

at which there was a significant decrease in $\dot{M}O_2$, lies between PO_2 levels of 8 - 2 kPa for limb formation embryos (one-way ANOVA, $P < 0.01$).

Figure 4.2c shows that eye phase embryos showed a marked and significant decrease in whole embryo $\dot{M}O_2$ during progressive hypoxia, for which the regression relationship was also highly significant (Regression, $P < 0.003$; $r^2 = .99$). $\dot{M}O_2$ were significantly lower at PO_2 of 8 kPa, with mean values decreasing from 213 ± 15 pmol embryo^{-1} h^{-1} at normoxia, to 168 ± 13 pmol embryo^{-1} h^{-1} (one-way ANOVA, $P < 0.016$). This represented a decrease of approximately 25%. The Pc can, therefore, be estimated to be higher than limb formation phase embryos, situated possibly between PO_2 range of 12 kPa and 8 kPa, implying an increased sensitivity to hypoxia. In severe hypoxia (2 kPa), the mean values for $\dot{M}O_2$ had decreased by 40% with respect to normoxic values, to 132 ± 12 pmol embryo^{-1} h^{-1}.

Finally, Figure 4.2d shows that hatching phase embryos observed the largest and most significant decrease in whole embryo $\dot{M}O_2$ during progressive hypoxia (regression, $P < 0.015$; $r^2 = 0.97$). Mean values ranged from 831 ± 57 pmol embryo^{-1} h^{-1} at normoxia, to 305 ± 24 pmol embryo^{-1} h^{-1} by 2 kPa PO_2, representing a 63% total decrease. $\dot{M}O_2$ were also significantly reduced by 8 kPa PO_2, with mean values approximately 32% lower than in normoxia, at 569 ± 35 pmol embryo^{-1} h^{-1} (one-way ANOVA, $P < 0.01$). In addition, although a mathmatically significant decrease in $\dot{M}O_2$ was not evident following a reduction from normoxia to 12 kPa, mean values were reduced by ~15% at 701 ± 45 pmol embryo^{-1} h^{-1}. Also, continued and significant reductions in $\dot{M}O_2$ were observed between 12 and 8 Kpa and between 8 and 2 Kpa ($P < 0.001$). As such it can be estimated that similar to the eye phase embryo, the Pc could potentially lie between the PO_2 region of 12-8 kPa, if not closer to 12 kPa.

*Note all statistical analysis performed in section 4.3.1 was completed using log transformed values.

As found during the determination of $\dot{M}O_2$ in normoxia, even at a PO_2 of 2 kPa development had a highly significant effect on whole-embryo $\dot{M}O_2$. The $\dot{M}O_2$ of hatching phase embryos were significantly higher, when compared with the other key phases in development (one-way ANOVA $P < 0.001$) (Figure 4.3a).

4.3.2. Oxygen Levels in the Egg Mass

The PO_2 level within the egg mass of ovigerous *C. pagurus* increased significantly during development (Figure 4.3b). At the blastula phase in development, the females were buried in the sand of the holding tanks and the PO_2 levels within the egg mass were significantly lower, at 3.5 ± .3 kPa, when compared with any of the other key phases in development (Mann-Whitney, $P < 0.05$). As development progressed the PO_2 level in the egg mass increased to 5.4 ± .6 kPa by limb formation, and then to 12.24 ± 1.6 kPa at the eye phase. By hatching phase, the mean PO_2 had reached its highest level at 15.2 ± 1 kPa, which was similar to that of the eye phase (Mann-Whitney, $P = 0.33$).

4.3.3. Rates of Protein Synthesis and Associated Variables

To determine whether amino acid uptake rates matched those seen in normoxia, specific activities in the free pools from each of the four key phases of development incubated in hypoxia (Figure 4.4a) were compared to the values recorded in normoxia (Chapter 3). These are both tabulated in Figure 4.4b. Hypoxia was found to have no effect on the incorporation of [^{3}H] phenylalanine into the embryonic free pools in *C. pagurus* during the 60 minutes labelling period at each of the key phases in development (Kruskal-Wallis, $P = .820$).

The effect of severe hypoxia (2 kPa) on rates of protein synthesis in *C. pagurus* embryos at 10°C is compared with normoxic rates of protein synthesis in Figure 4.5a. Hypoxic exposure for 60 minutes had a significant effect on k_s at the limb formation phase and the eye phase in development, where mean k_s in normoxia

decreased from 11.3 ± 3.1 to 4.95 ± 1.6 (one-way ANOVA, P = .03) and from the highly elevated value of 24.4 ± 5.1 to 5.62 ± 1 % day at 2 kPa (one-way ANOVA, P < .001), respectively. In light of this highly significant effect observed at the eye phase, the influence of hypoxia on k_s was examined more closely, by measuring k_s at the additional PO_2 levels of 10.1 kPa and 5.1 kPa (Figure 4.7). The mean values for k_s at these two PO_2 levels were 10.3 ± 3.8 % day and 5.9 ± 1.7 % day, respectively. Both mark a significant decrease in k_s with respect to normoxic values (one-way ANOVA, P = .019 and < .001, respectively). Although the mean k_s values between the PO_2 range 10 kPa and 2 kPa showed no significant differences (one-way ANOVA, P = .31), this was possibly influenced by the small sampling number (n = 4) used, but mainly the high variability between points. Overall, whole-embryo k_s during severe hypoxia (2 kPa) remained fairly constant, regardless of developmental phase, with mean values generally ranging between 5 - 6% day^{-1}. Despite this general trend, hypoxic k_s at the blastula phase, was significantly lower than at all other key phases in development, with mean values of 1.78 ± .56 % day (one-way ANOVA, P = < .05).

Absolute rates of protein synthesis (A_s) during development in hypoxia are shown in Figure 4.8 with the corresponding values in normoxia. Similar to the response observed in normoxia, A_s showed a marked increase over the total developmental period. However, this increase was not apparent during the early phases in development i.e. a marked increase occurred at the hatching phase. In light of this trend, hypoxia caused a significant reduction to A_s at both the limb formation and the eye phases in development, where respective A_s values were significantly reduced by 60% and 76% relative to normoxic values (one-way ANOVA, P = .04 and .001, respectively).

The effects of hypoxia on RNA:protein ratios and RNA activities (k_{RNA}) during development are compared with normoxic values in Figure 4.5b,c. Short-term exposure (60 minutes) to hypoxia (2 kPa) had no effect on the RNA:protein

ratios at any of the key phases in development (one-way ANOVA, $P < 0.05$). In contrast, k_{RNA} during hypoxia showed a similar response to hypoxic k_s values, owed to the strong correlation between the two variables (one-way ANOVA, $P = 0.03$) shown in Figure 4.6. At limb formation, hypoxia caused a significant decrease in k_{RNA} by 65% from 2.9 ± .72 in normoxia to 1 ± 0.3 μg protein.μg $RNA^{-1}.day^{-1}$ in hypoxia (one-way ANOVA, $P = 0.027$). At the eye phase, k_{RNA} was also significantly reduced from its normoxic value by 76% from 4.43 ± 1.3 in normoxia to 1.15 ± 0.22 μg protein.μg $RNA^{-1}.day^{-1}$ at 2 kPa (one-way ANOVA, $P = .003$). Mean values at the hatching phase did not change significantly in response to hypoxia, with values ranging from 1.14 ± .36 μg protein.μg $RNA^{-1}.day^{-1}$ in normoxia to .8 ± .2 μg protein.μg $RNA^{-1}.day^{-1}$ at 2 kPa (one-way ANOVA, $P = .34$). Additional PO_2 levels completed at the eye phase gave values of 2 ± .5 μg protein.μg $RNA^{-1}.day^{-1}$ at 10.1 kPa and 0.53 ± .16 μg protein.μg $RNA^{-1}.day^{-1}$ at 5.1 kPa. These values were significantly different from each other (one-way ANOVA, $P = .018$), however, the mean k_{RNA} value recorded at 10.1 kPa showed no significant difference to the k_{RNA} recorded in normoxia. In contrast, the mean k_{RNA} at 5.1 kPa was significantly different to mean k_{RNA} values in normoxia (one-way ANOVA, $P < .001$) but not to mean k_{RNA} values in 2 kPa hypoxia. Data at the blastula phase (normoxia) are not available due to data corruption.

*Note all statistical analysis performed in section 4.3.3 was completed using log transformed values.

4.3.4. Costs of Protein Synthesis

As described in Chapter 3, the relationship between rates of oxygen uptake and protein synthesis in *C. pagurus* embryos during development can be examined by plotting whole embryo $\dot{M}O_2$ against absolute rates of protein synthesis (As) i.e. the quantity of protein synthesised per unit time (Figure 4.9). In contrast to the poor relationship found in normoxia, regression analysis showed a much stronger positive correlation between mean $\dot{M}O_2$ and A_s in hypoxia ($r^2 = 0.99$, $P = .003$). Consequently, the estimated metabolic cost for protein synthesis in *C. pagurus* embryos during 2 kPa PO_2 hypoxia was constant throughout development at 149 ± 4 mmol ATP. g $protein^{-1}$.

The background energetics i.e. $\dot{M}O_2$ at zero protein synthesis for each phase in development showed the same relationship as that found in normoxia. Backgound metabolic costs therefore remained stable during development in hypoxia until the hatching phase, where they were significantly higher (ANCOVA, $P \leq 0.001$).

4.3.5. Whole Embryo Lactate Accumulation

Preliminary results for lactate production by hypoxic and normoxic embryos at the eye phase in development of embryonic *C. pagurus* are shown in Figure 4.10a and b, along with corresponding values for the velvet swimming crab, *Necora puber*. For both species, lactate production was not significantly different between normoxic and hypoxic treatments during a four hour incubation period at a PO_2 of 2 kPa and at 10°C.

4.4. Discussion

The results from the present study clearly show that early phases in development are subjected to bouts of severe hypoxia in the developing egg mass. Ovigerous crabs incubating blastula embryos remained continuously buried beneath the substratum, restricting any abdominal movement and, therefore, convective oxygen supply to the developing embryos. Female crabs with embryos at this phase in development remained buried, although, reversals in ventilation could have been a source of oxygen provision to the embryos (Wheatly, 1981; Shields et al., 1991). Despite this possibility, PO_2 levels in the egg mass during the blastula phase were severely hypoxic. This is consistent with reports of reduced abdominal flapping and prolonged hypoxia in the developing egg masses of other Cancrid crabs during the early periods of embryo incubation (Naylor et al., 1999; Fernández et al., 2000; Baeza and Fernández, 2002; Fernández and Brante, 2003).

Hypoxia is known to delay rates of growth and development in many marine invertebrates that develop their embryos in large egg masses, as these structures increase diffusion distances and limit the passive diffusion of oxygen into the centre of the mass (Strathmann and Strathmann, 1989; Chaffe and Strathmann, 1994; Booth, 1995; Strathmann and Strathmann, 1995; Cohen and Strathmann, 1996; Cancino et al., 2003). However, incubation of blastula phase embryos from *C. pagurus* at a PO_2 level similar to that found in the natural brooding egg mass at this phase in development (Fernández et al., 2000), had very little short-term effects on metabolic rate or on the rates of protein synthesis and RNA activity (k_{RNA}). Only metabolic rate appeared to be influenced by hypoxia. Despite this influence, the effect of progressive hypoxia on $\dot{M}O_2$ was not as clear as the effects observed at later phases in development, which showed a more linear decline in $\dot{M}O_2$, see below. Blastula embryos showed an initial increase in $\dot{M}O_2$ during progressive hypoxia, prior to a significant decrease between a PO_2 range

of 12 – 2 Kpa, although normoxic $\dot{M}O_2$ values were not significantly reduced during progressive hypoxia. Considered as a whole, these findings provide further evidence in support of a possible quiescent or diapause period at the blastula phase in development. Moreover, a similar brooding behaviour has been reported in *Heterozious rotundifrons*, which also exhibits a quiescent or diapause growth period at the blastula, due to the burial of the egg mass beneath the substratum by the ovigerous female. This probably represents a common brooding strategy, with the female crabs deliberately exposing the embryos to hypoxia, to reduce rates of growth and prolong embryonic development (see below) (Taylor and Leelapiyanart 2001).

4.4.1. Metabolic Rates in Progressive Hypoxia (2 kPa)

Although no previous studies have included both metabolic rate and rates of protein synthesis in brachyuran embryos for comparison with the present work, metabolic rate in blastula phase embryos of *Cancer setosus* have previously been shown to be unresponsive to changes in oxygen level, while *C. pagurus* blastulae have been found to be unresponsive to changes in temperature (Wear, 1974; Naylor et al., 1999; Fernández et al., 2003). Furthermore, rates of protein synthesis determined during embryonic development in species with well characterised diapause phases show similarities with the results reported here. For example, the killifish, *Austrofundulus heterocyclius*, and the diapause gastrula embryo of the brineshrimp, *Artemia franciscana*, show 93% and ~80% reductions in rates of protein synthesis, respectively, when compared with non-diapausing phases in development. In addition, metabolic rate is unresponsive to exogenous stimuli in both of these species, which is symptomatic of the endogenously controlled refractory period of diapause, known to last for several months or even years in certain species (Watson and Smallman, 1971; Clegg, 1993; Hairston et al., 1996; Clegg, 1997; Podrabsky and Hand, 2000; Irwin et al. 2001). This could explain why hypoxia had little effect on whole embryo $\dot{M}O_2$ and rates of protein synthesis in the blastula phase of *C. pagurus* embryos.

The ability of blastula phase *C. pagurus* embryos to withstand prolonged hypoxia may also be partly due to the spherical shape of the embryo and, therefore, the three dimensional surface area for oxygen uptake, along with a relatively large surface area to volume ratio. These properties could be sufficient to meet the low oxygen demand of the blastula embryo, since most of these cells are concentrated at the surface of the egg where they are in close contact with the external PO_2 levels. Furthermore, the diffusive conductance associated with the egg membranes is likely to be higher in these early phase embryos, since the number of embryonic membranes synthesised as a result of moulting, is less when compared with later phases in development (see Chapter 2, Goudeau and Lachaise, 1983; Helluy and Beltz, 1991). Recent experiments investigating the ovicapsule diffusive conductance of the gastropod *Fusitriton oregonensis*, show an increase in oxygen supply associated with a thinning of the capsule wall during development (Brante, 2005). Similar properties are also known in the embryos of frogs which also develop in egg masses (Seymour and bradford, 1987; Seymour et al., 1991). Despite this, it could also be argued that the burying behaviour of the ovigerous female, the tight packing arrangement and poor water circulation through the egg mass, which could clump *C. pagurus* embryos together and restrict the available surface area for gas exchange, and, therefore, increase the eventuality of hypoxia.

Future additions to this work could involve using embryos samples immediately after spawning, which would provide metabolic data during early cleavage through to the blastula phase, allowing a more complete and accurate metabolic profile of these early phase embryos to be obtained. This would help to determine whether similar patterns of developmental changes in $\dot{M}O_2$ occur to those found in *A. fundulus* and *A. franciscana*. In addition, a longer time course during the present experimental regime, particularly in normoxia, would have probably been more informative regarding the responses of the blastulae to changes in PO_2 level; since it still remains a possibility that the low metabolic rate could have been accounted for by the relatively small size of the blastula embryo, therefore, surviving hypoxia by having low metabolic demands, as reported in other marine invertebrates (Booth, 1995).

Whole-embryo metabolism, and in particular oxygen uptake rate in *C. pagurus*, following the blastula phase in development, appears to be closely related to the PO_2 level present in the egg mass. Similar findings have also been shown in other brachyurans and species with developing egg masses, encompassing both passive and active egg brooders (Booth, 1995; Naylor et al., 1999; Baeza and Fernández, 2002; Mitchell and Seymour, 2003).

The relationship between whole-embryo $\dot{M}O_2$ and progressive hypoxia at the limb formation phase implies that the naturally occurring PO_2 levels in the egg mass cause a significant reduction in normal embryonic metabolic rate. This is consistent with the relatively large proportion of the total developmental time spent at the naupliar phases in development (see below, and Chapters 2 and 3).

Overall, hypoxia in the egg mass appears to be responsible for reducing rates of development during the early embryonic phases in *C. pagurus*, and could also play an important role in regulating the quiescent period in development at the blastula phase. This has been shown in a number of gastropod molluscs, where embryos at the centre of the egg mass develop asynchronously since they are exposed to chronic hypoxia (< 0.5 kPa), and as a result are arrested at cleavage phases in development until oxygen levels increase (Booth, 1995; Strathmann and Strathmann, 1995; Cohen and Strathmann, 1996). A similar response, but to the whole embryo brood, may also occur during the onset of the blastula phase in *C. pagurus*, when female ovigerous crabs bury in the substratum and expose the embryos to hypoxia, so lengthening their development times. To this end the lithodid southern king crab *Paralomis granulosa* is known for asynchronous embryonic development, with delayed hatching of embryos from the inner egg mass, caused in part by the general absence of brooding behaviours at low temperatures (Lovrich and Vinuesa, 1993; Thatje et al., 2003). This gives a good indication of the effect of hypoxia on rates of growth and development in brachyurans in the absence of maternal oxygen provision. Furthermore,

apparently these embryos are unharmed by exposure to hypoxia, which is an interesting observation, particularly since very little is known about the mechanisms of cellular protection or hypoxic gene expression in brachyurans or marine invertebrates as a whole. Some of these aspects will be addressed in Chapter 5.

Whole embryo $\dot{M}O_2$ at both the eye and hatching phases in *C. pagurus* conformed to the progressive decline in PO_2 level. The Pc increased with development, and was associated with an increase in the PO_2 levels present in the egg mass. This supports the suggestion that the ovigerous female maintains egg mass PO_2 levels above the Pc to ensure continued development of the embryos (Wheatly, 1981; Naylor et al., 1999). Furthermore, at both the eye and hatching phases long periods of abdominal flapping was characteristic of maternal brooding behaviours, which appeared to be almost continuous during both the day and night. This behaviour is stereotypical and has thus been identified as the main mechanism for oxygen provision to the developing embryos in brachyurans (Naylor et al., 1999; Ruiz-Tagle et al, 2002; Fernández and Brante, 2003).

An increased sensitivity to hypoxia during development probably reflects the increase in oxygen demand by the developing embryos (see below). Whether the Pc was a true reflection of oxygen sensitivity at the hatching phase is difficult to ascertain, since determination of resting $\dot{M}O_2$ levels were difficult to achieve for reasons described in Chapter 3. However, hypoxic incubation of hatching phase embryos resulted in far fewer hatched zoea, which could be an indication of the lowered oxygen supply restricting highly energetic activities, such as exercise. With this in mind, one possible explanation for the maintenance of relatively high PO_2 levels in the egg mass during the hatching phases could have been to improve hatching competence and hatching synchrony for the entire embryo brood, in addition to supporting whole embryo metabolism. Although, hypoxia has also been reported as a stimulus for hatching in certain species of frog (Petranka et al., 2002; Warkentin, 2002; Warkentin, 2005) and in *Nephrops norvegica*, but in combination with reduced survivorship (Eriksson et al., 2006).

However, it is unclear as to whether the same function exists in *C. pagurus* embryos.

Insensitivity to hypoxia at the blastula phase in development could be a result of the relatively low metabolic demand, resulting from the relatively small size of the embryo and limited physiological complexity. In addition, biochemical metabolism in embryonic *Emerita asiatica* and *Hyas araneus* has also shown that metabolic activity is relatively low prior to the eye phase in development, after which lipid consumption and enzyme activities increase, followed by a general decline at the hatching phase (Subramoniam, 1991, Petersen and Anger, 1997). Furthermore, rates of protein synthesis and protein accumulation identified in Chapters 2 and 3 are also relatively low during the early period of development, in keeping with low rates of oxygen uptake.

Increased sensitivity to hypoxia during later phases in development occur due to an increase in size and as a consequence of the increased rates of growth, particularly at the eye phase (see below and Chapters 2 and 3). In addition, other important factors that influence sensitivity to low oxygen during development include ontogenetic changes, such as the formation and functioning of the neuromuscular and cardiovascular systems (Chapter 3; Naylor et al., 1999; Taylor and Leelapiyanart, 2001). The cardiovascular system enables circulation of haemocyanin in the haemolymph, to improve oxygen extraction and delivery (review, McMahon, 2001). This is particularly important at later phases in development, as the distance between the centre of the egg and its periphery increase as development progresses and the eggs increase in size (see Chapter 2).

Despite the development of the cardiovascular system, metabolic rate of the developing embryos conforms to the PO_2 level present in the developing egg mass. The main reasons for this are probably associated with the compressed nature of brachyuran embryogenesis, by which the metabolically demanding protozoea and prezoea embryonic phases remain bound within the egg

membranes. This is in contrast to many crustacean taxa that hatch as nauplius embryos (Dahms, 2000). As a consequence of this mode of development used by brachyurans, the supply of oxygen during development is restricted and demand increased. This is because the flow of fresh oxygenated seawater across the gills is prevented by the encapsulation of the embryo within the egg membranes. Furthermore, embryonic membrane formations following successive moult cycles probably further limit the diffusive conductance and, therefore, supply of oxygen to the developing embryo at later phases in development.

As development progresses, embryonic growth and metabolism become increasingly sensitive to hypoxia, and more dependent on maternal oxygen supply through brooding behaviours. Oxygen conformity in decapods has also been shown during the zoea phases of the Antarctic krill, *Euphausia superba* (Quentin & Ross, 1989), but a number of studies report a limited capacity for oxygen-regulation in embryonic brachyurans. For example, Wheatly (1981) showed that embryos (phase unknown) from the shore crab, *C. maenas,* are able to regulate and defend their $\dot{M}O_2$ in declining PO_2, and at increasing temperatures, albeit to a lesser extent, e.g. Pc = ~10.6 kPa at 20°C, as opposed to ~6.7 kPa at 11.5°C. Baeza and Fernández (2002) also showed similar results (although not reported) that indicate late phase embryos of *C. setosus* have a similar capacity for the defence of metabolic rate to an estimated Pc of 6.7 kPa at 14°C, which is more or less the PO_2 maintained in the egg mass. Furthermore, but in contradiction to the present study, Naylor et al (1999) reported eye phase embryos of *C. pagurus* as having a high degree of respiratory independence, showing regulation down to a Pc of ~4 kPa at 14°C. The reasons for the observed differences in oxygen sensitivity between studies are not clear, but the conditions inside the respirometer, differences in the measurement techniques and protocols may be important. For example, the quantities of embryos used for the oxygen uptake measurements were markedly different, being much less in the present study. By using only a small quantity of embryos it is possible to make sure that all of the embryos in each sample are representative of the specific developmental phase being examined. This is particularly important when sampling from the egg mass during relatively early periods of brooding, where

the developing embryos show evidence of asynchronous development. For example, naupliar phase embryos can be sampled from the inner regions of the egg mass at the same time as eye phase embryos. With this in mind, larger sample sizes may be complicated by such sampling issues.

Generally decapod crustaceans do show a considerable tolerance of hypoxia through various respiratory and metabolic adaptations, the necessity for which probably reflects the benthic lifestyles of the adults (Taylor, 1976; Reiber and McMahon, 1998; McMahon, 2001). Life history plays an important role shaping developmental differences in the oxygen affinity of haemocyanin in decapods. For instance, in the dungeness crab, *Cancer magister,* and the Norwegian lobster, *Nephrops norvegicus* (Spicer, 1995; Terwilliger and Brown, 1993; Durstewitz and Terwilliger, 1997; Terwilligr and Ryan, 2001). Both these species show a positive relationship between oxygen transport capacity in the haemolymph and developmental phase. The pelagic larval phases of these species disperse and feed in the water column, where they are unlikely to experience environmental hypoxia, compared to the adults that live amongst the benthos. This could be an important part of the explanation for why oxygen transport capacity increases during decapodan development.

4.4.2. Rates of Protein Synthesis in Hypoxia (2 kPa)

Developmental patterns in fractional rates of embryonic protein synthesis (k_s) in *C. pagurus* differed from those observed for $\dot{M}O_2$. Generally, k_s during development (excluding the blastula) were reduced following exposure to a five fold reduction in oxygen level, although these reductions were not always mathematically significant. The standard response is for rates of protein synthesis to decline during hypoxia (Land et al. 1993; Smith et al., 1996; Hand, 1997; Larade and Storey, 2002). However, despite phase specific responses of k_s to hypoxia in embryonic *C. pagurus*, there was little variation in the whole-embryo k_s values reached during hypoxia, with values being maintained at approximately 5% day^{-1} (see below).

At the limb formation phase, hypoxic k_s values were approximately half of those observed in normoxic embryos. This decline was significant, showing that there is potential for a decrease in protein synthesis rates and, therefore, growth at this developmental phase during natural development within the egg mass.

Eye phase embryos were more clearly influenced by prevailing PO_2 levels with significant differences from normoxic values reported after treatment at PO_2 levels of 10, 5 and 2 kPa. A similar response is observed in the embryonic cysts of *A. franciscana*, where rates of protein synthesis undergo approximately an 80% reduction during exposure to anoxia, in keeping with other hypoxia tolerant species (see below) (Hand, 1997). The decline in k_s at a PO_2 of 5 kPa suggests that the mean PO_2 present in the egg mass during incubation of eye phase *C. pagurus* (12.2 ± 1.6 KPa) is necessary to maintain continued protein accumulation, and, hence, growth.

At the hatching phase, rates of protein synthesis were not significantly affected by hypoxia. As discussed in Chapter 3, it is possible that hatching phase embryos had lowered cellular rates of protein synthesis in keeping with their transition from egg-bound embryos to free-living larvae. Following on from this argument, it is possible that at the cellular level, the effects of hypoxia were less acute due to the lowered cellular rates of synthesis and the directly associated decreases in oxygen demand, although total $\dot{M}O_2$ was higher, owed largely to an increase in embryo activity (see below).

On the whole, many vertebrates and invertebrates undergo a rapid down regulation of aerobic metabolic rate when in hypoxia to reduce and rebalance metabolic ATP supply and demand (Guppy and Withers, 1999; Boutilier and Pierre, 2000; Boutilier, 2001). Downregulation of protein synthesis to reduce metabolic expenditure is key to this strategy, since protein synthesis accounts for a large proportion of basal metabolic rate (Houlihan et al., 1989; Houlihan et al.,

1990; Pannevis and Houlihan, 1992 Whiteley et al., 1996). Whole body rates of protein synthesis in C. *carassius* show a 40% net reduction in anoxia, due to tissue specific changes in the rates of protein synthesis (Smith et al., 1996). A similar relationship has also been reported in adult *C. maenas*, with a number of large reductions to in-vivo k_s observed in response to hypoxic challenge (Mente et al., 2003).

It is important, however, to acknowledge the more subtle differences within these metabolic strategies to hypoxia, in order to further appreciate the metabolic response shown in embryonic *C. pagurus*. For instance, rates of protein synthesis in anoxia tolerant turtles become undetectable during extended anoxia (> 1 h), whereas general rates of protein synthesis are not entirely inhibited during the same treatment in *C. carassius*, particularly in the brain, where rates of protein synthesis are maintained through a high dependence on anaerobic metabolism (Smith et al., 1996; Smith et al., 1999). This difference is also apparent in the behaviour patterns between the two species during anoxia, as *C. carassius* remains relatively active whereas the anoxia tolerant turtles become quiescent (Nilsson, 2001; Fraser et al., 2001).

In light of the results reported here, the full picture of the metabolic response in embryonic *C. pagurus* to hypoxia remains unclear. Although it is tempting to suggest that the maintenance of a common hypoxic k_s value may represent tissue specific regulation of protein synthesis, as found in *C. carassius* and *C. maenas*. This strategy seems more likely as embryos viewed under a light microscope following extended exposure to hypoxia e.g. 8 hour treatments of PO_2 levels of 2 kPa, showed rhythmic heartbeat and tail flicking activity. Interestingly, there were also indications of bradycardia in many but not all embryos at later phases in development (S. J. McCleary, unpublished observations), which has also been shown in a number of other species during developmental hypoxia, such as lobster, crayfish and fish embryos (Reiber, 1997; Barrioneuvo and Burggren, 1999; Harper and Reiber, 2004; Eriksson et al., 2006). Alternatively, and as

found in the turtle *Trachemys scripta elegans*, it is possible that protein synthesis may continue in hypoxia for a limited period using internal oxygen stores, which could be another reason for the common k_s value in hypoxia (Fraser et al., 2001). In embryonic *C. pagurus* this could occur through oxygen circulating in the haemolymph and/or oxygen present in the internal water of the egg and the egg mass. The use of oxygen stores to support metabolism is exploited by the cephalopod, *Nautilus pompilus* (Boutilier, 1996). A similar capacity in embryonic *C. pagurus* seems unlikely, as shown by the linear decline in metabolic rate during hypoxia.

An additional part of the hypoxic response shown in *C. pagurus* may involve activation of oxygen inducible genes and protein expression. This is known in a number of species, and could account for some of the k_s levels measured in hypoxia in the present study (Land and Hochachka, 1995; Gorr et al., 2004; Larade and Storey, 2002; Cheng et al., 2003). As the eggs in the present study were only exposed to hypoxia for one hour, a logical extension to the work would be to prolong the incubation times to study the long-term effects of hypoxia, which are likely to be different to those observed here.

4.4.3. Changes in RNA:protein ratios and RNA Activities in hypoxia (2 kPa)

As found with normoxic k_s values reported in Chapter 3, short-term changes in embryonic k_s in *C. pagurus* following exposure to hypoxia were not brought about by modifications to RNA:protein ratios. This response is not unusual because RNA capacity does not change in various tissues of *C. maenas*, and also in the rat and *Trachemys. s. elegans* exposed to hypoxia or anoxia (Preedy et al., 1985; Fraser et al., 2001; Mente et al., 2003). Bulk changes in tissue RNA reflecting modifications to cellular synthetic capacity, are more characteristic of long-term metabolic adjustments. For example, seasonal modification of metabolism in ectotherms, such as adaptation to low temperatures, although changes in RNA:protein have been reported in response to SDA in *Litopennaeus*

vannamei (Mathers et al., 1993; McCarthy et al., 1999; Mente et al., 2001; Fraser et al., 2004).

In the present study, changes in embryonic k_s were caused by changes to k_{RNA}, as shown by the strong correlation between the two variables at constant RNA:protein ratios. This relationship has also been demonstrated in a number of studies looking in to factors causing short-term adjustments to rates of protein synthesis (Preedy et al., 1985; Fraser et al., 2001; Whiteley et al., 2001; Mente et al., 2003; Storch et al., 2003). For example, the Q10 for k_{RNA} in fish hepatocytes following incubation at various temperatures was > 2 at constant RNA:protein (Pannevis and Houlihan, 1992). In addition, elevated protein synthesis rates during the moult cycle in adult *Homarus americanus* following injection of 20-hydroxyecdysone was mirrored by elevations to k_{RNA}, and not RNA:protein ratios (El Haj et al. 1996). Despite a downregulation of k_{RNA} to reduce protein synthesis in hypoxia, RNA synthesis was not downregulated. This has been shown in recent experiments using *C. carassius*, where Smith et al (1999) reported huge tissue specific changes in mRNA synthesis (+132%) in the heart, and (+871%) in the liver following a 48 hour exposure to hypoxia. Furthermore, transcript levels in *A.franciscana* remain stable following anoxia (Hardewig et al., 1996) indicating that protein synthesis is not inhibited through anoxic degradation of mRNA, but rather by translational inhibition. This theory is further supported by the fact that addition of mRNA to *A. franciscana* embryos in similar experiments was not sufficient to recover protein synthesis rates during anoxia (Hofmann and Hand, 1992). An increase in mRNA stability during anoxia is also a feature of a number of hypoxia inducible genes such as erythropoietin and vascular endothelial growth factor (VEGF), amongst others (review article, Ebert and Bunn, 1998). This is thought to facilitate a rapid resumption of protein synthesis, following the return of normal oxygen conditions in hypoxia tolerant species (Smith et al., 1999).

4.4.4. Costs of Protein Synthesis

The relationship between rates of protein synthesis and whole embryo $\dot{M}O_2$ was re-assessed using the values obtained from embryos subjected to one hour hypoxia. Hypoxic whole embryo $\dot{M}O_2$ and rates of protein synthesis showed a much closer correlation when compared with normoxic values in the previous chapter. The main difficulty observed in the previous chapter was that resting rates of $\dot{M}O_2$ during the hatching phase were not easy to record due to high embryo motility in the egg membranes. As stated above, the hatching of zoea were reduced in 2 kPa hypoxia and, therefore, $\dot{M}O_2$ was considered to be closer to resting values. Although and despite this fact, embryonic background energetics remained significantly elevated at the hatching phase, when compared to earlier phases in development, for reasons discussed in Chapter 3.

The theoretical cost of protein synthesis in hypoxic embryos was taken to be fixed during development, at 149 ± 4 mmol ATP. g protein^{-1}. Pace and Manahan (2006) also found a fixed cost relationship for protein synthesis during development in a sea urchin, despite a highly variable range in the rates of protein synthesis. In addition, when this cost is re-calculated from the mean values to match the calculation method used in the present study, the metabolic costs are very similar at ~110 mmol ATP. g protein^{-1}. However, the difference in *C. pagurus*, was that both $\dot{M}O_2$ and A_s values were reduced to a common value during hypoxia, negating the variation in A_s that was thought to be partly responsible for the poor relationship between $\dot{M}O_2$ and A_s in normoxic embryos (see Chapter 3). However, these are only preliminary conclusions, which are limited by the fact that rates of protein synthesis were influenced by hypoxia, and so do not give a true indication of its relation to normal metabolic rate. The use of a protein synthesis inhibitor may be more successful, although the problem of achieving resting metabolic rate at later phases in development still remains.

4.4.5. Preliminary Results: *Lactate accumulation at the eye phase*

Overall, results from this preliminary study appear to suggest that metabolic rate in brachyuran embryos during hypoxia is not defended through the activation of

anaerobic metabolism. This is in contrast to adult decapodans that show significant and large increases in blood lactate levels (Hagerman and Szaniawska 1986; Hagerman and Vismann, 1995; Mente et al., 2003). A low accumulation of lactate in progressive hypoxia or anoxia is indicative of a high tolerance to reduced oxygen level, and or a low metabolic rate. This is consistent with the linear reductions to whole embryo $\dot{M}O_2$ at the eye and hatching phases in *C. pagurus*, and also the reductions to rates of protein synthesis during progressive hypoxia at the limb and eye phases. Wang and Widdows (1991, 1993) have shown similar responses in bivalve larvae and spat of *Mytilus edulis*, which regulate metabolism at a reduced and stable level, but only in severe hypoxia (≤ 1 kPa). Similarly, early life phases are more tolerant in *M. edulis* owing to their large surface area to volume ratios and accompanying small diffusion distances for oxygen uptake. However, these physical benefits in developing eggs are limited by the egg membranes and the size of the egg mass.

Typically species that defend normal metabolic rate in declining oxygen levels, as found in adult brachyurans, accumulate anaerobic end products, such as lactate, more rapidly when compared with species that undergo a pronounced decline in metabolic rate (Hoback et al., 2000; Kolsch et al., 2001). However, anaerobic metabolism may play a limited role in metabolic response to hypoxia in embryonic phases as basal lactate levels only showed moderate increases in *C. pagurus* and *N. puber* embryos. One potential problem for the embryos, with regards to lactate accumulation, is its toxicity. Either the embryos have very effective mechanisms for removing lactate, or lactate accumulation is avoided because of the consequent effects on intracellular pH and on rates of protein synthesis, as reported in the extreme case of *A. franciscana*, where gastrula cysts endure high lactate levels for prolonged periods of time (Busa et al., 1982; Kwast et al., 1995; Kwast and Hand, 1996).

4.4.6. General Conclusion

In summary, this Chapter shows that the developing embryos of *C. pagurus* show phase specific differences in their metabolic responses to hypoxia. Blastula phase

embryos were unresponsive to hypoxia, possibly relating to metabolic quiescence. $\dot{M}O_2$ in limb formation phase embryos was more independent of changes in PO_2, compared to later phases which were strict oxygen conformers. This was likely to be due to an increase in oxygen demand following an increase in embryo size, an increased energy demand following organogenesis, and the restricted surface area for gas exchange at later phases in development.

Embryonic rates of protein synthesis were maintained at approximately 5% day in short term hypoxia at all developmental phases (excluding the blastula), although at the eye phase this value is representative of a 5-fold reduction since protein synthesis rates were highly elevated in normoxia. Overall, embryonic metabolism and, therefore, growth and development in *C. pagurus* is dependant on the level of oxygen present within the egg mass during brooding, which is ultimately controlled by the female crab. This is consistent with other studies that have investigated the relationship between rates of development and the PO_2 level in developing egg masses of marine invertebrates. Maternal control of development rate through oxygen provision could be important for reproductive synchronicity. For instance, metabolic quiescence at the blastula phase may limit the more serious implications of brooding hypoxia on embryonic development, such as retarded growth and uncontrolled cell death, as reported in hypoxia sensitive species, but also permit timing of the zoea larvae hatching period to coincide with exogenous food resources. Synchronicity of development in the embryo brood is also important to minimise the effects of predation, since observations of hatching in captivity showed that benthic scavengers were attracted to ovigerous females during the hatching period. The impact of this predation could be offset due to the sheer numbers of larvae released at any one time.

In addition, a delayed rate of development as a result of hypoxia has potential implications to fecundity. For example, less energy investment (yolk) is presumably required in each embryo per unit time of the total development period. This is because in species that exhibit a quiescent phase in development,

embryonic metabolic rate is low during this time period (Petersen and Anger, 1997; Taylor and Leelapiyanart, 2001). Furthermore, the effect of this would also influence the parental costs associated with embryo brooding, since ovigerous *C. pagurus* have also been shown to be hypometabolic during resting periods (Naylor et al., 1997).

Finally, the tight regulation of PO_2 levels present within the egg mass close to or at the Pc for embryonic metabolic rate indicates that the developing embryos have a limited capacity to regulate metabolism during development in the egg mass (Wheatly, 1981; Naylor et al., 1999; Baeza and Fernández 2002). The absence of metabolic defence in hypoxia could save a greater proportion of the available endogenous energy for growth, which may improve larval fitness by maximising growth and, therefore, size at hatching (Lonsdale and Levinton, 1985; Lee and Strathmann, 1998). The restricted embryonic surface area, high competition for oxygen uptake, and convective restrictions to oxygen supply would severely limit the success of metabolic defence in the embryo mass of brachyurans, and similarly in other marine invertebrates. The molecular mechanisms that may assist the physiological changes during hypoxia, such as cellular defence and hypoxia inducible gene expression will be investigated in the final chapter.

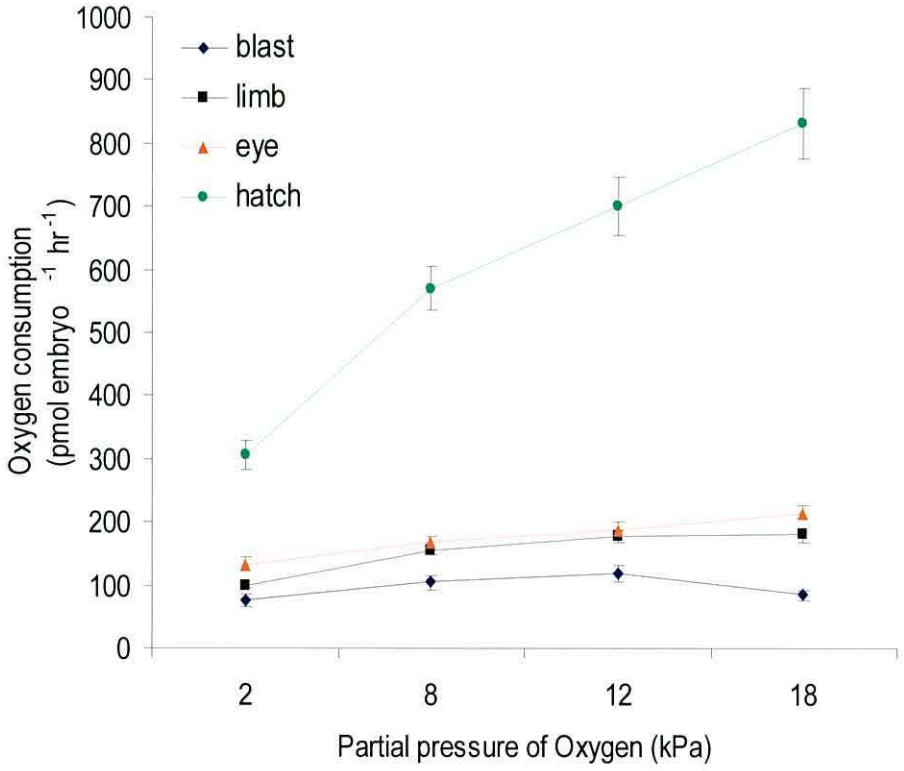
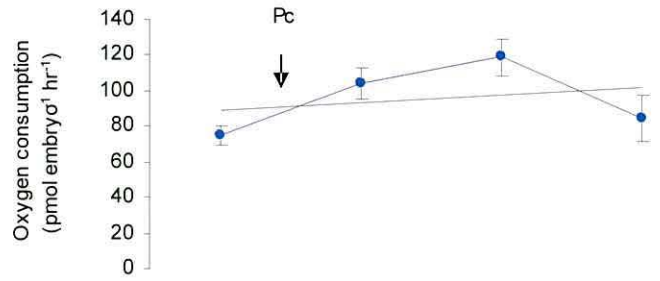
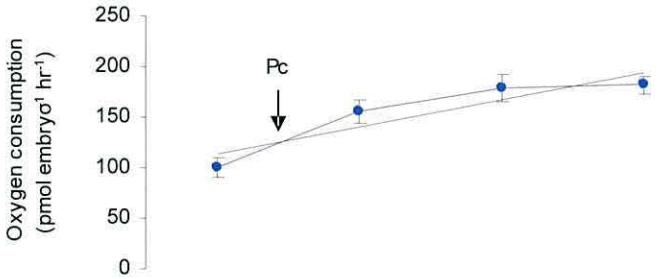


Figure 4.1. Effects of progressive hypoxia on whole embryo rates of oxygen uptake ($\dot{M}O_2$) at each of the key phases in embryonic development (Blastula, Limb formation, Eye, Hatching) of *Cancer pagurus* at 10°C. Values are means ± SE (n = 6 except for the blastula, where n = 5).

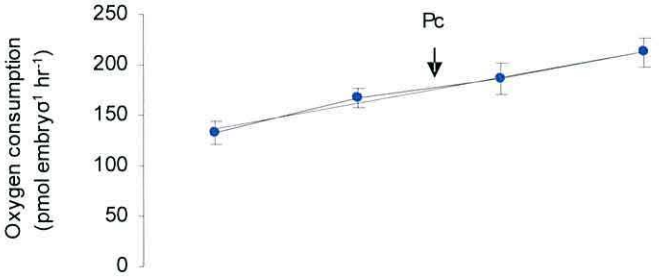
Figure 4.2. Effects of progressive hypoxia on whole embryo rates of oxygen uptake ($\dot{M}O_2$) and critical partial pressure (Pc) at each of the key phases in embryonic development of *Cancer pagurus* at 10°C. The broken line on each plot is the relationship between $\dot{M}O_2$ and progressive hypoxia, which are described by the following equations: (a) Blastula ($y = 4.5x + 84.5$, $r^2 = 0.08$; $P = .72$); (b) Limb formation ($y = 27x + 86.5$, $r^2 = .84$; $P = .08$); (c) Eye ($y = 25.99x + 109.95$, $r^2 = .99$; $P = .003$); (d) Hatching phase ($y = 171.23x + 173.75$, $r^2 = .97$; $P < .015$). Values are means ± SE (n = 6 except for the blastula, where n = 5).


Pc
Oxygen consumption (pmol embryo^{-1} hr^{-1})
140
120
100
80
60
40
20
0

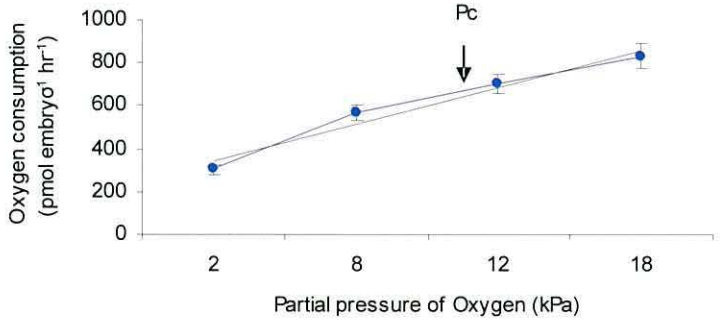
a


Pc
Oxygen consumption (pmol embryo^{-1} hr^{-1})
250
200
150
100
50
0

b

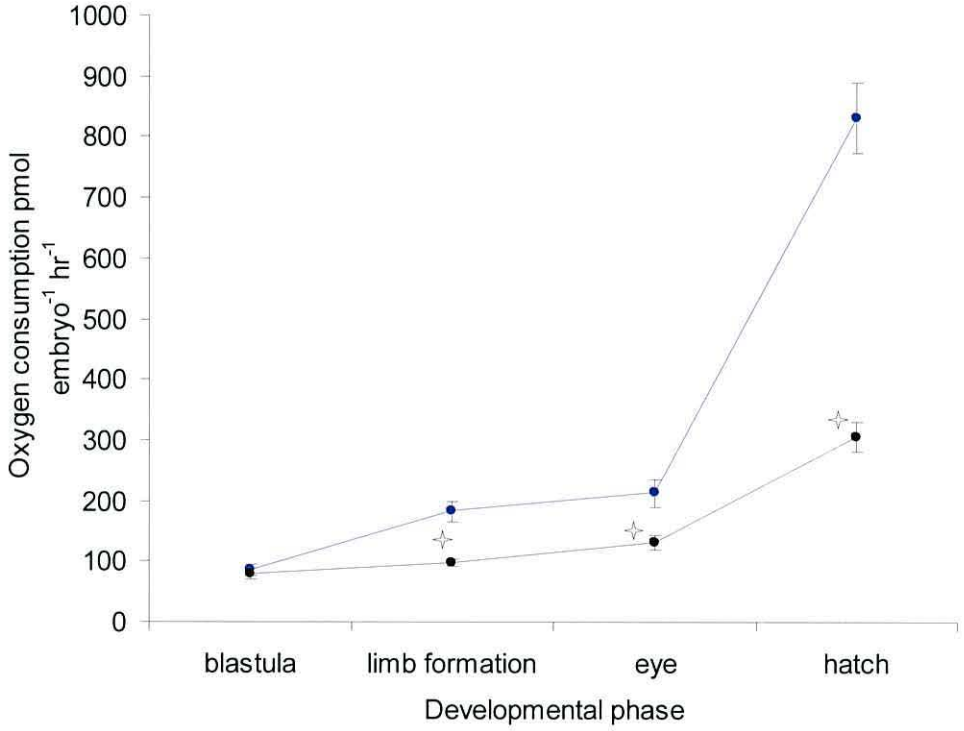

Pc
Oxygen consumption (pmol embryo^{-1} hr^{-1})
250
200
150
100
50
0

c


Pc
Oxygen consumption (pmol embryo^{-1} hr^{-1})
1000
800
600
400
200
0
2
8
12
18
Partial pressure of Oxygen (kPa)

d

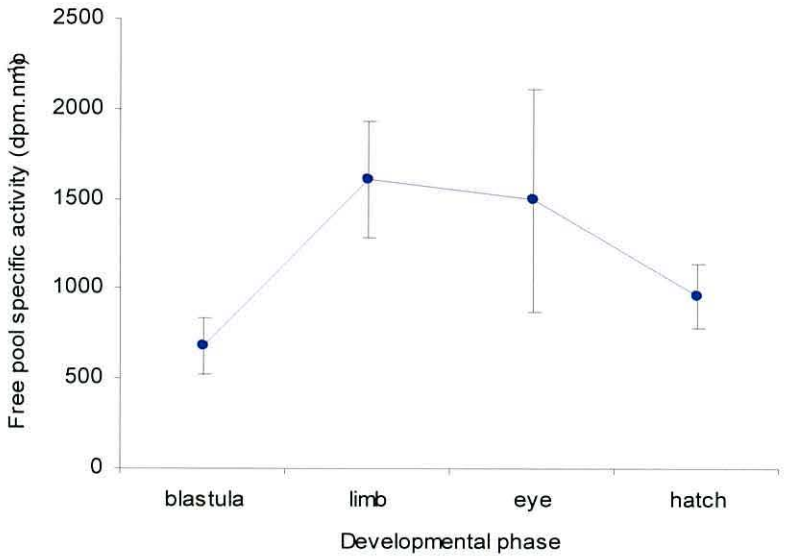
Figure 4.3. (a) Changes in whole embryo rates of oxygen uptake ($\dot{M}O_2$) in normoxia, and after exposure to hypoxia (2 kPa) at all key phases in embryonic development (Blastula, Limb formation, Eye, Hatching) in *Cancer pagurus* at 10°C. Hypoxic means significantly different from normoxia represented by an asterisk (one-way ANOVA, $P < 0.05$). Values are means ± SE (n = 6 except for the blastula, where n = 5). (b) Tabulated mean values of oxygen partial pressure (PO_2) present at the centre of the developing egg mass at each of the four key phases in development of *Cancer pagurus*. Values are means ± SE (n = 6 at the blastula and hatching phases, n = 7 at limb formation, and n = 9 at the eye phase).



a

Developmental phase	PO_2 (kPa) at the centre of the egg mass
Blastula	3.5 ± .3
Limb formation	5.4 ± .6
Eye	12.2 ± 1.6
Hatching	15.2 ± 1

b



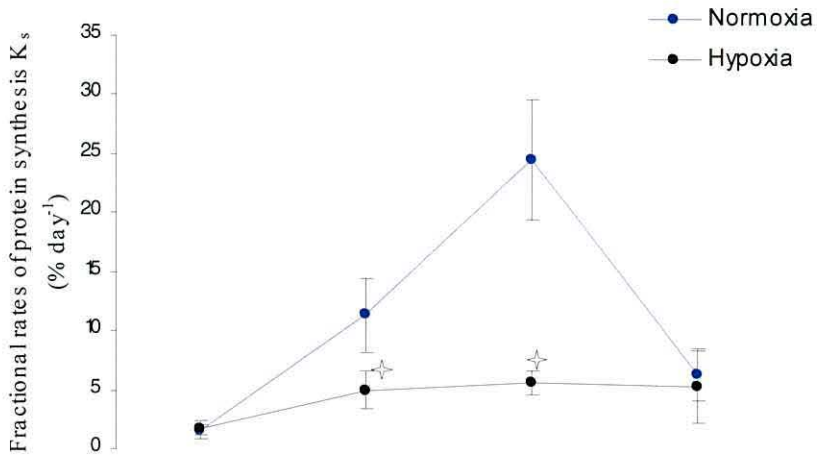
a

Development	Sa, dpm.nmol Phe (Normoxia)	Sa, dpm.nmol Phe (2 kPa)
Blastula	1431 ± 381	683 ± 159
Limb formation	1176 ± 188	1609 ± 320
Eye	603 ± 61	1492 ± 620
Hatch	790 ± 83	959 ± 181

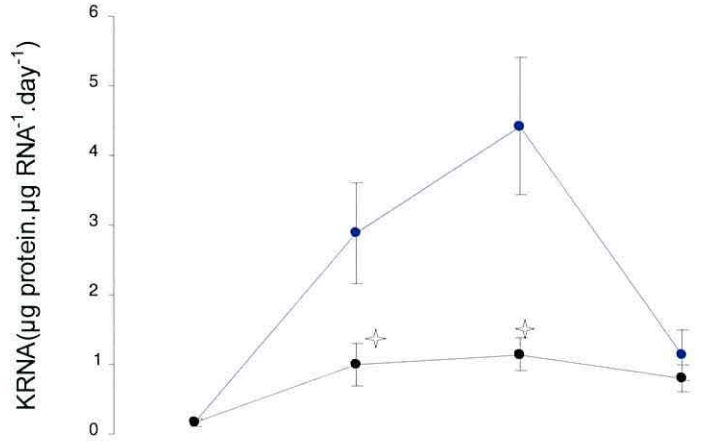
b

Figure 4.4 (a) Specific activities of the free-pool phenylalanine at all key phases in embryonic development (Blastula, Limb formation, Eye, Hatching) in *Cancer pagurus*, exposed to hypoxia (2 kPa) after incubation in (^{3}H) phenylalanine at 10°C. Values are means ± SE (n = 6 except at the hatching phase, where n = 7). (b) Tabulated specific activities of the free-pool phenylalanine from key phases in embryonic development of *C. pagurus*, after exposure to normoxia, or hypoxia.

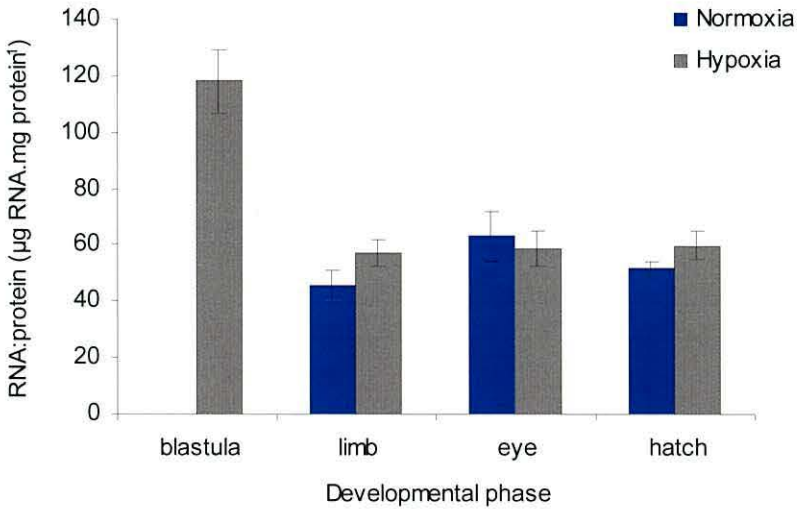
Figure 4.5. Changes in whole embryo fractional rates of protein synthesis (K_s, % day^{-1}) (a), RNA activity (k_{RNA}) (b), and RNA:protein ratios (c), during normoxia and after exposure to hypoxia (2 kPa), at each of the key phases in development of *C. pagurus* at 10ºC. Hypoxic means significantly different from normoxia represented by an asterisk (one-way ANOVA, $P < 0.05$). Values are means ± SE (n = 6 except at the hatching phase, where n = 7).


Normoxia
Hypoxia
Fractional rates of protein synthesis K_s (% day^{-1})
35
30
25
20
15
10
5
0

a


KRNA(µg protein.µg RNA^{-1}.day^{-1})
6
5
4
3
2
1
0

b


Normoxia
Hypoxia
RNA:protein (µg RNA.mg protein^{-1})
140
120
100
80
60
40
20
0
blastula
limb
eye
hatch
Developmental phase

c

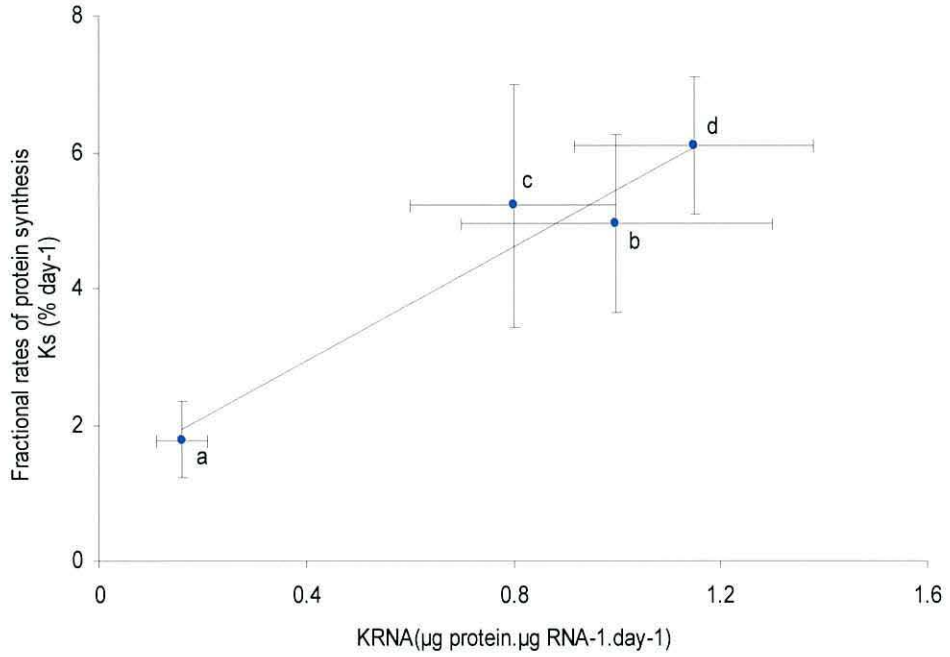


Figure 4.6. The relationship between mean RNA activity (k_{RNA}) and mean fractional rates of protein synthesis (K_s, % day^{-1}) during development (a = Blastula, b = Limb formation, c = Eye, d = Hatching) after exposure to 2 kPa hypoxia in *C. pagurus* at 10ºC. The broken line is the regression of k_{RNA} and K_s, and is described by the linear regression equation y = 4.19x + 1.25, r^2 = .94; P = .031. Data at the blastula represents substituted values recorded in 2 kPa hypoxia (see Chapter 4).

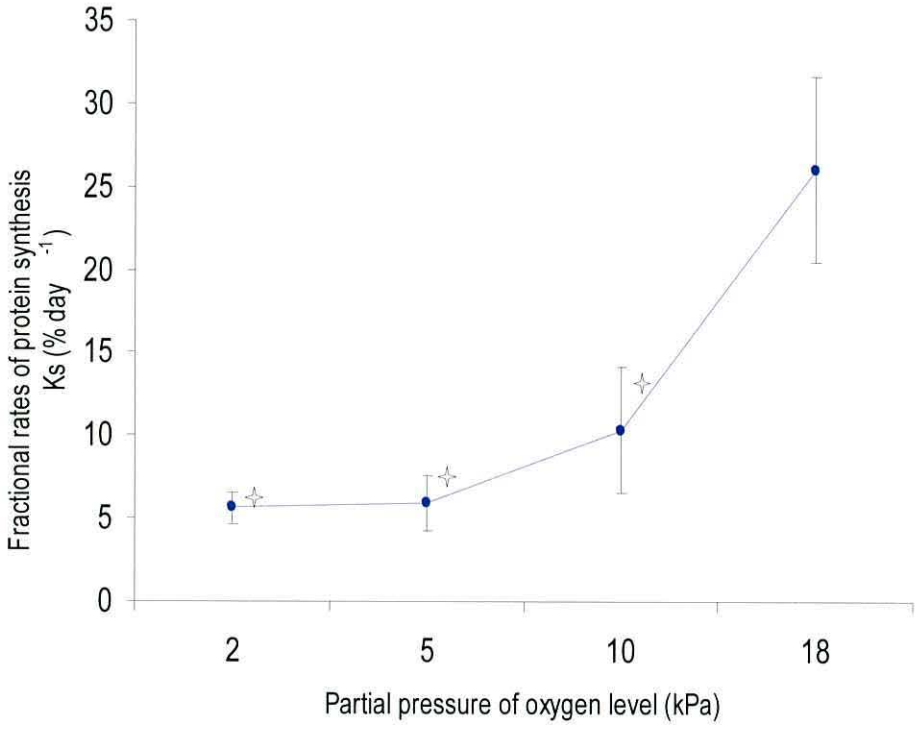


Figure 4.7. Effect of progressive hypoxia on whole embryo fractional rates of protein synthesis (K_s, % day^{-1}) at the eye phase in development in *Cancer pagurus* at 10°C. Hypoxic means significantly different from normoxia represented by an asterisk (one-way ANOVA, $P < 0.02$). Values are means ± SE, n = 6 at 18 and 2 kPa, and n = 4 at 10 and 5 kPa. The broken line is from the regression equation y = 6.6x + 4.5, $r^2 = .78$; $P = .262$.

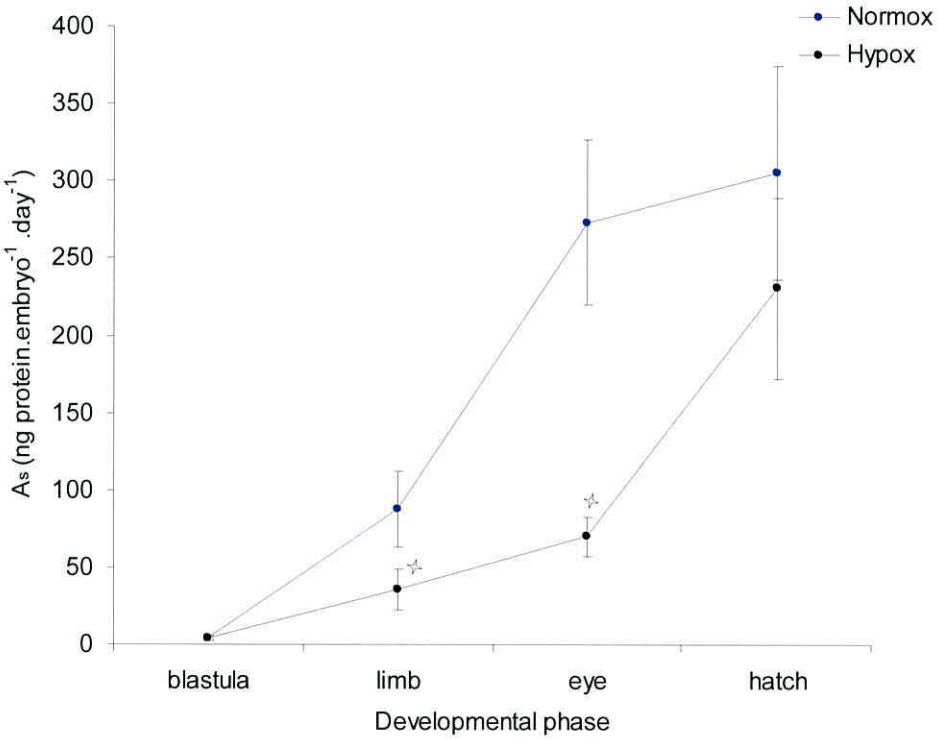


Figure 4.8. Changes in whole embryo absolute rates of protein synthesis (*As*) in normoxia, and after exposure to hypoxia (2 kPa) at all key phases in embryonic development (blastula, Limb formation, Eye, Hatching) in *Cancer pagurus*, after incubation in (^{3}H) phenylalanine at 10ºC. Hypoxic means significantly different from normoxia represented by an asterisk (one-way ANOVA, $P < 0.05$). Values are means ± SE (n = 6 except at the hatching phase, where n = 7).

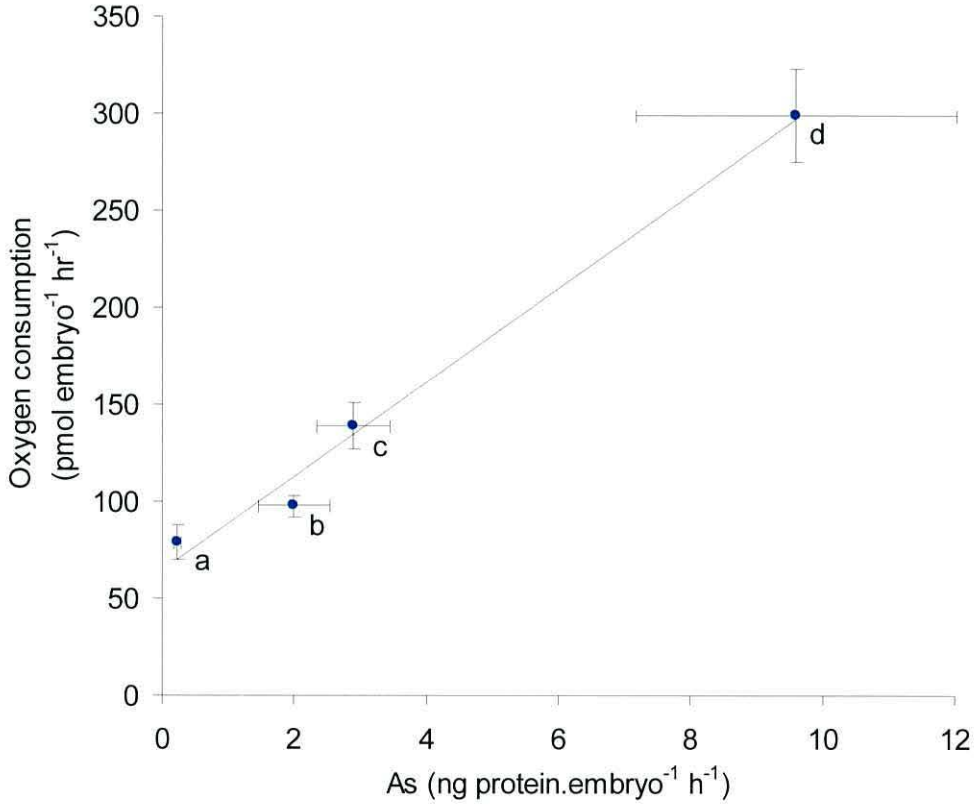


Figure 4.9. The relationship between mean absolute rates of protein synthesis (A_s) and mean whole embryo rates of oxygen uptake ($\dot{M}O_2$) during exposure to hypoxia (2 kPa) at each of the key phases in development of *C. pagurus* at 10°C. The broken line is from the regression equation y = 24.9x + 64.6, $r^2 = 0.99$; $P = .003$. Values are means ± SE (n = 6 except at the hatching phase, where n = 7)

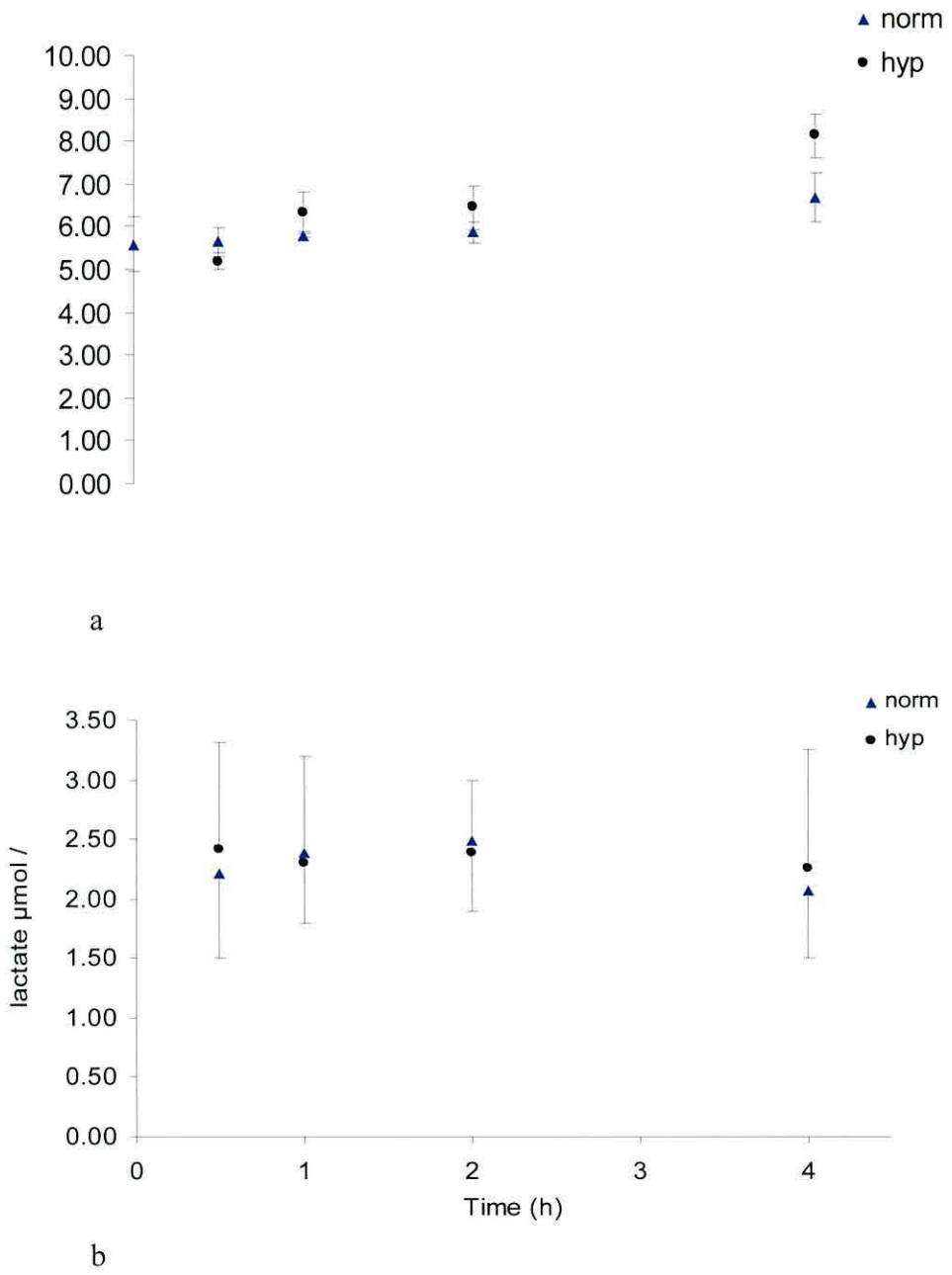


Figure 4.10. Changes in whole embryo lactate levels of the eye and chromatophore phase in development of: (a) *Necora puber*; (b) eye phase in development of Cancer pagurus, during exposure to normoxia, and hypoxia at (2 kPa) at 10ºC. Values are means ± SE (n = 5 in (a), and 3 in (b).

Chapter 5

Cellular Responses to Hypoxia during embryonic development in *Cancer pagurus*

Evidence was given in Chapter 4 to suggest that early phases in the embryonic development of *Cancer pagurus* survive natural brooding hypoxia possibly by entering quiescent periods at early developmental phases, and through oxygen conformation of metabolic rate and protein synthesis at more advanced phases in development. Cellular protection against hypoxic damage also appears to form a crucial aspect of the oxidative stress tolerance mechanism employed during embryonic development in *C. pagurus*. Hsp 70 and related homologues were found at all of the key phases in development following Western blotting, although the response was greatly attenuated by the hatching phase. ^{35}S methionine/cysteine protein labelling of newly synthesised protein classes also showed evidence of these hsps', and interestingly other protein classes were both induced and suppressed in response to progressive hypoxia.

Transcriptional responses to hypoxia, and also modification of the cell cycle were other potential mechanisms of cellular protection against hypoxia that were investigated in embryonic *C. pagurus*. In particular, the use of Bromodeoxuridine to label newly synthesised DNA during the S-phase of cell division, provided preliminary evidence of a phase specific response to hypoxia. Collectively, the physiological and molecular responses to oxidative stress during embryonic development in *C. pagurus* appears to be an appropriate defence against the natural brooding hypoxia associated with growth and development in a large egg mass.

5.1 Introduction

Many species have been identified as having an innate capacity to survive a range of environmental conditions, including changes in temperature, salinity, UV light, anthropogenic xenobiotics, and environmental hypoxia or anoxia. However, exposure of an organism to these environmental parameters is indiscriminate and, therefore, occurs at any phase in the life cycle, including the most vulnerable embryonic phases in development.

The capacity of a species to tolerate exposure to environmental stress is usually related to its natural history. Many marine invertebrates produce embryos that are highly resistant to environmental stress. This is necessary for both broadcasting and egg brooding reproductive strategists, although the types and level of risk are different for each (Strathmann, 1985; Booth, 1995; Lee and Strathmann, 1998; Strathmann et al., 2002). Broadcasted planktonic embryos and larvae drift in the highly changeable surface waters, owing to their positive buoyancy and phototactic behaviour (Vuorinen et al., 1999). As a result, many species show a range of coping strategies such as the presence of natural sunscreens, additional egg membranes, and rapid rates of growth and development, amongst others (McClintock and Baker, 1997; Epel, 2002; Strathmann et al., 2002). Alternatively, those marine invertebrates that spawn their embryos in egg masses, albeit regarded as being well protected when compared with planktonic species, the developing embryos still face a barrage of environmental stressors, possibly the most notable of which is the exposure to hypoxia (Booth, 1995; Cohen and Strathmann, 1996; Naylor et al., 1999; Fernández et al., 2000; Baeza and Fernández, 2002).

Hypoxia is an associated feature of egg masses, and is known to have a range of effects from reduced or retarded growth rates to increased mortality rates (Wickland and Sundelin, 2001; Frazier et al., 2001; Steer et al. 2002). Many

strategies are employed to increase oxygen supply in egg masses including: 1) different shaped egg masses, such as the spiralled ribbon egg capsules of certain gastropods; 2) foamy egg masses of anuran frogs that contain air bubbles as an immediate oxygen store for the embryos; 3) the selection of well oxygenated and cool environments for oviposition; 4) and ultimately direct oxygen provision through parental care (Burggren, 1985; Booth, 1995; Seymour and Loveridge, 1994; Lee and Strathmann, 1998; Ruiz-Tagle et al, 2002; Fernández and Brante, 2003; Green and McCormick, 2005).

Despite the diversity of these different reproductive strategies, many embryos that develop within egg masses, including brooded embryos, are exposed to hypoxia, and sometimes for prolonged periods of time (Booth, 1995; Naylor et al., 1999; Fernández et al., 2000). The ontogeny of physiological adaptations to hypoxia is unclear. There is accumulating evidence that the ability of many species to withstand hypoxia is tolerated through mechanisms that have been well described in adults. Physiological and metabolic responses to hypoxia in adult life phases have been well documented (Taylor, 1976; Butler et al, 1978; Taylor, 1982; Reiber and McMahon, 1998; McMahon, 2001). In addition, the latter can generally be categorised into the activation of anaerobic metabolism, which is useful as a short term solution, or depression of metabolic rate, which is the most effective strategy for tolerance of prolonged hypoxia, and has also been shown in a number of species during embryonic development (Clegg, 1993; Clegg, 1997; Naylor et al., 1999; Baeza and Fernández, 2002). In addition to both the biological design of egg masses, and the physiological mechanisms utilised to limit the effects of hypoxia, there are also molecular responses to hypoxia, which are receiving more attention as hypoxia sensitive gene regulation is shown to be wide-spread (Land and Hochachka, 1995; Gracey et al, 2001; Larade and Storey, 2002; Cheng et al., 2003; Gracey and Cousins, 2003; Gorr et al., 2004; Semenza, 2004 and references within). The interest in the latter has gained momentum since the development of genomic technologies.

Molecular responses to environmental stress have been studied in a number of model organisms such as the fruit fly, *Drosophila melanogaster,*, diapause embryos of the brineshrimp, *Artemia franciscana*, and embryos of the African clawed toad *Xenopus laevis* (Arrigo and Tanguay, 1991; Clegg et al., 1995; Clegg, 1999; Heikkila et al., 1997; Douglas et al., 2001; Douglas et al., 2005). A combination of strategies appears to be important for improving tolerances to hypoxia and other environmental stressors, which includes cellular protection with heat shock proteins (Hsps), hypoxia inducible gene expression, and modification of the cell cycle in hypoxia. Initial experiments were carried out to investigate whether one or all of these modifications have a role to play in the survival of *Cancer pagurus* embryos during severe hypoxia, and whether or not these responses, if they indeed exist, change with development.

The expression of Hsps has been demonstrated during embryonic development, and in response to environmental stress in a number of marine invertebrates, including crustaceans. Similarly, hypoxia inducible gene expression, which could be representative of Hsp expression, has also been reported in marine invertebrates (Larade and Storey, 2002). Despite this, and even though many invertebrates develop in hypoxic egg masses, little is known about the mechanisms that safeguard the cell against hypoxia and other environmental stress. Furthermore, in *X. laevis* it appears that maternal small molecular weight Hsps are responsible for protecting early phase embryos against stressful episodes (Heikkila et al., 1987; Davis and King 1989). In later phases, less Hsps are synthesised meaning that embryos are potentially more susceptible to stress, suggesting phase specific differences in the ability of the *X. laevis* embryos to survive environmental stress.

Exposure to hypoxia could invoke a comparable cell defence strategy in the embryonic phases of *C. pagurus*, especially when the embryos are subjected to prolonged hypoxic episodes at early phases in development and when they are also potentially the most vulnerable. Patterns of Hsp expression were, therefore, examined in the same 4 phases of *C. pagurus* development as those examined in

the previous chapters, to determine if phase specific differences in Hsp response exist in *C. pagurus*. Experiments will concentrate on Hsp70, the major family of heat shock proteins, and one that is known to respond to hypoxia (Airakinsinen et al., 1998; Cheng et al., 2003; Scott et al., 2003). As Hsps are ubiquitous, Hsp70s in a diverse range of species will cross react with monoclonal antibodies raised against the human form of Hsp70, making immuno-detection by Western blotting the desired method for identifying Hsp70 protein synthesis patterns.

In addition, general protein synthesis patterns will be investigated by exposing *C. pagurus* embryos to a metabolic label (^{35}S-methionine/cysteine) and separation of the labelled proteins by one-dimensional SDS PAGE for visualisation by autoradiography. This technique has been successfully applied to the lecithrotrophic embryos of the gastropod *Haliotis rufescens* (Varvra and Manahan, 1999), and should enable comparison of one-dimensional protein expression profiles of embryonic *C. pagurus* following incubation in normoxia with those produced in hypoxia. Hopefully this will also enable the identification of both hypoxia inducible and suppressible gene expression during development.

To gain an appreciation of the specific effects of hypoxia at the level of gene transcription, as opposed to the translational level outlined above, initial molecular studies will be carried out to amplify a cDNA for the alpha subunit of hypoxia inducible factor 1 (HIF-1α) in *C. pagurus* embryos. HIF-1α forms the hypoxia effective subunit comprising the heterodimer hypoxia inducible factor 1 transcription factor, responsible for the transcriptional activation of many hypoxia inducible genes important for oxygen homeostasis (review, Semenza, 2004). A range of studies have shown that HIF-1α has been found in a number of vertebrates and invertebrates inclusive of crustaceans, and also that HIF-1α plays a major role in the formation of the cardiovascular system in mammalian embryos (Iyer et al., 1998; Bacon et al., 1998; Soitamo et al., 2001; Jiang et al., 2001; Gorr et al., 2004).

Finally, the effects of hypoxia on cell division during embryonic development in *C. pagurus* will be investigated by following the incorporation of the thymidine analog bromodeoxyuridine (BrdU) into the cellular DNA during the S-phase of mitosis. Recent experiments using *D. melanogaster* have effectively applied this technique to developing embryos incubated in either normoxia or hypoxia (Douglas et al., 2001; Douglas et al., 2005). This has proved a useful approach, enabling the effect of hypoxia on cell division, and by association, growth and development, to be visualised using confocal microscopy. A number of studies have also applied the same technique to study neurogenesis in developing embryos of certain decapods (Harzsch et al., 1998). In light of this previous work, it would be interesting to see if a similar approach could be applied to further study the influence of hypoxia on the progression of growth and development in *C. pagurus*.

5.2. Material and Methods

5.2.1. Animal Maintenance and Egg Collection

Female *Cancer pagurus* were collected and maintained in aquaria at the University of Wales, Bangor as outlined in Chapter 2. The sampling procedures used for egg collection during all of the experiments outlined in this chapter matched those described previously. In addition, ovigerous *Carcinus maenas* were collected from Menai Straits, Anglesey (53.23°N, 4.16°W), and maintained in the same holding system. Embryos from *C. maenas* were included for the characterisation of heat shock expression patterns.

5.2.2. Heat Shock Protein 70

5.2.2.1. *Heat Shock Protein 70 Sample Preparation and Experimental Regime*

Typically 300-400 mg of crab embryos were subdivided into four equal groups of approximately 100 mg each. Three of these sub-samples were placed into separate incubation chambers (6x3cm), and left to acclimate at 10°C in gently aerated seawater for 30 minutes. The remaining sub-sample was flash frozen in liquid nitrogen and stored at -20°C. Each treatment group was started at 5 minute intervals to allow for sample processing time following the completion of the incubation period. After sealing the incubation chambers, a small needle connected to either a gas mixing pump (Wösthoff SA27/3-F, Bochum, Germany) for treatment with hypoxia, or an air pump (Whisper 400) for normoxic incubation, was inserted through the lid, to bubble the appropriate gas mixture into the seawater. This also served to gently mix the eggs to ensure an even distribution of the prevailing PO_2 level. All incubation chambers were then placed into a water bath (Grant Y22) and submerged almost entirely to maintain constant temperature. Each sample was incubated for 2 hours, which was the

time found necessary to observe a heat shock response in previous experiments using embryonic *Carcinus maenas* (N. M. Whiteley, unpublished observations).

Where appropriate, heat shock control experiments using adults of both *C. pagurus* and *C. maenas* were completed by placing animals into separate glass tanks (45x30cm), containing aerated re-circulating seawater and heated by a water heater. The experiment was started for animals designated as heat shock positive controls, when the flow of recirculating seawater was stopped by closing a valve on the tank inflow pipe, and the temperature of the holding water increased by 10ºC. For negative controls, adult *C. maenas* were fished from shallow water and processed immediately on the shore, and gill tissue dissected and kept on ice, until storage at -20ºC. Adult *C. pagurus* selected as negative controls were left undisturbed overnight in the experimental tanks, and processed by dissection of gill tissue, and stored as for *C. maenas*.

5.2.2.2. ***Embryonic Heat Shock Protein 70 kDa (Hsp70) Experiments***

Tissue from both embryonic *C.pagurus* and from embryonic *C. maenas* were analysed for heat shock protein 70 (Hsp70) synthesis. Embryos of *C. pagurus* were heat shocked at each of the four key phases in development used in the control experiments. Sub-samples of embryos were incubated as follows: negative control, 10°C in normoxia (18-20 kPa); heat shock, 23°C in normoxia (18-20 kPa); hypoxic, 10°C in (2 kPa) PO_2 . Following incubation, the embryos were removed from the incubation chambers, drained and instantly snap frozen in liquid nitrogen, for storage at -20°C. In addition, all key phases excluding the blastula phase in *C. maenas* embryos were subjected to the same period of normoxia and hypoxia, but were not heat shocked. Each experiment was completed in duplicate for *C. pagurus*, and singularly for *C. maenas*, and the presence of Hsp70 detected by Western Blotting using a monoclonal antibody against Hsp70 (Affinity BioReagents, MA3-006).

5.2.2.3. ***Analysis of Heat shock Protein Synthesis***

Frozen samples were ground under liquid nitrogen into a fine powder and placed into sample buffer (62.5mM Tris, pH6.8) and kept on ice for protein separation according to Laemmli (1970, leigh thesis). A 20 µl sub-sample was assayed for total protein as described in Chapter 2. Following total protein quantification, the samples were boiled for five minutes in 20 µl SDS buffer (62.5mM Tris pH6.8, 10% Glycerol, 2% SDS, 5% 2-mercaptoethanol and bromophenol blue) and loaded according to protein content into each well of a 12% polyacrylamide SDS-PAGE gel. Each gel was calibrated with a pre-stained molecular transfer marker (Sigma Rainbow marker 14300-220000Da). Electrophoresis was completed at a constant voltage of 200 V.

Following completion of electrophoresis, the polyacrylamide gel was prepared for Western blotting. The gel was placed on top of a nitrocellulose membrane (Hybond-C extra, Amersham) and electro-blotted over night, at 50 mA in transfer buffer using a standard procedure (25mM Tris pH7, 0.2M Glycine, 20% Methanol).

Subsequently the nitrocellulose membrane was taken through a series of washes for the detection of transferred proteins. Firstly the membrane was blocked for non-specific protein transfer, in a 2% milk powder phosphate buffered saline Tween20 (PBS-tween) for 60 minutes, followed by three 5 minute washes in PBS-tween. Next the membrane was bathed for 120 minutes in primary antibody diluted 1/500 in PBS-tween, followed by 120 minutes in secondary antibody (Goat anti-mouse IgG (H+L)/PO) diluted 1/1000. This was used to specifically target the transfer of Hsp70 present on the transfer membrane. Washes, both before and after the secondary antibody incubation with PBS-tween were the final preparatory steps prior to colorimetric visualisation of immunoreactive transferred proteins. For visualisation of the hybridised bands, the membrane was bathed in 20mls TBS buffer pH 7.8, and then placed into developer solution (4ml Methanol, 20mg Chloronapthol) containing 10 µl of hydrogen peroxide and left for 20 minutes for the colour to develop. After drying a digital image of the

Western blot was captured using a Gel Documentation System (Biorad Gel Doc 2000).

5.2.2.4. *Protein Identification (Mass Spectrometry)*

Preliminary experiments were completed to identify heat shock protein bands using mass spectrometry. This was attempted by running a 12% polyacrylamide SDS-PAGE gel (see above) using sub-samples known to produce immunoreactivity with Hsp70 (Affinity BioReagents, MA3-006). Following one dimensional SDS-PAGE protein separation with 12% polyacrylamide, the resulting gel was stained overnight in Coomassie brilliant blue G250. The gel was then de-stained for 4-5 hours after which an image was captured using a (Biorad Gel Doc 2000) camera. Images from Coomassie staining and western blotting were then compared and the desired protein bands were selected for excision from the gel. The digital image of the Coomassie stained gel was also used to compare total protein profiles with those of the newly synthesised protein pools of the embryos (see Section 5.2.3). Protein bands of interest were removed using a sterile scalpel blade and placed into eppendorf tubes ready for tryptic digestion. A known positive control (Myoglobin) and a negative control (piece of protein-free polyacrylamide gel) were also taken through the digestion protocol. Each sample band was de-stained using 50mM Ambic:50% acetonitrile (ACN) for 10 minutes, twice, before being reduced in dithiothreitol for 30 minutes. Samples were then alkylated using iodoacetamide in the dark for 30 minutes, and finally dehydrated in 100% ACN. The samples were later digested overnight in trypsin, and the reaction stopped by the addition of 2.6 mol l^{-1} formic acid. Following evaporation in a vacuum centrifuge, the samples were rehydrated in 0.1% trifluroacetic acid (TFA), mixed 1:1 with matrix solution (αCyano-4-hydroxy cinnamic acid 1:2 TFA 0.1%) and spotted onto a MALDI target for peptide ion analysis.

5.2.2.5. *MALDI-TOF Analysis*

Samples were analysed using a Bruker Reflex IV Mass spectrometer and peptide mass profiles / sequences exported into Mascot sequence database (www.matrixscience.com).

5.2.3. Metabolic Labelling

5.2.3.1. *Sample Preparation*

Approximately 100mg (wet weight) of washed embryos were placed into aerated seawater for 30 minutes and then sealed with 1.1 ml of incubation medium. The incubated embryos were held at 10°C in a temperature controlled water jacket, and the incubation medium was continuously, but gently stirred. A gas mixing pump (Wösthoff SA27/3-F, Bochum, Germany) was used to maintain a constant PO_2 of either 18-20 kPa, 5.1 kPa, and 2 kPa throughout the incubation medium during incubation.

5.2.3.2. *In-vivo Metabolic Labelling*

To test for newly synthesised proteins at each of the selected developmental phases, *C. pagurus* embryos were metabolically labelled with [^{35}S] methionine and cysteine (TRAN^{35}S-LABEL™ ICN Biomedicals Inc., sp. Act. 1175 Ci.mM^{-1}) at 90 μCi ml^{-1}, in temperature acclimated U.V. treated and double filtered sterile seawater. Two key phases in development, limb formation and eye phase, were chosen for these experiments as they represented an early and a relatively late stage in development. In addition, fractional rates of protein synthesis were at their highest in eye phase embryos and 2-times higher compared with limb phase embryos, as detailed in Chapter 3. Embryos were also metabolically labelled at the 'eye and chromatophore' phase (see Chapter 2), which represented the most advanced phase in development available at the time these studies were carried out.

5.2.3.3. *Experimental Regime*

Due to the high specific activity of the radionuclide needed in each experiment, it was decided not to complete a time course for label uptake in embryonic *C. pagurus*. Instead, each group of embryos were incubated for 2 hours, which is the time period used in previous investigations on adult crustaceans at relatively low temperatures (Faulkner, 2001), and is the standard time interval used in many heat shock experiments, including the present investigations using *C. pagurus* embryos (see Section 5.2.2.1). Embryos were divided into three treatment groups, each treated with a different level of PO_2 for the 2h incubation period: normoxia at 18-20 kPa; mild hypoxia at 5.1 kPa; and severe hypoxia at 2 kPa. After incubation the embryos were washed four times in 2 ml of U.V. filtered seawater and flash frozen in liquid nitrogen for storage at -80°C. Newly synthesised proteins were detected using SDS-PAGE, followed by autoradiography as described below.

5.2.3.4. *Analysis of Newly Synthesised Proteins*

Frozen samples were ground under liquid nitrogen into a fine powder, placed in sample buffer (62.5mM Tris, pH6.8) and kept on ice to reduce proteolysis. The specific activity of a small sub-sample of embryo homogenate was determined by scintillation counting in an Optiphase 'Hisafe' liquid scintillant, using a Wallac WinSpectral 1414 scintillation counter. This was completed in duplicate for each sample to standardise samples according to specific activity ready for loading onto the SDS gels. Each sample (3 kBq, 18 x 10^4 dpm) was then boiled for five minutes in 20 µl SDS buffer (62.5mM Tris pH6.8, 10% Glycerol, 2% SDS, 5% 2-mercaptoethanol and bromophenol blue) and loaded on a 12% polyacrylamide SDS PAGE gel. Each gel was standardised by loading a (^{14}C) labelled molecular marker (Amersham 14300-220000 Da), and electrophoresis completed at a constant voltage of 200 V.

Following the completion of electrophoresis, the polyacrylamide gel was removed and fixed in a mixture of isopropanol:H_2O:Acetic acid (25:65:10) for 30 minutes. The gel was then impregnated with an autoradiographic enhancer

Amplify™ for 20 minutes and dried under vacuum for 2 hours. Finally, the dried gel was exposed to pre-flashed Hyperfilm™ for a minimum of 72 hours at -80C.

5.2.3.5. *Autoradiogram Analysis*

Images of each autoradiogram were recorded using a (Biorad Gel Doc 2000) camera and the image exported into ImageJ imaging software (version 1.34s), and analysed for metabolic labelling intensity. The [14C] labelled molecular marker (Amersham 14300-220000Da) was used to determine approximate molecular mass of the main protein bands and to act as a means of standardising readings to compare the density of bands between the wells and between gels.

5.2.4. Methods HIF-1α

5.2.4.1. *Sample Preparation and Experimental Regime*

Approximately 100 mg of washed embryos (Limb formation, eye, and hatching phase) were placed into a small glass vial (6 x 3 cm) and left to acclimate in gently aerated seawater maintained at 10°C for 30 minutes. The experimental apparatus was as described in Section 5.2.2.1. Each experiment started once the embryos had been sealed inside the experimental glass vial and the PO_2 levels reduced to 2 kPa. The incubation seawater was maintained at 2 kPa using a gas mixing pump (Wösthoff SA27/3-F, Bochum, Germany). Embryos were sampled after 5, 6, and 7 hours at 2 kPa. At each sampling time-point the embryos were rapidly removed, blotted and stored in RNAlater (Qiagen) at -80°C.

Adult *C. pagurus* and *C. maenas* were aerially exposed to induce systemic hypoxia by placing crabs into separate dark opaque tanks (45 x 30cm), lined with paper saturated in seawater and covered with a dark cloth. Animals were left for 16 hours, after which time, gill tissue was dissected out and rinsed in sterile

seawater and stored as described for the embryos below. These samples acted as controls.

5.2.4.2. *Primer Design*

Published sequences for hypoxia inducible factor one alpha subunit (HIF-1α) from the following species: *Mus musculus* (accession number MMY09085); *Rattus norvegicus* (accession number AF057308); *Homo sapiens* (accession number ah006957); *Xenopus laevis* (accession number XLA277829); *Oncorhynchus mykiss* (accession number AF304864); *Danio rerio* (accession number BC046875) were accessed in GenBank using the Blast software program available at the National Centre for Biotechnology Information website. These sequences were alligned using Biology Workbench (version 3.2) software, and regions of the HIF-1α molecule with high DNA sequence homology, and encompassed or were close to the oxygen dependant domain or the helix loop helix domain, were chosen as target regions for HIF-1α primer design. These selected sequence regions were then transformed according to codon usage in *C. pagurus* (www.kazusa.or.jp/codon/), and optimal primer pair sequences were generated based on the following criteria: number of base pairs; melting temperature (C+G content); and the size of the target product. Nine primer pairs were designed to target HIF-1α in genomic and cDNA templates taken from embryonic and adult *C. pagurus*, and also adult *C. maenas* (see below). Primers were ordered from Sigma-Genosys Ltd.

5.2.4.3. *Extraction and Thermal-Cycling*

Total RNA was extracted from embryos and from adult gill samples using TRI reagent (Sigma) according to Ausubel et al., (1992). Briefly, samples were thawed, blotted, and homogenised using a polytron homogeniser in TRI Reagent. The soluble RNA fraction was removed from the tri-phasic separation following chloroform extraction. Finally the aqueous RNA was precipitated with isopropanol. All steps were performed on ice. A sub-sample of RNA was quantified according to Ashford and Pain (1986) as outlined in Chapter 3. After precipitation of the RNA, genomic DNA was removed by incubation in DNase 1

(37°C, 60 mins), and mRNA isolated with Dynal Dynabeads according to the manufacturer's instructions.

Approximately 2 ug of mRNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase with random hexamers (Quiagen) to generate cDNA for HIF-1α, and amplified using the polymerase chain reaction (PCR).

The PCR conditions were optimised for each set of primer pairs. Amplification of HIF-1α cDNA was achieved using a gradient thermocycler (Techne Touchgene Gradient). Optimisation involved changing the number of cycles, using a range of $MgCl_2$ concentrations (1, 1.25, 1.5, 2, 3mM) and changing the annealing temperatures for each primer. Optimal conditions for PCR were those that increased the yield of PCR product as viewed after separation by horizontal gel electrophoresis (see below). In addition, a range of different Taq polymerases were trialled including: standard Taq (Promega) and platinum Taq (Invitrogen).

The optimal conditions for all primer pairs were as follows: Initial denaturation at 95C for 45 sec; annealing at 55C for 30 sec; extension at 72°C for 60 sec; for 30 cycles; followed by a final extension time of 15 min. A $MgCl_2$ concentration of 1.25 mmol l-1 was used in all reactions. PCR controls included crab genomic DNA and mRNA amplified with primers specific to actin (forward primer: 5' – GGI/TT(C/T)/GCI/GGI/GA(C/T)/GCI/CC-3', Reverse primer:5' –GG(A/G)/TG (C/T)/TC(C/T)/TCI/GGI/GCI/ACI/CG-3'.

5.2.4.4. *Analysis of PCR Products*

5 µl of each PCR product was loaded with sample buffer (Novagen) onto horizontal 1% agarose gels and stained with ethidium bromide. Each gel was calibrated with a DNA molecular weight ladder (Novagen 100bp) and

electrophoresis was completed at constant voltage (80 V). Images were viewed under UV light and captured using a Biorad GelDoc 2000 photo system.

DNA products of interest were excised from the agar gel under UV light using a sterile scalpel blade. PCR products were isolated from the gel slices using micro columns (Amicon Ultrafree). PCR products were sequenced commercially by MWG Biotech Ltd.

5.2.5. Bromodeoxyuridine (BrdU) Labelling

5.2.5.1. *Sample Preparation and Experimental Regime*

Approximately 200mg of washed embryos of *Cancer pagurus* were divided equally into two sub-samples, one for normoxia and one for hypoxia (2 kPa). In both cases, embryos were left to acclimate in gently aerated temperature acclimated seawater held at 10°C for 30 minutes. Bromodeoxyuridine (BrdU) stock incubation medium in sterile seawater was heated to 37°C to improve BrdU solubility, and also to reduce the possibility of BrdU precipitation during the incubation period. After the initial 30 minute period, each sub-sample was placed inside a glass vial (4 x 2.5cm) containing 2 ml of incubation medium. Experiments were started when the embryos were sealed inside the sample vials. Samples were held at 10°C in a temperature controlled water jacket, and the incubation medium was continuously, but gently aerated and stirred in the case of normoxic embryos, and for hypoxia, embryos were subjected to PO_2 levels of 2 kPa maintained by a gas mixture of nitrogen and air, delivered via a gas mixing pump (Wösthoff SA27/3-F, Bochum, Germany). In addition, a small sub-sample of washed embryos were fixed in Stephaninis' fixative, without *in-vivo* labelling with BrdU, and processed as below. These were used as negative controls.

5.2.5.2. *Concentration Titre and Time Course Experiments*

The optimal uptake rate for BrdU was tested by exposing eye phase *C. pagurus* embryos to different concentrations of BrdU (0.2, 0.4, 0.6 mg ml^{-1}) for 4 hours at 10°C. These initial experiments showed that the various BrdU concentrations had little effect on the uptake of BrdU at the eye phase. Subsequently, all incubations were carried out at 0.2 mg ml^{-1} which is the concentration used by Harzsch et al (1998) to investigate BrdU labelled neurogenesis in crustacean embryos. Preliminary time course experiments were also carried out on eye phase embryos in order to determine optimal labelling times at 10°C. A small sub-sample of embryos were quickly removed from the incubation chamber every 60 minutes for 6 hours and stored as below. No major differences were found in BrdU labelling between the various time intervals.

5.2.5.3. *In-vivo Labelling with BrdU*

To test for BrdU labelled cell division at all key phases in embryonic *C. pagurus*, embryos were incubated for a total period of 240 minutes in fully aerated seawater (normoxia), with a sub-sample of embryos removed every 60 minutes. A separate sub-sample of embryos was simultaneously subjected to hypoxia by supplying a nitrogen/air mixture via a gas mixing pump to maintain PO_2 at 2 kPa. In hypoxia, embryos were also sampled every 60 minutes. In both cases, and at all sampling intervals, a small sub-sample (approx 50 ug wet weight) of embryos were quickly removed, blotted and then rinsed in Stephaninis' fixative to remove residual BrdU solution, re-blotted and stored in the fixative overnight at room temperature.

5.2.5.4. *Immunofluorescent Detection of BrdU*

Following overnight fixation at room temperature a sub-sample of embryos were removed and checked for successful primary fixation. To this end, the vitelline and embryonic membranes were removed from the embryo under a dissecting microscope (Leica MZ APO) using pulled glass capillaries, at a rate of approximately 40-60 embryos per hour. If the yolk mass remained intact after removal of the embryonic membranes, then the fixation was deemed to be successful. After the egg membranes had been removed the embryos were fixed

again at room temperature using 2% paraformaldehyde for 4 hours. Following this secondary fixation, the embryos were stored overnight in storage buffer (PTX: 0.1 mol l^{-1} phosphate buffer; Triton x-100; 0.5 mol l^{-1} sodium azide). PTX was also used in additional washing steps throughout the protocol, which were at least 30 minutes in length with a minimum of 3 changes. Once removed from the storage buffer, the embryos were incubated at room temperature in 2 N hydrochloric acid in 0.1 mol l^{-1} pH = 7.2 phosphate buffer for 30 minutes at room temperature. HCl was used to denature cellular DNA, and subsequently expose the incorporated BrdU for indirect detection using specific antibodies. Next the embryos were washed and then blocked using Image-IT™ signal enhancer (Molecular Probes, I36933) for 30 minutes at room temperature, washed again and blocked in blocking solution (Triton x-100; 5% normal goat serum; 0.2% Bovine serum albumin in PTX) for 2.5 hours at RT. Blocking steps were carried out to reduce non-specific antibody binding, which was especially important considering the large proportion of yolk present in whole embryos. The embryos were subsequently washed and then incubated for 20 hours in 1/100 primary anti-BrdU monoclonal (Molecular Probes, A21300) suspended in PTX at 4°C, followed by a double washing step. Next the embryos were incubated at 4°C overnight with a fluorescent tagged secondary antibody, Alexa 488 (Molecular Probes, A21121), at a dilution of 1/400 in PTX. The optimal antibody concentration was determined by testing embryos from the time course experiments and examining the most effective fluorescent signal. Labelling was improved by extending antibody incubation periods and by maintaining the embryos at low temperatures as this reduced background interference. Finally the samples were washed twice and mounted on glass slides using Vector shield mountant (Vector labs). In addition, dissected and fixed hatching phase embryos, from both normoxic and hypoxic treatment groups were washed in PTX overnight at 4°C as described above, and prepared for tissue sectioning. Embryos were then dehydrated with a graded alcohol series (50 – 100%), cleared in methyl benzoate, followed by toluene, and added to molten wax (Paraplast) overnight at 60°C. Samples were orientated where possible, blocked out, and sections cut at 10 µm using a rotary microtome. Serial sections were subsequently treated as described for whole mount preparations, and kept in a small container humidified with a saturated sponge lining the base of the container to prevent sections from

drying out during incubation steps.

5.2.5.5. *Visualisation and Documentation*

Samples were viewed under a confocal microscope (Zeiss LSM510). Images were captured using LSM510 software and exported using LSM510 image browsing software (version 2.8.0.0).

5.3. Results

5.3.1. Heat Shock Protein 70

5.3.1.1. *General Hsp70 Reactivity in Cancer pagurus and Carcinus maenas*

The monoclonal antibody against human Hsp70 (Affinity BioReagents, MA3-006) successfully cross reacted with crustacean heat shock proteins, both in the embryos and adults of *C. pagurus* and *C. maenas*. Figure 5.1a shows the general heat shock protein pattern recorded at all embryonic phases in *C. pagurus* following an incubation period of 2 hours. Heat shock protein expression patterns for *C. maenas* are shown in Figure 5.2b.

5.3.1.2. *Patterns of Hsp70 Synthesis during Embryonic Development*

In general, cross reactivity appearing as a diffuse reaction was observed for Hsp70 at all phases of embryonic development in *C. pagurus*, and for the limb formation phase embryos of *C. maenas*. The cross reactivity was most evident in *C. pagurus*, particularly at developmental phases which preceded the hatching phase.

5.3.1.3. *Normoxia*

In *C. pagurus,* blastula phase embryos showed very diffuse but also intense reactivity with anti-Hsp70, along with cross reactivity with further molecular weight protein classes (see Figures 5.1a,b). This mainly included a high molecular weight protein class (< 100 kDa), a 70 kDa protein class, and two low molecular weight protein classes (< 30 kDa), the latter consistently displayed the highest cross reactivity. These figures also show that the same protein profile was also observed at both the limb formation and eye phases. Hatching phase embryos showed a more limited staining reactivity with anti-Hsp70 in both species examined, especially in *C. maenas* (see Figures 5.1a,b). However, faint banding patterns also revealed three protein classes located within 60 to 70 kDa at the hatching phase, and two other protein classes between 30 and 40 kDa. The

reactivity from the low molecular weight protein classes was lost at the hatching phase. In addition, all samples and at all phases in development, including controls removed from the egg mass, showed evidence of a constitutive population of Hsp70 in the protein pool (see Figure 5.1c).

In *C. maenas* only the limb formation, eye and hatching phases were analysed for cross reactivity with anti-Hsp70. In this species, cross reactivity with anti-Hsp70 (MA3-006) was generally more specific, particularly at the eye phase in development, when compared with the reactivity observed with *C. pagurus* embryos (see Figure 5.2b). In addition, a diffuse cross reactivity for Hsp70 only occurred at the limb formation phase of development in *C. maenas*. Anti-Hsp70 reactivity at the eye phase showed a marked increase when compared with all samples, including both the embryonic and adult control samples of both species. At hatching phase, there was very limited evidence of cross reactivity with the anti-Hsp70 antibody. Finally, in contrast to embryonic *C. pagurus,* there was no evidence for additional anti-Hsp70 cross reactivity with proteins at other molecular weights. In particular, no cross reactivity was observed in the low molecular weight classes.

5.3.1.4. ***Effects of Hypoxia and Heat Shock Treatment***

Hypoxia had no noticeable effect on the heat shock protein patterns observed in normoxic embryos in either *C. pagurus* or *C. maenas* (see Figures 5.1a, b, c and 5.2b). Similarly, heat shock had no effect on the presence or patterns of synthesis of Hsp70 at any of the developmental phases in *C. pagurus* (Figure 5.2a). In contrast, Figure 1b shows that gill tissue from adult *C. pagurus* showed cross reactivity with Hsp70 antibody, with three relatively discrete protein classes visible between 60-70 kDa . Also, similar to the situation in *C. pagurus* embryos, a protein class of lower molecular weight >45 kDa cross reacted with anti-Hsp70 following a heat shock.

5.3.1.5. *Further Characterisation of Low Molecular Weight Protein Classes*

Low molecular weight protein bands excised from polyacrylamide gels and targeted for mass spectrometry failed to show any significant positive matches when compared with known sequences on Mascot sequence database.

5.3.2. Metabolic Labelling

5.3.2.1. *Comparison of Existing and New Protein Pools*

Figure 5.3 shows an SDS PAGE gel with existing protein pools stained with Coomassie Blue G250 displaying the developmental phases of interest in normoxia and in hypoxia. The protein pools found for early phases in development (blastula and limb formation) were dominated by 2 protein bands of approximately 66 kDa and ≤ 130 kDa. As development progressed through to the eye and hatching phases, these 2 protein bands subsequently decreased in staining intensity. This was most apparent at the hatching phase, where these two bands were much less diffuse, producing discrete protein bands. A similar change to the staining intensity of a number of other protein classes during development were also observed at: 45 kDa; 30 kDa; and < 21 kDa.

Comparison of the gels stained with Coomassie Blue (total protein) at each of the key phases in development, with the autoradiographs of metabolically labelled (newly synthesised) protein showed marked differences. The dominant high molecular weight protein classes (66 kDa and ≤ 130 kDa) were not part of the newly synthesised pool of proteins at any of the developmental phases used in these experiments (see below).

5.3.2.2. *Developmental Profiles of Specific Protein Synthesis*

Figure 5.4a is an autoradiograph of an SDS PAGE gel showing the incorporation of ^{35}S-methionine and ^{35}S-cysteine into embryonic protein at the three phases of development after exposure to various partial pressures of oxygen (PO_2). Figure 5.4b shows the protein synthesis profiles for the limb formation and eye phase only. The number of protein bands (^{35}S-methionine/cysteine incorporation over the 2h incubation period) present at each developmental phase increased with development in all treatments. For example, there were fewer protein bands at the limb formation and the eye phases, compared with the 'eye and chromatophore' phase in development. However, despite differences in relative labelling intensity, a number of protein classes were common to each of the three developmental phases, and across all treatments including: a series of relatively high molecular weight proteins (85, 73, 68 kDa); and a low molecular weight protein class of 19 kDa. In contrast, there was also evidence of phase specific changes in protein synthesis patterns. Most of these changes were due to the increased number of protein classes found at the 'eye and chromatophore' phase. However, a very distinct protein band corresponding to a molecular weight of 17 kDa was observed at all three phases in hypoxia, except for the eye phase. Similarly, a 21 kDa protein class was only present at the two later phases in development, i.e. eye and the 'eye and chromatophore' phases.

5.3.2.3. *Hypoxia Inducible Protein Synthesis*

The prevailing PO_2 levels present during metabolic labelling had a varying effect on the protein profile, depending on the phase in development. During limb formation, hypoxia strongly suppressed proteins at 85 kDa in size. In contrast, protein classes at 68 kDa, 19 kDa, and 17 kDa appeared more densely labelled during hypoxia than proteins of the same molecular weight during normoxia. During the eye phase, a range of protein classes (72, 68, 41, 30 kDa) were more densely labelled during hypoxia compared with normoxia, although a 21 kDa was constitutively synthesised.

In contrast to the eye phase, the protein class at 72 kDa was expressed in the 'eye and chromatophore' phase embryos at all three PO_2 treatments. However,

expression of 68 kDa and 21 kDa protein classes increased during hypoxia, the former of which was also observed in the eye phase embryos. In addition, a suite of other low molecular weight protein classes (21, 20, 19, 17, 15, 14 kDa) were also up-regulated during hypoxia at the eye and chromatophore phase.

5.3.3. Characterisation of HIF-1 in *Cancer pagurus*

5.3.3.1. *Cloning of Cancer pagurus and Carcinus maenas HIF-1α*

Reverse transcription experiments were completed using embryonic and adult tissue samples of *C. pagurus*, and also adult *C. maenas*, subjected to systemic hypoxia (2 kPa). Unfortunately, none of these preliminary investigations were successful in detecting the brachyuran HIF-1α homologue reported in other species (Head and Terwilliger, 2005).

Attempts were made to optimise the polymerase chain reactions (PCR) conditions, such as by trial of a range of primer annealing temperatures, and $MgCl_2$ concentrations. Incubation time periods were also varied, and selected in accordance with time courses reported in the literature for successful HIF-1α induction (Wang et al., 1995; Jiang et al., 1996). In addition, genomic DNA extracted from adult *C. pagurus* and *C. maenas* was also analysed with the same primer range, however none of these modifications produced positive HIF-1α PCR product. Finally, contact was made with Prof. Nora Terwilliger (Oregon Institute of Marine Biology) to organise a collaborative experiment using primers sequenced for HIF-1 in *Cancer magister*, although this was not possible in the end.

5.3.4. Bromodeoxyuridine (BrdU) Labelling

5.3.4.1. *General Overview of BrdU Incorporation*

In-vivo BrdU labelling of cell division in embryonic *C. pagurus* was met with a variable amount of success. Firstly, the blastula phase embryos incubated during preliminary experiments were later found to be unsuitable for tissue processing and immuno-detection procedures. This was because it was never possible to dissect the blastula embryo out of the egg membranes without destroying the embryo first. In light of this problem, only the three remaining key phases in development were selected for *in-vivo* incorporation studies with BrdU. Secondly, there was a high degree of variation in BrdU uptake at the eye phase with some embryos intensely stained and others not stained at all. Consequently, many specimens were processed to try and optimise conditions to obtain consistent results. Finally, it was only possible to section the embryos at the hatching phase, because the other phases weren't suitable for wax embedding and sectioning.

5.3.4.2. *BrdU Labelling in Normoxic Embryos*

At the limb formation phase the embryos sampled at each time point (every 60 minutes for 240 minutes) generally showed very little evidence of BrdU incorporation, with fluorescence in approximately 5% of the total embryos screened. When present, these cells appeared in the developing limbs and also in a concentrated area in the dorsal region of the embryo, which could be the developing heart (Figures 5.5 a, b). Figure 5.5c is a negative control of the limb formation phase embryo.

In-vivo labelling and visualisation of eye phase embryos with BrdU is shown in Figures 5.6 and 5.7. There was evidence of discrete whole mount labelling in the eye phase embryos compared with the other two developmental phases. Labelled cell clusters were predominantly found on the ventral surface of the embryo

situated between the yolk droplets (see Figure 5.6 a-e). These were present in samples incubated from the 2 h time course (Figure 5.6 a) and mainly also following the 6 h time course (Figure 5.6 b, c). Many distinct ventral processes were also observed in the eye phase embryo, and the eyes were often also intensely stained (Figure 5.7). However, at 10-20x magnification it was not possible to see discrete cells in either the eyes or the ventral processes that showed fluorescence.

Hatching phase embryos showed very little evidence of whole mount *in-vivo* incorporation of BrdU (Figure 5.9 a, b). Sections through the hatching phase embryos were more revealing and showed cell labelling particularly in developing appendages and in cells around the eyes (Figures 5.8 a - e). However, as found at other phases in development, there was a great deal of variability in labelling intensities between batches of slides.

5.3.4.3. ***BrdU Labelling in Hypoxic Embryos***

Due to the limited incorporation of BrdU at the limb formation and the hatching phases these developmental phases did not provide sufficient data to analyse the influence of hypoxia on cell proliferation. To determine if hypoxia had an effect on BrdU labelled embryos at the eye phase where incorporation was high, a total of 50 embryos were counted at each time point and the number of embryos with and without fluorescence was recorded. This method, however, was met with limited success, owing to the large amount of variability in labelling. In addition, a series of intensity measurements of the resulting fluorescent signal in the eyes was made using LSM510 Image software. Again, this attempt to quantify the differences produced highly variable results. In light of this it was not possible to determine if hypoxia had a quantifiable effect on BrdU labelled cell division in the eye phase embryos of *C. pagurus*. At both the eye and the hatching phases, discrete BrdU labelled cell divisions were apparent. At the eye phase this was only found after 6 hours incubation in hypoxia (Figure 5.6 d, e), and at the hatching phase in sections cut from embryos incubated for 4 hours hypoxia (2 kPa) (Figure 5.8 c, d).

5.4. Discussion

Evidence was given in Chapter 4 to suggest that early phases in the embryonic development of *Cancer pagurus* survive hypoxia by regulating metabolic rate down to a low critical oxygen partial pressure, and possibly by entering quiescent periods. In contrast, metabolic rates in the later phases of development conform to a decline in PO_2 and show a rapid reduction in rates of protein synthesis to save on energy demand. From the initial experiments carried out in this chapter, preliminary evidence would suggest that molecular mechanisms of hypoxia tolerance have a role to play in *C. pagurus*, such as hypoxia inducible gene expression, cell protection due to heat shock protein function, and hypoxic modification of cell division.

5.4.1. Developmental Changes to the Heat Shock Response in *C. pagurus*

Hsp70 was found at all of the key phases in embryonic development in *C. pagurus*, although to much less of a degree at the hatching phase. Hsp70 expression during embryogenesis is ubiquitous and has been found in invertebrates including: flies; crustaceans; echinoids; and also in vertebrates, such as frogs and mammals. Interestingly, it is amongst the first products synthesised by the embryonic genome (Bensaude and Morange, 1983; Heikkila et al., 1997; Spees et al., 2003; Geraci et al., 2004).

The presence of Hsp70 reactivity at the blastula in *C. pagurus* is a good indication of its importance and immediate requirement, possibly for protein chaperoning at fertilisation and cleavage, and also potentially for cellular protection against hypoxia within the embryo mass (see below). The attenuation of this response at the hatching phase could relate to the increased availability of oxygen in the water surrounding the egg mass and, therefore, the reduction in natural exposure to hypoxic periods as development proceeds (see Chapter 4;

Naylor et al., 1999; Fernández et al., 2000, Baeza and Fernández, 2002; Fernández et al., 2002). Alternatively, the developmental specificity of the Hsp70 response, shown by this attenuated response at the hatching phase in embryonic *C. pagurus* may suggest a maternal origin for Hsp70 in *C. pagurus*. Pre-gastrula embryos of the African clawed toad, *Xenopus laevis,* are known to have maternal isoforms of Hsp70, but these only function until genome competence part way through blastulation (Heikkila et al., 1987; Davis and King 1989). Further support for a maternally derived Hsp70 in embryonic *C. pagurus* can be found when comparing the immunoreactive expression profile found during development, with that found in embryonic *Carcinus maenas*. For example, the diffuse immunoreactivity associated with anti-Hsp70 during early development in *C. maenas* was similar to the general response observed in the 70 kDa protein class region for *C. pagurus* embryos (excluding hatching phase). In contrast, at the eye phase a discrete, and intense protein band was produced in *C. maenas*, more similar to stress inducible responses of Hsps characterised and reported in other species (see Figure 5.2b) (Cimino et al., 2002; Scott et al., 2003). Furthermore, this was much more intense than the reactivity observed at any of the key phases in *C. pagurus* embryos that consistently produced a diffuse reactivity. The reasons for this disparity are not clear, however, differences in natural history between the adults may be an important factor. For instance, ovigerous *C. maenas* can be found stranded in rock pools along the intertidal zone (S. J. McCleary, personal observation), where they are exposed to highly changeable environmental variables, such as temperature, UV light, oxygen and salinity. On the other hand, *C. pagurus* is subtidal and, therefore, exposure of the embryos to many of these environmental variables is generally avoided.

Environmental factors are well known to induce Hsp70 expression in a range of intertidal fauna, including embryonic phases in the lifecycle (Sharp et al., 1994; Sharp et al., 1997;Geraci et al., 2004) Furthermore, a similar Hsp response across the intertidal environmental gradient has been shown in marine molluscs (Sanders et al., 1991; Tomanek, 2002). Despite the differences reported here, ^{35}S methionine protein labelling experiments in embryonic *C. pagurus* also showed constitutive protein expression of a 68 kDa protein class. This was evident at the

limb formation phase, and also the eye phase where it appeared to be upregulated by hypoxia (see below) (Figure 5.4a). It is possible that this protein class is in the same family as Hsp70, showing the upregulation of multiple forms of the Hsp70 family in embryonic *C. pagurus*. ^{35}S methionine protein labelling in the trout, *Oncohynchus mykiss*, and following heat shock in the gastropods *Tegula brunnea* and *Tegula funebralis*, also showed evidence of the possible upregulation of multiple Hsp70 members in response to hypoxia (Airaksinen et al., 1998; Tomanek, 2002). However, the results reported here remain a preliminary observation and await the completion of more comprehensive experiments with larger sample sizes.

^{35}S methionine/cysteine labelling data in the current study was generated with a sample number of only two at each of the developmental phases, although each sample was characterised by 3 sub-samples of embryos that were treated at different PO_2 levels (discussed below). Sampling with ^{35}S methionine/cysteine was restricted due to the large volume of radionuclide required to complete each experimental treatment and due to its short half-life, requiring all experiments to be carried out within a 2 week period. An additional problem was that blastula and hatching phase embryos were subsequently unavailable for specific protein expression work. Such information would have been useful for comparison of protein expression profiles with the immunoreactive Hsp70 results completed at all key phases in development; in particular at the hatching phase where the attenuated immunoreactive response may have been traced to changes in the 68 kDa protein class.

Future improvements to this experiment could involve the use of 2D gel protein separation, followed by immunodetection with anti-Hsp70. Subsequent comparison of immunoreactive Hsp70 profiles found at each developmental phase with ^{35}S methionine/cysteine labelled 2D protein profiles, would potentially improve resolution of specifically synthesised protein classes in the 70 kDa region, allowing more accurate identification of proteins following the isoelectric protein separation. In addition, these investigations could also utilise northern

blot analysis, or real time PCR to examine embryonic messenger expression levels of Hsp70 during development. This may provide a more accurate determination of the origin of Hsp70 in embryonic *C. pagurus*. Furthermore, female oocyte analysis, similar to the experiments completed on *X. laevis*, could also be used to analyse the vitellogenic component for maternal Hsps in maturing oocytes prior to fertilisation (Ohan and Heikkila, 1995; Ali et al., 1996; Gordon et al., 1997).

Interestingly, anti-Hsp70 cross reactivity with proteins at different molecular weights showed the presence of a number of potential Hsp70 homologues, two of which were highly conspicuous and discrete low molecular weight (Mw) proteins (< 30 kDa). Both homologues showed the same patterns of developmental specificity, along with an attenuated response at the hatching phase, as described for the Hsp70 response (Figure 5.1a,b). Also, similar protein classes were synthesised during ^{35}S methionine/cysteine labelling at the limb formation and eye phases in development. These protein classes also appear to be part of the general protein pool in embryonic *C. pagurus* (Figure 5.3), suggesting that they are constitutively expressed during early phases in development. Anti-Hsp70 has also shown cross reactivity producing low molecular weight proteins in other species where it an important aspect of the cellular stress response (Sharp et al., 1994).

The role of small Hsps during embryogenesis is possibly best known and most widely researched in diapause embryos of *Artemia franciscana*. In *A. franciscana* a small maternally derived heat shock/ α-Crystallin protein (P 26) has been shown to undergo nuclear translocation in response to a range of physiological stressors, but notably in response to anoxic stress during diapause (Clegg et al., 1994; Clegg et al., 1995; Willsie and Clegg, 2001). A diverse range of functions for P 26 have been identified, including protein chaperoning, protein stabilisation, and inhibition of apoptosis (Haas et al., 1991; Clegg et al., 1999; Villeneuve et al., 2006). It is important, however, to note firstly that P 26 appears to show limited cross reactivity outside of the Genus Artemia, but has been found

in scleratinian corals (Branton et al., 1999), although not as yet in brachyurans. Despite this, a role for small Hsps has also been implicated in embryonic *Drosophila melanogaster*, where small Hsps are involved with neuronal protection during hypoxia (Pauli et al., 1990; Haas et al., 1991). The presence of small Hsps in addition to Hsp70 during embryonic development in *C. pagurus* may further enhance the cell protection and rescue response to hypoxia, experienced at early phases of development. The early constitutive expression, and also presence of these proteins amongst the main protein classes characterised in the existing protein pools is further indication of their possible importance during embryonic development. To this end, it would be particularly interesting to examine the response of Hsp70 and its homologues in embryonic *C. pagurus* immediately following fertilisation, and during early cleavage for instance, at the morula phase.

Cellular protection at the early and most vulnerable phases in development is vital to safeguard against hypoxia induced and heritable damage during development in the egg mass. A number of constitutively expressed mid range protein bands (30-40 kDa), specific to the hatching phase, also showed cross reactivity with anti-Hsp70 in *C. pagurus*. These potential Hsp70 homologues may be representative of basal Hsp expression by the zoea larvae in preparation for the planktonic phase in the lifecycle, where environmental conditions can be highly changeable in the surface waters (Epel, 2003; Mann and Lazier, 2006).

In general, Hsp expression and function in response to hypoxia appears to be a common feature of the molecular mechanisms used to protect and rescue cells in a range of different animals, and at different life phases (Hutter et al., 1994; Mestril et al., 1994; Chang et al., 2000; Scott et al., 2003). Although hypoxia did not appear to induce Hsp70 in *C. pagurus* embryos, control Hsp70 levels were relatively high, suggesting that these proteins are already expressed and at sufficient levels to protect against cellular hypoxic damage. Raised levels of Hsp 72 and Hsp 73 have previously been reported in the western painted turtle, *Chrysemys picta belli*, which regularly experiences hypoxia during diving in its

natural environment (Scott et al., 2003). Also, seasonal elevations in basal levels of Hsps have been found in a range of eurythermal species, in anticipation of increased exposure to environmental stress during the summer (Huey and Bennett, 1990).

It is feasible that maternally derived, and/or high basal levels of Hsps could also occur in embryonic *C. pagurus*, to protect against the likelihood of hypoxic stress. A high constitutive population of Hsps, as opposed to an increase in the Hsp response, could be beneficial. The metabolic cost of protein synthesis is known to represent a substantial proportion of basal metabolic rate, with the suggestion that Hsp synthesis is a metabolically expensive response, which inhibits general protein synthesis rates when Hsp synthesis is induced (Calow, 1991; Krebs and Loeschcke, 1994). If some Hsps were derived from the mother during vitellogenesis, then this would act to reduce energy expenditure in the embryos at a time when general protein synthesis rates are on the increase. In addition, an improved tolerance to hypoxia during development has been found previously in the rat heart, in diapause embryos of *A. franciscana* and tardigrade species, and also in various somatic tissues and eggs of the garter snake, *Thamnophis sirtalis parietalis*. In all cases, increased tolerance to hypoxia was thought to be related to the presence of high basal levels of Hsps (Mestril et al., 1994; Clegg et al., 1999; Storey and Mosser, unpublished observations; Ramlov and Westh, 2001; Schill et al., 2004).

5.4.2. Effects of Hypoxia upon Protein Expression in Embryonic *C. pagurus*

Hsp expression is one of many mechanisms available for an organism to employ against environmental stress. Many other genes are also induced in response to hypoxia, and many are targeted for transcription by hypoxia inducible factor 1 (HIF-1), which has been identified as the master regulator of cellular oxygen homeostasis (review, Semenza, 2004). Preliminary studies were used to assess the expression of the alpha subunit of HIF-1 in embryonic phases of *C. pagurus,* but these experiments had limited success.

There may be a number of reasons for why these experiments were not successful, most of which probably reflect experimental design. A range of experiments show that both time course and incubation PO_2 level are very important for characterisation of HIF-1α expression (Wang et al., 1995 in Jiang et al., 1996; Garayoa et al., 2000; Stroka et al., 2001). This is potentially a crucial aspect that may require further consideration in future experiments using embryonic *C. pagurus*. In addition, it is equally as probable that there were failings not just with experimental regime, but also with the techniques employed to target HIF-1α detection. Firstly, the short half life of HIF-1α requires rapid tissue processing to avoid normoxic reductions to HIF-1α cell copy number. This is particularly important in embryonic samples, considering the relatively small cell volumes, low cell number and the concomitant effect of these properties on total messenger levels, when compared with adult cells. With this in mind these experiments would also benefit from more sensitive methods of messenger detection for example, the use of real time PCR (Dr Stuart Egginton, personal communication). This has previously been used successfully to detect messenger transcripts in crustacean embryos (Chung and Webster, 2004). Alternatively, immunoreactive experiments could be trialled for HIF-1 protein detection, using anti-HIF-1 proteins that show high species cross reactivity. This could be used as another approach to determine if HIF-1α is expressed during embryonic development in *C. pagurus* (Dr Mike Cross, personal communication). Finally, and interestingly, a homologue of HIF-1 in the closely related dungeness crab *Cancer magister* has reportedly been sequenced by N. Terwilliger and colleagues, and its response to hypoxia analysed (Head and Tewillger, 2005). Unfortunately, this sequence is yet to be published, but would be of great value for future studies into HIF-1α expression in embryonic experiments with *C. pagurus*.

Although experiments with HIF-1 were unsuccessful in embryonic *C. pagurus*, ^{35}S methionine/cysteine labelling experiments were supportive of hypoxia inducible gene expression. Preliminary data suggests that a range of different

protein classes were induced by hypoxia. In addition, certain protein classes also appeared to be suppressed by hypoxia, with both inducible and suppressible protein classes showing developmental specificity. A number of studies encompassing a range of hypoxia tolerant and intolerant species show modified gene or protein expression profiles in hypoxia (Land and Hochachka, 1995; Gracey et al., 2001; Larade and Storey, 2001; Larade and Storey, 2002; English and Storey, 2003; Brouwer et al., 2004). More extensive investigations have applied gene microarrays to observe the effects of hypoxia on gene expression, giving a more comprehensive overview of the response of the genome to hypoxia (Gracey et al., 2001; Ton et al., 2003). From these experiments, specific genes or groups of gene systems can be identified and their expression patterns and adaptive significance analysed. Gene expression studies on the goby, *Gillichthys mirabilis*, which is known to be particularly hypoxia tolerant, identified the upregulation of anaerobic gene pathways in response to prolonged hypoxia. This is part of a global response to hypoxia in this species, whereby general rates of protein synthesis are also down regulated and animal behaviour altered to conserve and maintain cellular energy homeostasis (Gracey et al., 2001).

To gain a more informed appreciation of the complete physiological response to hypoxia during development in *C. pagurus*, larger scale gene/protein expression studies could have been used. For instance, further analysis and improved resolution of protein profiles from all key phases in embryonic development between normoxic and in hypoxic embryos could be very informative. This would involve 2D gel electrophoresis experiments comparing normoxic with hypoxic responses, and selection of specific protein spots that are only found in hypoxia for identification by mass spectrometry. The use of 2D gels in the present study would have improved the chances of protein identification with mass spectrometry, due to the further separation of protein classes through isoelectric focusing (Prof Rob Beynon, personal communication).

One interesting aspect from the ^{35}S methionine labelling experiments was the large number of protein classes synthesised over the labelling period, particularly at the 'eye and chromatophore' phase. Identification of the various

protein bands and comparisons between the different developmental phases would provide an overview of the changes in protein expression patterns during embryogenesis. Again these individual proteins could be identified by mass spectrometry.

The final salient feature of this work is the large and dominant high molecular weight protein classes present in the normoxic protein pools, which were absent in the newly synthesised protein pools. It is highly likely that these two protein classes represent polypeptide subunits from the two main yolk proteins vitellin 1 and vitellin 2. For example, in the developing embryos of *Emerita asiatica*, SDS PAGE analysis identified two polypeptide subunits of 105 and 109 kDa for vitellin 1, and 6 smaller units ranging between 42 – 65 kDa for vitellin 2 (Tirumalai and Subramoniam, 2001). Similar findings have also been reported in a number of other studies (Baert et al., 1991; Shapiro et al., 2000), including the freshwater crayfish *Cherax quadricarinatus*, where polypeptide subunit molecular weights were found to vary during development (Pinto et al., 2002). Furthermore, in the present study these two protein classes showed a clear reduction in staining intensity during development, which coincides with the decrease in the embryonic yolk proportion following its conversion into metabolising tissue. This decline is also consistent with the reduction in basal levels of hsp in embryonic *C. pagurus*, and the increased number of newly synthesised protein classes found during development. The latter being a potential indication of the degradation of yolk proteins in support of new embryonic protein synthesis (Varvra and Manahan, 1999).

5.4.3. Effects of Hypoxia BrdU Labelled Cell Division during Development

The theme of this thesis is to study the ability of *C. pagurus* embryos to survive hypoxia, and to investigate the underlying physiological and molecular mechanisms, responsible for any developmental differences. A final series of

preliminary experiments were carried out to determine if hypoxia had an effect on cell proliferation during development. The results from this set of experiments are limited mainly because of the high lot to lot variability in label uptake found at all phases in development, and possible evidence of auto-fluorescence. For example, there were a number of instances (excluding the eye phase) where most of the embryos visualised on a single slide showed little or no evidence of BrdU labelled cell proliferation, and with just a small fraction of embryos showing BrdU incorporation. Attempts were made to allow for this: firstly by visualising at least 50 embryos at each time point used; secondly by repeating the experiments using embryos from a different ovigerous crab. Although, this sample number was still too small for any conclusive observations to be made. In the light of these issues, the resultant data can only provide a preliminary insight into the effects of hypoxia on cell division in *C. pagurus* embryos. These experiments, however, did go some way to establishing the BrdU technique as a useful tool for detecting cell division in crustacean eggs, showing that BrdU uptake is possible across the egg membranes and in species producing relatively small embryos, at least in marine decapodans. The final improvement to this protocol simply requires development of tissue sectioning at early developmental phases to enable visualisation at the cellular level (see Figures 5.8 b-e).

A number of recent studies have shown that one component of hypoxia tolerance in certain species can be attributed to an inhibition of the cell cycle at certain check points during exposure to hypoxia (Douglas et al., 2001; Douglas et al., 2005). BrdU labelling was used as a means of assessing whether a similar mechanism was present in *C. pagurus* embryos, especially at developmental phases experiencing regular hypoxia within the egg mass. Limb formation embryos showed very limited evidence of BrdU labelled cell division during exposure to normoxia or hypoxia, suggesting that cell division either did not occur, or occurred at a relatively low rate over the time frame of the labelling period. A cessation of the cell cycle in *D. melanogster* embryos, and the occurrence of suspended animation in developing embryos of the zebra fish, *Danio rerio* and at all phases of the lifecycle in *Caenorhabditis elegans,* have also been reported, but in response to anoxia (Padilla and Roth, 2001; Padilla et

al., 2002; Douglas et al., 2001). However, hypoxic (~10% ambient PO_2) induction of genes responsible for cell cycle repression have been found after 8 hours in adult *G. mirabilis* (Gracey et al., 2001).

The inhibition of cell division during low oxygen is believed to be an important part of the cellular defence mechanism by reducing mutations caused by cell toxicants, either during hypoxia, or during oxygen re-perfusion following hypoxia (Epel, 2003). The physiological advantages to this strategy include the regulation and reorganisation of cellular energetics, at least in hypoxia tolerant species, where energy demand and supply are known to be downregulated in hypoxia to reduce metabolic expenditure (Guppy and Withers, 1999; Boutilier and Pierre, 2000; Boutilier, 2001). The former may be particularly important at very early phases in development, such as during periods of early cleavage, where a mutation to a single cell has the potential to have severe repercussions, including developmental aberrations and death (Geraci et al., 2004; Bonaventura et al., 2006). An alternative strategy, however, has been shown in planktonic marine invertebrates during cleavage. These animals undergo a very rapid cell cycle to reduce the duration and, therefore exposure time of the more vulnerable periods in development (Strathmann et al., 2002). In light of the results reported here in limb formation phase *C. pagurus* embryos, BrdU uptake and, therefore, cell division in normoxic embryos was also relatively low, and could instead be associated to the low rates of growth and protein synthesis reported at this phase (Chapters 2 and 3). Clearly more experiments are required, and with much longer incubations time e.g. ≥24 hours combined with tissue sectioning (see below).

In contrast to the results observed at the limb formation phase embryo, BrdU uptake at both the eye and the hatching phases in *C. pagurus* appeared to suggest the presence of cell division during normoxia and interestingly in hypoxia also. With respect to the eye phase, cell division at a PO_2 of 2 kPa was only apparent after a period of 6 hours incubation, when compared with cell division observed in normoxia after 2 hours. Although, with respect to the latter, this observation

was found in far fewer embryos, when compared with the cell division observed in embryos incubated with 6 hours of hypoxia.

At the hatching phase cell division was only present in samples incubated for a period of 4 hours regardless of the PO2 level, and with much of the cell division located in the abdomen and maxillipeds. Localised mitotic activity in the maxillipeds has also been found in the spider crab *Maja squinado* at hatching (Harzsch et al., 1998) and is thought to reflect neurogenesis in the embryonic appendages. Considered as a whole, it is tempting to suggest that the duration of the cell cycle in hypoxia is extended at the eye phase, even at a time when protein synthesis rates and growth rates are relatively high. For example, Strathmann et al (2002) reported cell cycle times at 10°C that generally varied between ~0.5 – 2 h in planktonic invertebrate embryos, and ~1 – 4 h in brooded marine invertebrate embryos. However it is important to point out that these examples represented normal cell division from the 2-cell to 4-cell developmental phase, which are known to be amongst the shortest cell divisions recorded for animals, and also that these examples were not inclusive of decapod species (Strathmann et al., 2002).

Inhibitions of the cell cycle during hypoxia, or cellular protection with heat shock proteins are just two of multiple strategies that have been identified as a safeguard against DNA damage, which also includes DNA repair and apoptosis (see below). It also appears that DNA synthesis continues during hypoxia, albeit at a reduced rate. For example, in human cardiac fibroblasts hypoxic modulations of gene expression results in the release of growth factors, which negate the inhibitory effects of hypoxia, and enable the continuation of DNA synthesis for angiogenesis (Agocha et al., 1997; Zhao and Webb, 2001). Similarly, recent evidence in adult *Carassius carassius* exposed to long term hypoxia has shown continued but reduced cell division, also combined with apoptosis in the gill tissue (Sollid et al., 2003). This response in *C. carassius* supports gross morphological changes to the gill lamellae, functioning to increase surface area for improved gas exchange in hypoxia.

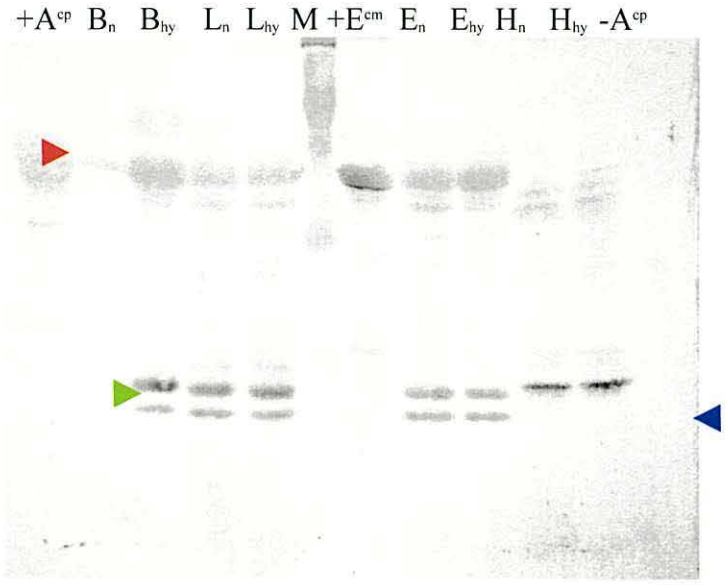
Ultimately, the continuation of DNA synthesis in hypoxia is associated with gene transcription, modulated in most cases by HIF-1, since growth factors are known gene targets of HIF-1, and, therefore its role in angiogenesis is currently of great interest (Jiang et al., 1997; Carmeliet et al., 1998; Vincent et al., 2000; Kelly et al., 2003). Furthermore, the response of the cell cycle to hypoxia in embryos of *D. melanogaster* has recently been shown to undergo a dose specific response, whereby mild hypoxia causes an extension to the cell cycle duration, compared with severe hypoxia and anoxia that cause a complete inhibition (Douglas et al., 2005). These embryos also synthesise a number of proteins during hypoxia and anoxia that mediate these modifications to cell division. Cell division at later phases in development of *C. pagurus* could also be triggered by hypoxia inducible changes to gene expression, as found from the uptake of ^{35}S methionine/cysteine, and by the fact that general rates of protein synthesis were also not entirely inhibited by hypoxia (see Chapter 4). The successful characterisation of HIF-1 in brachyurans would further support this argument. Equally, it is likely that disruption to cell division during hypoxia in embryonic *C. pagurus* could be dose responsive to hypoxia, permitting cell cycle progression but at a reduced rate. This would agree with the oxygen conformation shown for metabolic rate and rates of protein synthesis at the eye phase (see Chapters 3 and 4). Furthermore, this could form an additional part of the explanation for why growth rates are reduced at early phases in development in the naturally hypoxic egg mass.

Apoptosis is another facet of cellular protection utilised by animals that undergo rapid cell cycles during hurried periods of development in order to progress through vulnerable phases of development (Epel, 2003). Cell division in late phase embryos could be uncharacteristic and represent cells targeted for apoptosis rather than representing an increase in cell numbers. This programmed cell death has also been found in a number of other studies (Meier et al., 2000; Hensey and Gautier, 1998; Goda et al., 2003) Future experiments using TUNEL (TdT-mediated dUTP digoxigenin nick end labelling) could help to determine if the former were present during hypoxia in embryonic *C. pagurus*.

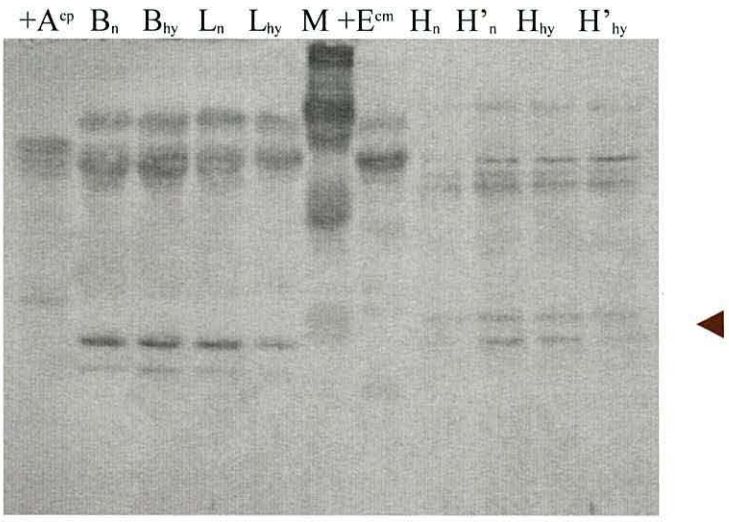
5.4.4. General Conclusion

Collectively, these studies provide some initial evidence for the involvement of molecular mechanisms in enabling *C. pagurus* embryos to survive hypoxia, an associated feature of development in large egg masses. Such mechanisms may be common survival strategies for other species of egg brooders and egg layers. In *C. pagurus*, changes in expression of a variety of protein classes occurred in response to progressive hypoxia, although most of these were not identified. Interestingly, the presence of high basal levels of Hsps at all phases in development, except for the hatching phase, were found. This strategy appears to be an appropriate defence against established periods of hypoxia in the brooding egg mass during embryonic development. Also, the indication of a possible arrest in cell division in response to hypoxia during these vulnerable early periods would also be beneficial to prevent inheritable errors from damaged DNA replication being passed on to later phases in development. In general, these mechanisms could potentially limit irreversible cellular damage from hypoxic exposure that could impair embryo fitness, whilst enabling reproductive synchrony to be controlled by maternal changes in oxygen provision to the developing embryos.

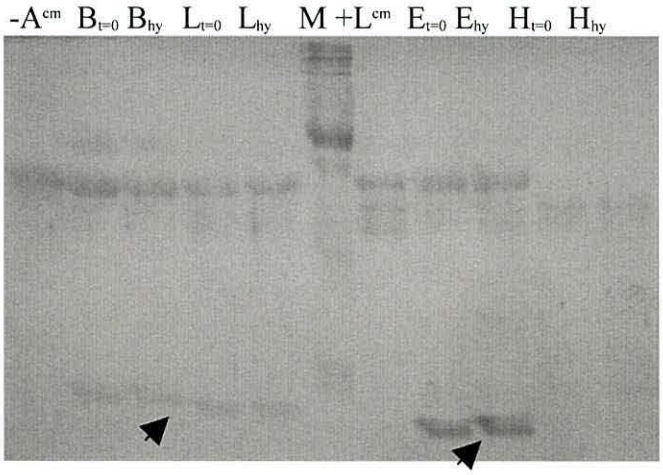
Figure 5.1. (a) Expression of heat shock protein 70 (Hsp70) during the key phases in embryonic development (B, Blastula; L, limb formation; E, eye; H, hatch) of *Cancer pagurus*, after incubation for 2 hours at 10°C in (n, normoxia and hy, hypoxia). Red arrowhead shows representative expression of Hsp70 at each phase in development and after each treatment following SDS PAGE and Western blotting. Green arrowhead show representative expression of low molecular weight (MW) Hsp70 homologues at each phase in development. Blue arrowhead highlights the absence of 17 kDa hsp70 homologue at the hatching phase in development. (b) Maroon arrowhead shows constitutively expressed mid range hsp70 homologues present at the hatching phase. H'_{n} and H'_{hy} are replicates of hatching phase samples. (c) Representative expression levels of heat shock control samples (embryos removed and processed directly from the egg mass) at each phase in development. Also shown are samples incubated in hypoxia at each developmental phase. Black arrowheads show compression of low MW Hsp70 homologues caused by both poor separation and transfer of proteins. n = 1 for each treatment lane in a,b,c. Control samples: $+A^{cp}$ (heat shock, adult *C. pagurus*); $+A^{cm}$ (heat shock, adult *Carcinus maenas*); $+L^{cm}$ (hypoxia 10°C, limb formation phase *C. maenas*) $+E^{cm}$ (hypoxia 10°C, eye phase *C. maenas*); $-A^{cp}$ (negative, adult *C. pagurus*); $-A^{cp}$ (negative, adult *C. maenas*).


+A^cp B_n B_hy L_n L_hy M +E^cm E_n E_hy H_n H_hy -A^cp

a

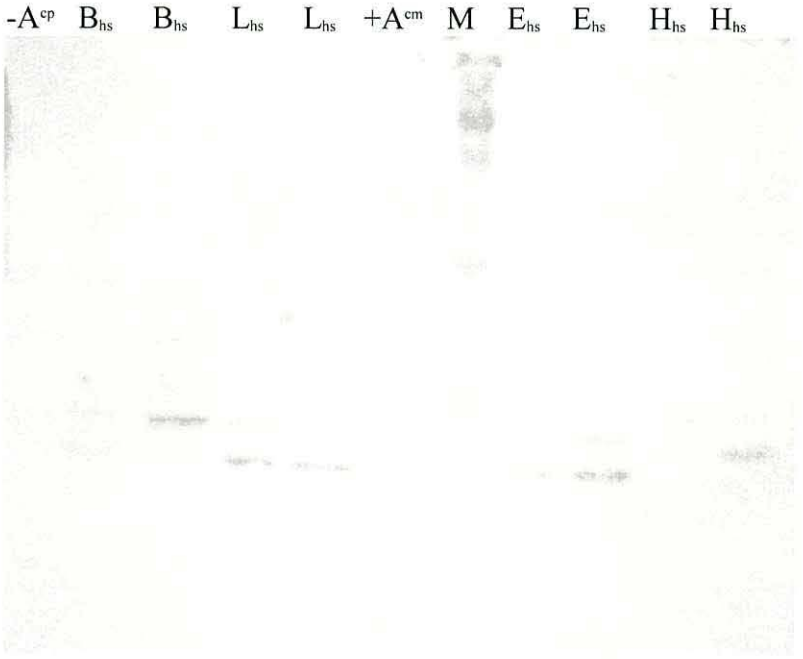

+A^cp B_n B_hy L_n L_hy M +E^cm H_n H'_n H_hy H'_hy

b

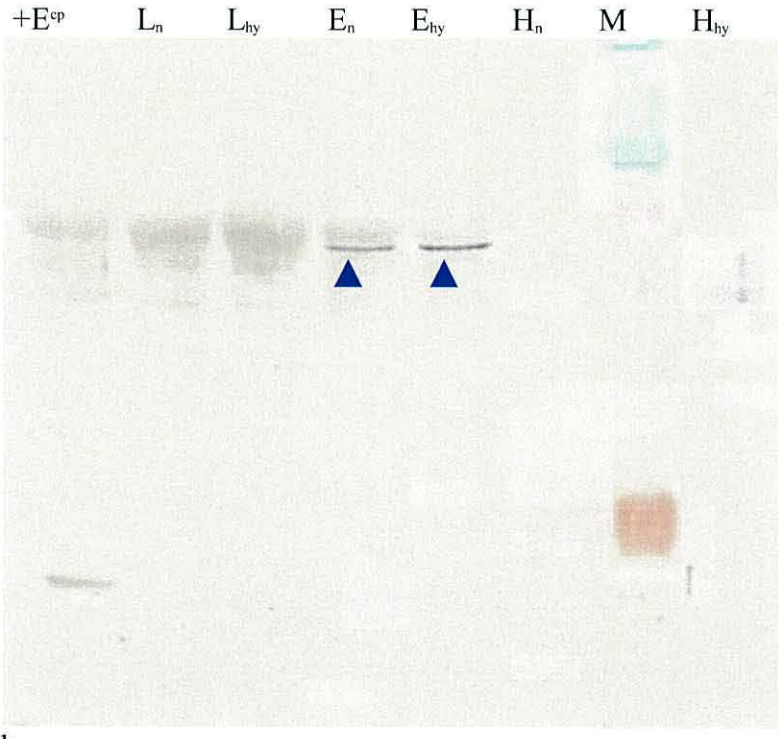

-A^cm B_t=0 B_hy L_t=0 L_hy M +L^cm E_t=0 E_hy H_t=0 H_hy

c

Figure 5.2. (a) Representative expression of Hsp 70 and respective homologues at each key phase in development after heat shock (23ºC) incubation for 2 hours in normoxia in *Cancer pagurus*. Note the relatively poor transfer of proteins. (b) Representative expression of Hsp 70 and respective homologues during development (L, limb formation, E, eye, H, hatch) in *Carcinus maenas*, after incubation for 2 hours at 10ºC in (n, normoxia and hy, hypoxia (2 kPa)). Blue arrowheads show intense constitutive expression of Hsp 70 at the eye phase in development. Note the relative absence of Hsp 70 at the hatching phase. n = 1 for each treatment lane in a,b. Control samples: $+A^{cm}$ (adult *Carcinus maenas*); $-A^{cp}$ (negative, adult *C. pagurus*); $+E^{cp}$ (eye phase embryonic *C. pagurus*, hypoxia 10ºC).


-A^cp
B_hs
B_hs
L_hs
L_hs
+A^cm
M
E_hs
E_hs
H_hs
H_hs

a


+E^cp
L_n
L_hy
E_n
E_hy
H_n
M
H_hy

b

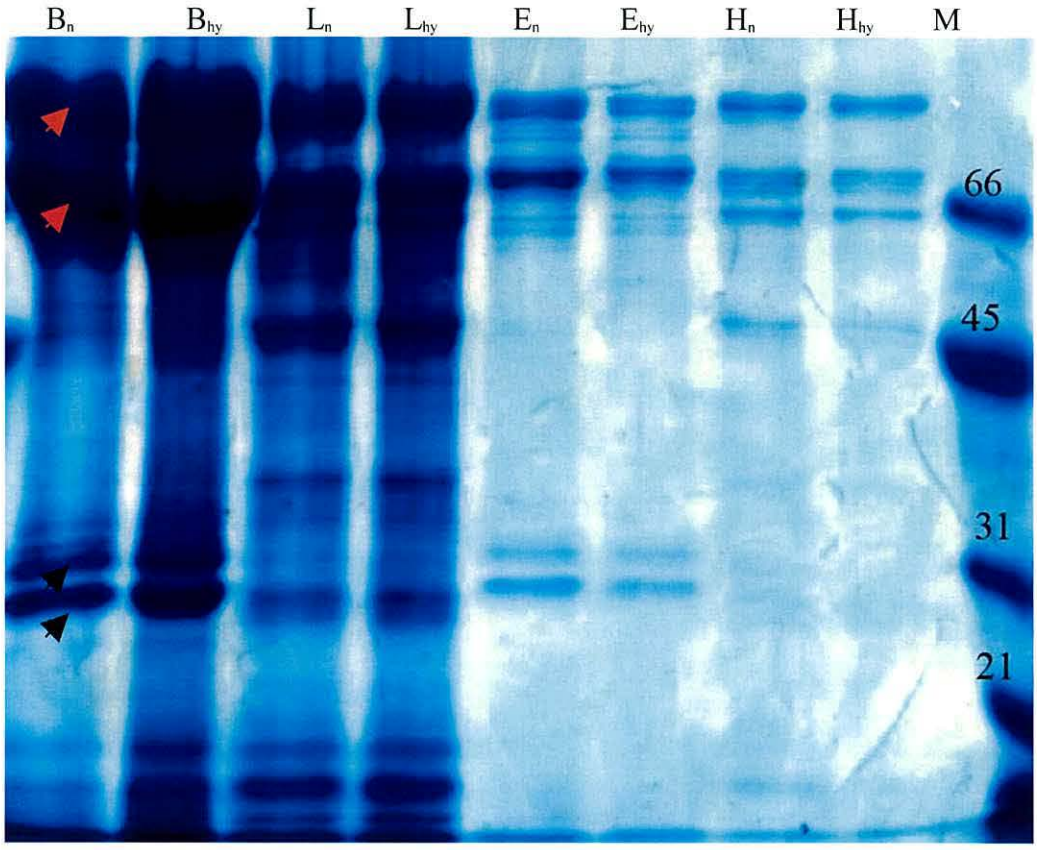
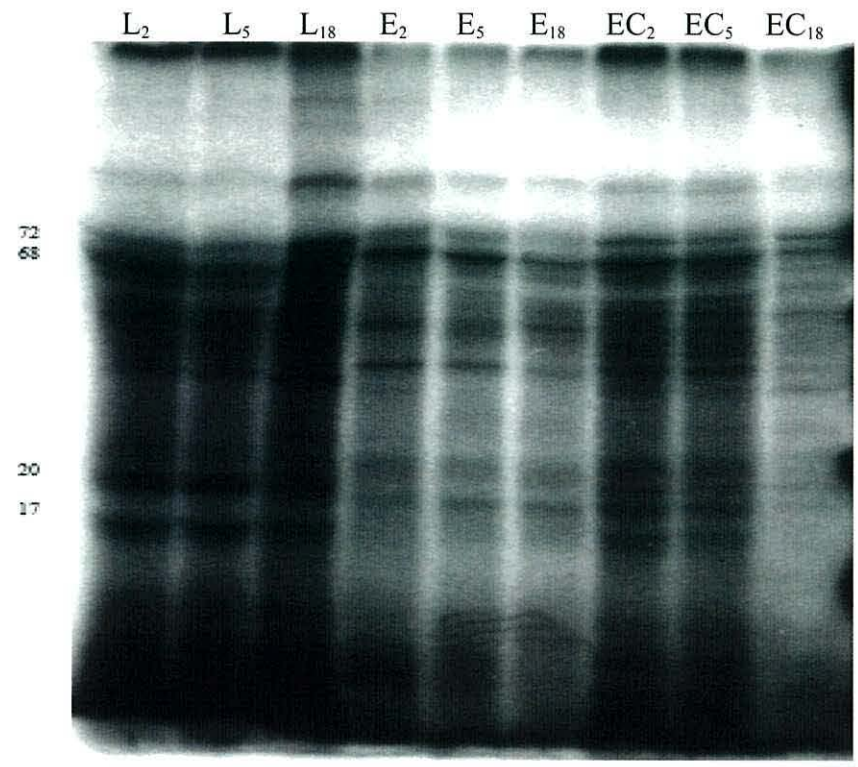
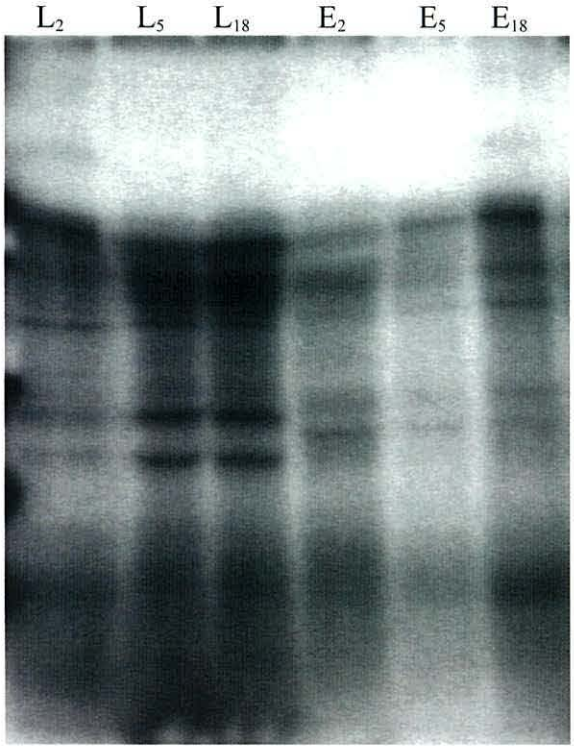


Figure 5.3. Coomassie stained gel showing location of the main protein classes in the existing protein pools at all the key phases in development of *Cancer pagurus*, after incubation for 2 hours at 10°C in (n, normoxia and h, hypoxia (2 kPa).

Figure 5.4. (a) Autoradiograph showing the incorporation of ^{35}S-methionine/cisteine into the newly synthesised protein pools during development (L, limb formation, E, eye, EC eye and chromatophore phase) in *Cancer pagurus* after incubation for 2 hours at 10ºC in 18, 5, 2 kPa. n = 1 for each treatment lane. Note the incorporation into the 72, 68, 20, and 17 kDa protein classes throughout development and after exposure to progressive hypoxia. (b) Autoradiograph showing the incorporation at the limb formation and eye phases only.

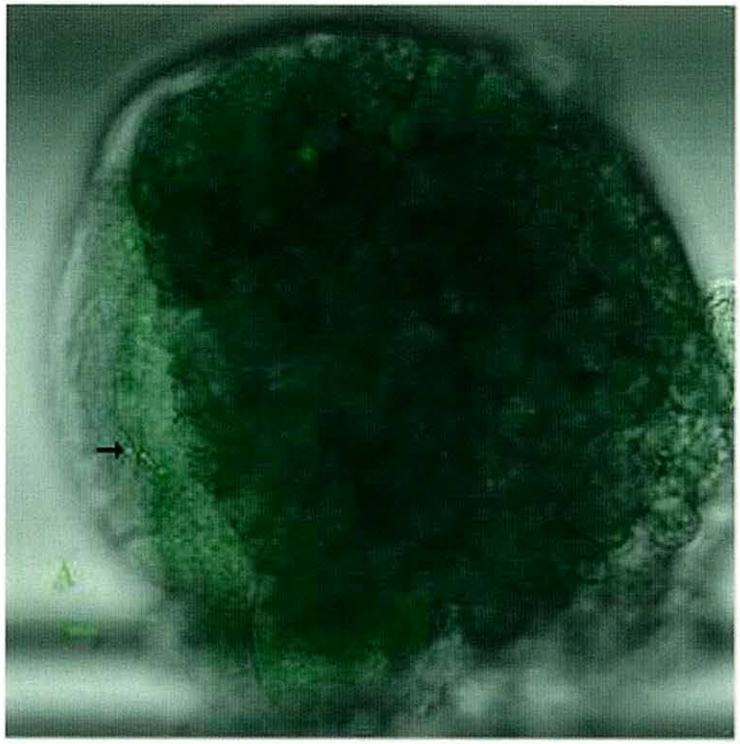


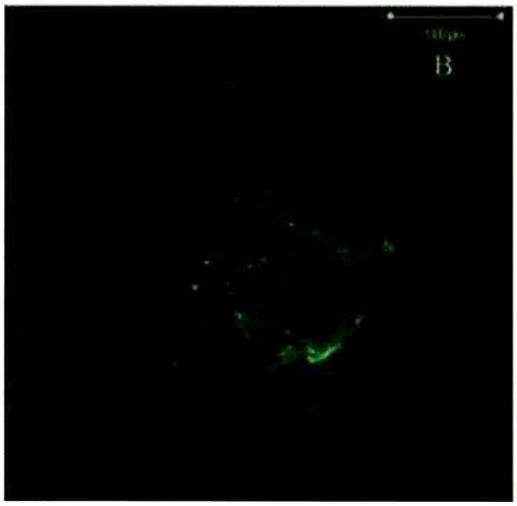
a



b

Figure 5.5. Bromodeoxyuridine (BrdU) labelled cell division at S-phase in the developing limbs and abdomen of the limb formation phase embryo of *Cancer pagurus* (whole mount preparation). (a) Arrow showing labelled cells in what could be the limb buds of the maxillipeds, after 4 hours incubation in normoxia. (b) Higher magnification of staining pattern observed at the surface of the yolk in a dorsal region of the embryo that could be the heart. (C) Negative control embryo, incubated in the absence of BrdU.




B

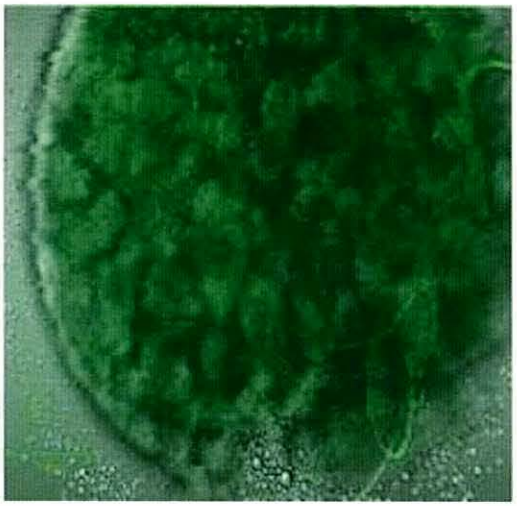
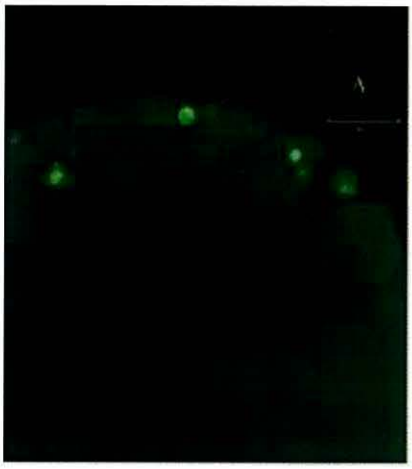
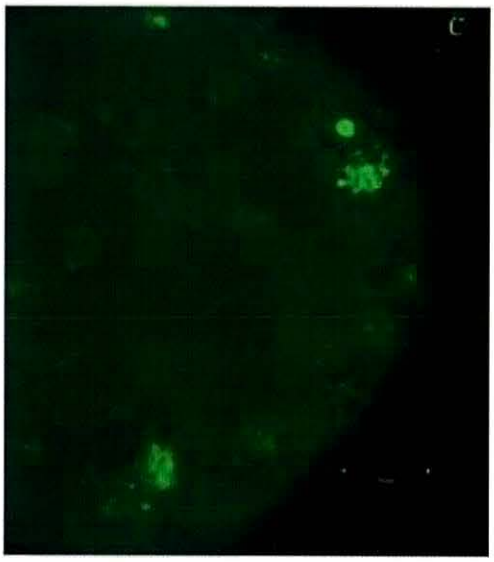
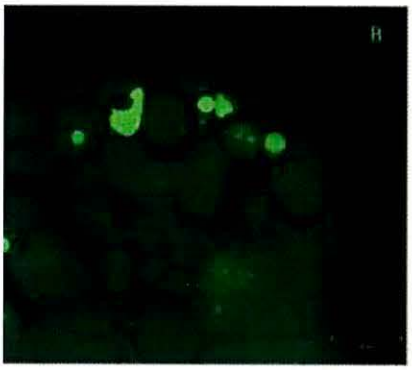
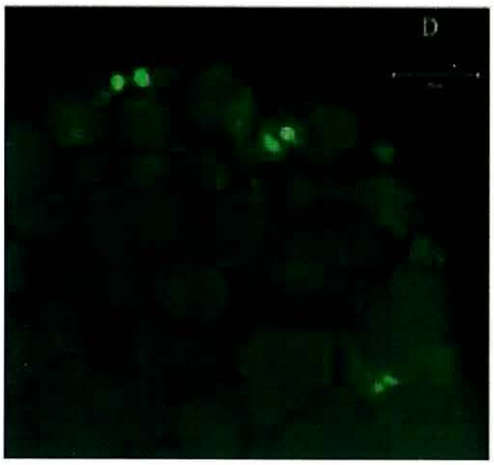


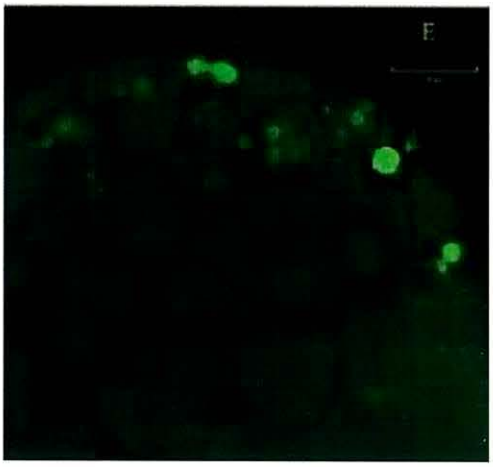
Figure 5.6. Bromodeoxyuridine (BrdU) labelled cell division at S-phase in the eye phase embryo of *Cancer pagurus* (whole mount preparation). (a) Labelled cells at the surface of the embryo after 2 hours incubation in normoxia (18-20 kPa). (b & c) Labelled cells after 6 hours incubation in normoxia. (d & e) Cell proliferation after 6 hours incubation in hypoxia (2 kPa).


A


C


B


D


E

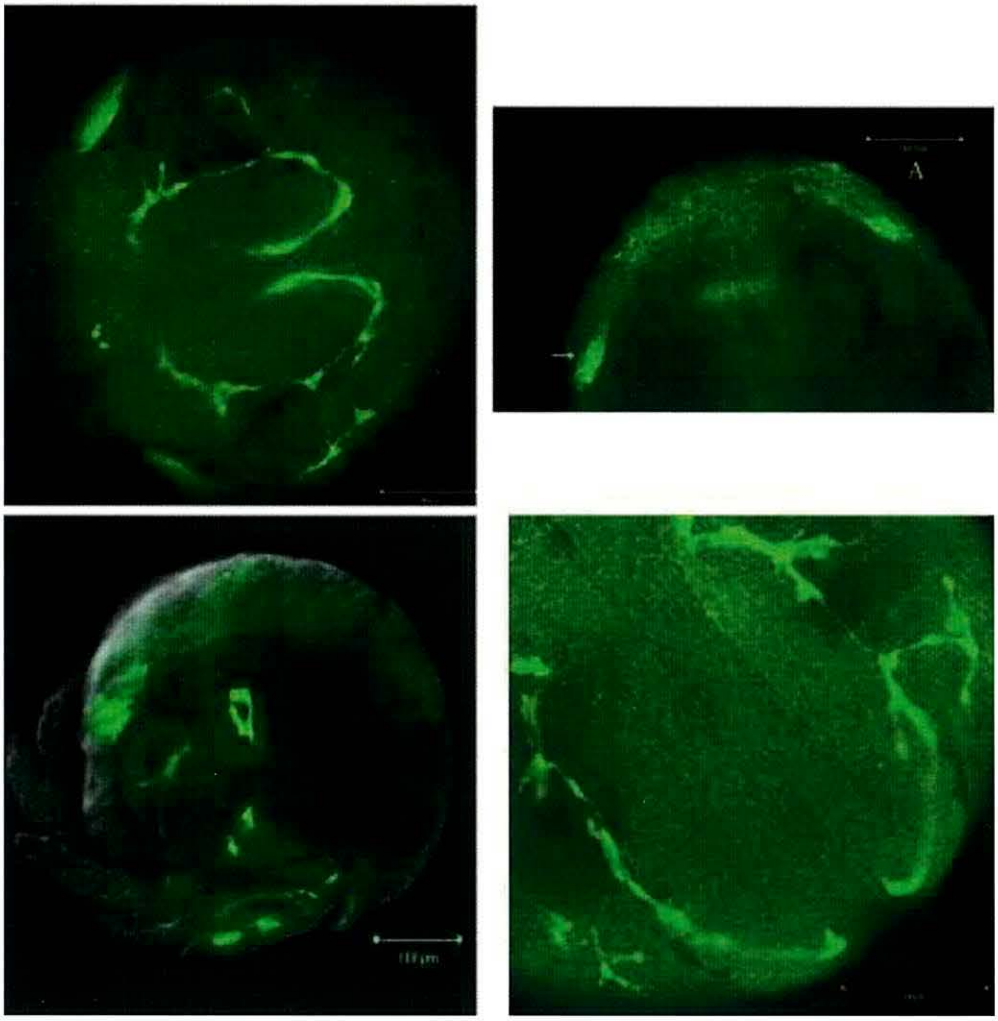


Figure 5.7. Natural auto-fluorescence in the ocular lobes of the eye phase embryo of *Cancer pagurus* (whole mount preparation). (a) Arrow showing natural fluorescence marking the developing omatidia. Staining patterns of tubular processes found on the ventral surface of the abdomen after incubation in normoxia and hypoxia for 1-6 hours.

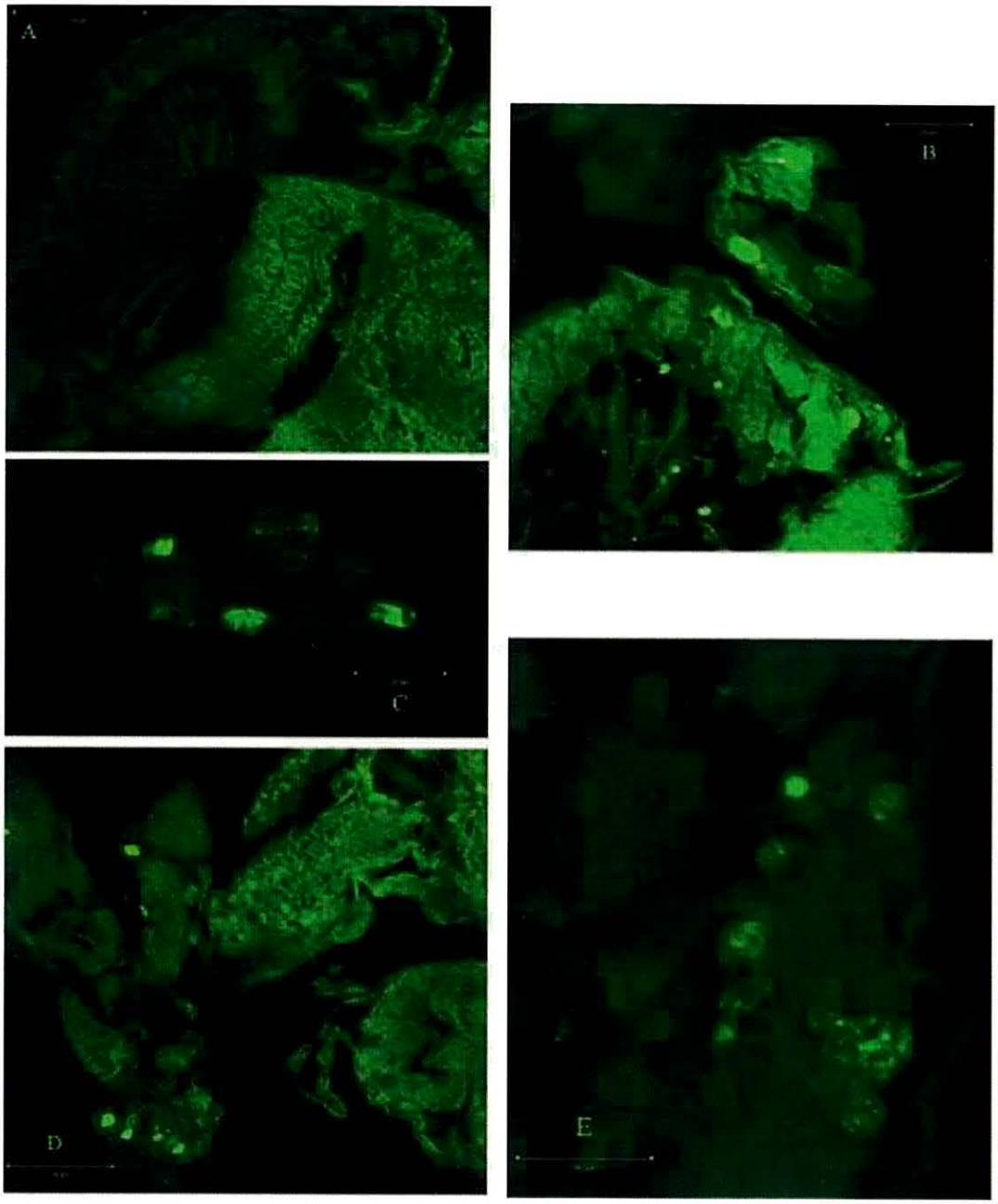


Figure 5.8. Bromodeoxyuridine (BrdU) labelled cell division at S-phase in the hatching phase embryo of *Cancer pagurus* (microtome longitudinal sections 10μm). (a) Negative control section through the ocular lobe, prepared without incubation in BrdU. (b) Labelled cells present in the ocular lobe (black arrow), in the end region of a maxilliped (white arrow), and in the base of the abdomen (e) high magnification after 4 hours incubation in normoxia. Note the labelled cells in the abdomen show a variation in BrdU incorporation, which could indicate differences in potential mitotic activity (c) Labelled cells present in a maxilliped, and also a labelled cell cluster at the base of the abdomen (d), after 4 hours incubation in hypoxia.

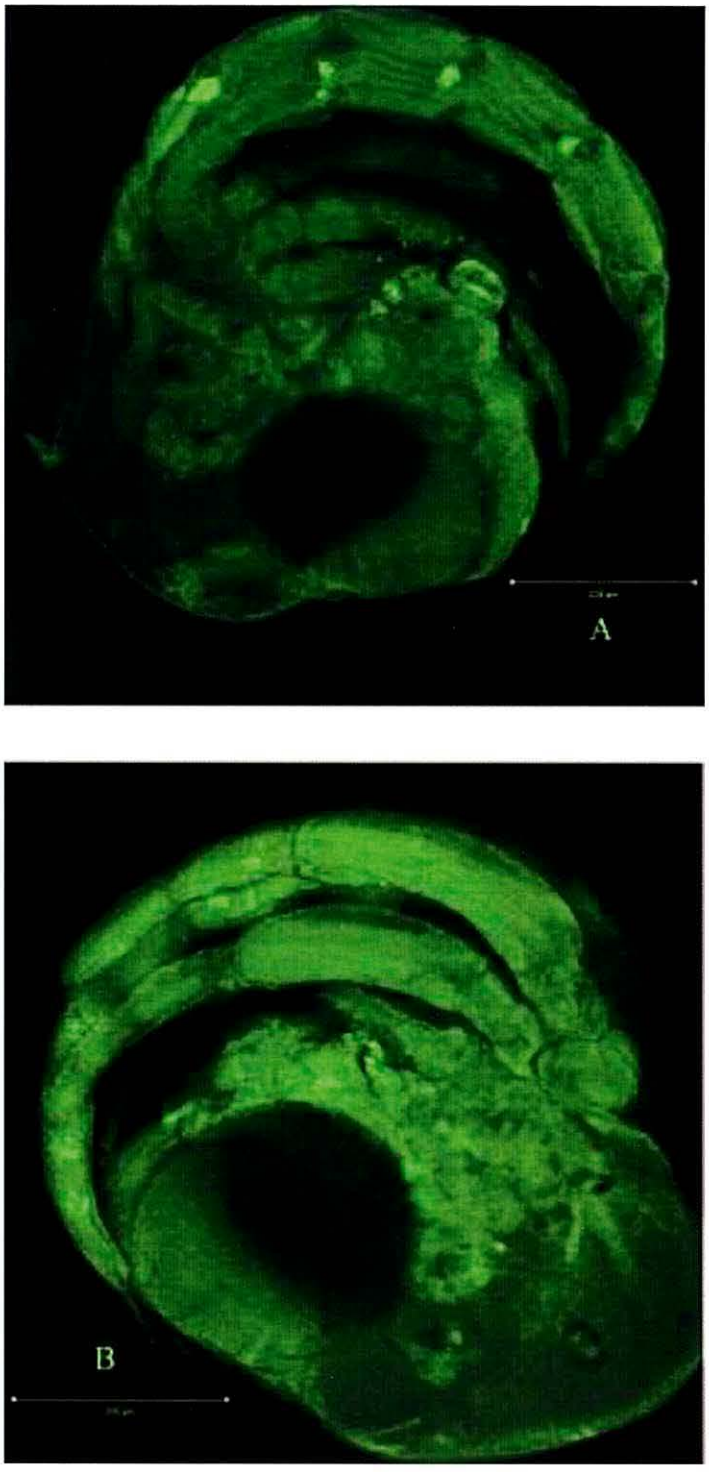


Figure 5.9. Natural auto-fluorescence in the hatching phase embryo of *Cancer pagurus* (whole mount preparation). (a) Negative control prepared without incubation in BrdU. (b) BrdU labelling and or natural fluorescence in the maxillipeds observed after 1-4 hours incubation in normoxia and hypoxia.

References

Adiyodi, R. G. and Subramoniam, T., (1983). Arthropoda-Crustacea. In, *Reproductive Biology of Invertebrates:* Vol: **1** *Oogenesis, Oviposition and Oosorption*. (Adiyodi, K. G. and Adiyodi, R. G., eds). *John Wiley and Sons Ltd. Chichester, NY.* Pp 443-495.

Agocha, A., Lee, H-W. and Eghbali-Webb, M. (1997). Hypoxia regulates basal and induced DNA synthesis and collagen type I production in human cardiac fibroblasts: Effects of transforming growth factor-β1, thyroid hormone, angiotensin II and basic fibroblast growth factor. *J Mol Cell Cardiol.* **29**, Pp 2233-2244.

Airaksinen, S., Rabergh, C. M., Sistonen, L. and Nikinmaa, M. (1998). Effects of heat shock and hypoxia on protein synthesis in rainbow trout. *J. Exp. Biol.* **201**, Pp 2543-2551.

Airriess, C. N. and McMahon, B. R. (1994). Cardiovascular adaptations enhance tolerance of environmental hypoxia in the crab *Cancer magister. J. Exp. Biol.* **190**. Pp 23-41.

Ali, A., Krone, P. H., Pearson, D. S. and Heikkila, J. J. (1996). Evaluation of stress-inducible hsp90 gene expression as a potential molecular biomarker in *Xenopus laevis. Cell stress and chaperones*. **1**(1), Pp 62-69.

Alwes, F. and Scholtz, G. (2004). Cleavage and gastrulation of the euphausiacean *meganyctiphanes norvegica* (crustarea, Malacostraca). *Zoomorphology*. **123**, Pp 125-137.

Anderson, J. B., Hedrick, M. S. and Wang, T. (2002). Cardiovascular responses to hypoxia and anaemia in the toad *Bufo marinus. The journal of experimental biology*. **206**, Pp 857-865.

Anger, K. and Dawirs, R. R. (1982). Elemental composition (C,N,H) nad energy in growing and starving larvae of *Hyas araneus* (Decapoda, Majidae). *Fish. Bull. U.S.* **80**, Pp 419-433.

Anger, K. (1983). Temperature and larval development of *Hyas araneus* L. (Decapoda: Majidae); extrapolation of laboratory data to field conditions. *J. Exp. Mar. Biol. Ecol.* **69**, Pp 203-215.

Anger, K. and Hirche, H. J. (1990). Nucleic acids and growth of larval and early juvenile spider crab *Hyas araneus. Mar. Biol.* **105**, Pp 403-411.

Anger, K. (1998). Patterns of growth and chemical composition in decapod crustacean larvae. *Invertebrate reproduction and development*. **33**, Pp 2-3.

Arrigo. A. P. and Tanguay, R. M. (1991). Expression of Heat Shock proteins during development in *Drosophila. Results Probl. Cell Differ.* **17,** Pp 106-119.

Ashford, A. J. and Pain, V. M. (1986). Effect of diabetas on the rates of synthesis and degradation of ribosomes in rat muscle and liver *in vivo* . *Journal of Biological Chemistry*. **261**, Pp 4059-4065.

Bacon, N. C. M., Wappner, P., O'Rourke, J. F., Bartlett, S. M., Shilo. B., Pugh, C. W. and Ratcliffe, P. J. (1998). Regulation of the drosophila bHLH-PAS protein sima by hypoxia: Functional evidence of homology with mammalian HIF - 1α. *Biochemical and Biophysical Research Communications*. **249**, Pp 811-816.

Baert, J. L., Britel, M., Slomianny, M. C., Delbart, C., Fournet, B., Sautiere, P. and Malecha, J. (1991). Yolk protein in leech identification, purification and characterization of vitellin and vitellogenin. *Eur. J. Biochem.* **201**, Pp 191-198.

Baeza, J. and Fernández, M. (2002). Active brood care in *Cancer setosus* (Crustacea: Decapoda): the relationship between female behaviour, embryo oxygen consumption, and the cost of brooding. *Funct. Ecol.* **16,** Pp 241-251.

Basu, N. Todgham, A. E. Ackerman, P. A. Bibeau, M. R. Nakano, K. Schulte, P. M. and Iwama, G. K. (2002). Shock protein genes and their functional significance in fish. *Gene.* **295**, Pp 173-183.

Bekker, A., Holland, H. D., Wang, P. L., Rumble, D., Stein, H. J., Hannah, J. L., Coetzee, L. and Beukes, N. J. (2004). Dating the rise of atmospheric oxygen. *Nature*. **427,** Pp 117-120.

Benninger, P. G., Elner, R. W., Foyle, T. P. and Odense, P. H. (1988). Functional anatomy of the male reproductive system and the female spermatheca in the snow crab *Chinoecetes opilio O. Fabricius* (Decapoda: Majidae) and a hypothesis for fertilization. *J. Crust. Biol.* **8**, Pp 322-332.

Bensaude, O. and Morange, M. (1983). Spontaneous high expression of heat-shock proteins in mouse embryonal carcinoma cells and ectoderm from day 8 mouse embryo. *EMBO*. **2**, Pp 173-177.

Berschick, P., Bridges, C. R. and Grieshaber, M. K. (1987). The influence of hyperoxia, hypoxia and temperature on the respiratory physiology of the intertidal rockpool fish *Gobius-cobitis pallas*. *J. Exp. Biol*. **130,** Pp 369-387.

Bickler, P. and Buck, L. (1998). Adaptations of vertebrate neurons to hypoxia and anoxia: maintaining critical Ca2+ concentrations. *J. Exp. Biol*. **201**, Pp 1141-1152.

Biesiot, P. M. and Perry H. M. (1995). Biochemical composition of the deep-sea red crab *Chaceon quinquedens* (Geryonidae): organic reserves of developing embryos and adults. *Marine Biology*. **124**, Pp 407-416.

Bligh, E. G. and Dyer, W. J. (1959). Canadian journal of biochemistry and physiology. *Can. J. Biochem. Physiol*. **37**, Pp 912-917.

Bliss, D. E. (1968). Transition from water to land in decapod crustaceans. A. M. *Zoologist*. **8**, Pp 355-392.

Bonaventura, R., Poma, V., Russo, R., Zito, F. and Matranga, V. (2006). Effects of UV-B radiation on development and Hsp70 expression in sea urchin cleavage embryos. *Marine Biology*. **149**, Pp 79-86.

Booth, D. (1995). Oxygen availability and embryonic development in sand snail (*Polinices sordidus*) egg masses. *J.Exp. Biol*. **198**, Pp 241-247.

Bosch, I. and Slattery, M. (1999). Costs of extended brood protection in the Antarctica seastar, *Neosmilaster georgianus* (Echinodermata: Asteroidea). *Marine biology*. **134**, Pp 449-459.

Boutilier, R. G., West, T. G., Pogson, G. H., Mesa, K. A., Wells, J. and Wells, M. J. (1996). *Nautilus* and the art of metabolic maintenance. *Nature*. **382**, Pp 534-536.

Boutilier, R. G. and St-Pierre, J. (2000). Surviving hypoxia without really dying. *Comparative Biochemistry and Physiology Part A*. **126**, Pp 481-490.

Boutilier, R. G. (2001). Mechanisms of cell survival in hypoxia and hypothermia. *J. Exp. Biol*. **204**, Pp 3171-3181. *Comp. Biochem. Physiol A*. **126**, Pp 481-490.

Bradford, S. M. and Taylor, A. C. (1982). The respiration of *Cancer pagurus* under normoxic and hypoxic conditions. *J. Exp. Biol*. **97**, Pp 273-288.

Bradford, D. F. and Seymour, R. S. (1985). Energy conservation during the delayed-hatching period in the frog *Pseudophryne bibroni*. Physiol zool. **58**, Pp 491-496.

Brante, A. (2005). An alternative mechanism to reduce intracapsular hypoxia in ovicapsules of *Fusitrition oregonensis* (Gastropoda). *Marm Biol*. **149**(2), Pp 269-274.

Branton, M. A., Macrae, T. H., Lipschultz and Wells, P. G. (1999). Identification of a small heat shock/a-crystallin protein in the scleractinian coral *Madracis mirabilis* (Duch and Mitch). *Can, J. 2001*. **77**(5), Pp 675-682.

Bricker, S. (1997). *Estuarine research federation Newsletter*. **23**, Pp 20-21.

Broekhuysen, G. J. (1936). On development, growth and distribution of *Carcinides maenas* (L). *Archives Neerlandaises de Zoologie*. **2**, Pp 257-399.

Brouwer, M., Larkin, P., Brown-Peterson, N., King, C., Manning, S. and Denslow, N. (2004). Effects of hypoxia on gene and protein expression in the blue crab, *Callinectes Sapidus. Marine Environmental Research*. **58**, Pp 787-792.

Buck, L. T., Land, S. C. and Hochahcka, P. W. (1993b). Anoxia-tolerant hepatocytes: model system for study of reversible metabolic suppression. *Am. J. Physiol.* **265**, R49-R56.

Buckley, L. J. (1979). Relationships between RNA-DNA, ratio, prey density and growth rate in cod (*Gadus* morhua L.) larvae. *J. Fish Res. Bd Can*. **36**, Pp 1497-1502.

Buckley, L. J. (1982). Effects of temperature on growth and biochemical composition of larval winter flounder, *Pseudopleuronectes americanus*. *Mar. Ecol. Prog. Ser*. **8**, Pp 181-186.

Buckley, L. J. (1984). RNA-DNA ratio: an index of larval fish growth in the sea. *Marine Biology*. **80**(3), Pp 291-298.

Buckley, L. J., Turner, S. S., Halavik T. A., Smigielski, A .S., Drew, S. M. and Laurence, G. C. (1984). Effects of temperature and food availability in growth, survival and RNA-DNA ratio of larval sand lance (*Ammodytes americanus) Mar. Ecol. Progr. Ser*. **15**, Pp 91-97.

Buckley, L., Caldarone, E. and Ong, T. L. (1999). RNA-DNA ratio and other nucleic acid-based indicators for growth and condition of marine fishes. *Hydrobiologia*. **401**, Pp 265-277.

Bunn, H. F. and Poyton, R. O. (1996). Oxygen sensing and molecular adaptation to hypoxia. *Physiol. Rev.* **76**, Pp 839-885.

Burggren, W. W. (1985). Gas exchange, metabolism and "ventilation" of gelatinous frog egg masses. *Physiol Zool*. **58**, Pp 503-514.

Burnett, L. (1992). Integrated function of the respiratory pigment hemocyanin in crabs. *Amer. Zool*. **32**, Pp 438-446.

Burnett, L. (1997). The challenges of living in Hypoxic and Hypercapnic Aquatic Environments. *Amer. Zool*. **37,** Pp 633-640.

Busa, W. B., Crowe, J. H. and Matson.G. B. (1982).Intracellular pH and the metabolic status of dormant and developing *Artemia* embryos. *Archs Biochem, Biophys.* **216**, Pp 711-718.

Busa, W. B. (1985). How to succeed at anaerobiosis without really dying. *Molec. Physiol.* **8**, Pp 351-358.

Butler, P.J, Taylor, E.W. and McMahon, B. R. (1978). Respiratory and circulatory changes in the lobster (Homarus vulgaris) during long term exposure to moderate hypoxia. J. Exp. Biol. **73**, Pp 131-146.

Caceres, C. E. and Tessier, A. J. (2004). Incidence of diapause varies among populations of *Daphnia pulicaria*. *Oceologica*. **141**, Pp 425-431.

Camargo, J. A. and Ward, J. V. (1995). Nitrate ($NO3^-N$) toxcity to aquatic life: a proposal of safe concentrations for two species of nearctic fresh water invertebrates. Chemosphere. **31**, Pp 3211-3216.

Camargo, J. A., Alonso, A. and Salamanca, A. (2005). Nitrate toxicity to aquatic animals: a review with new date for freshwater invertebrates. Chemosphere. **58**, Pp 1255-1267.

Campbell, R. W. and Dower, J. F. (2003). Role of lipids in the maintenance of neutral buoyancy by zooplankton. *Mar. Ecol.Prog. Ser.* **263**, Pp 93-99.

Cancino, J. M., Gallardo, J. A. and Torres, F. A. (2003). Combined effects of dissolved oxygen concentration and water temperature on embryonic development and larval shell secretion in the marine snail *Chorus giganteus* (Gastropoda: Muricidae). *Marine biology*. **142**, Pp 133-139.

Carmeliet, P., Dor, Y., Herbert, J. M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., Koch, C. J., Ratcliffe, P., Moons, L., Jain, R. K., Collen, D., Keshert, E. and Keshet, E. (1998). Role of HIF-1α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature.* **394**, Pp 485-490.

Carter, C. G. and Brafield, A. E. (1992). The relationship between specific dynamic action and growth in grass carp, *Ctenopharyngodon idella* (Val.). *journal of Fish Biology*. **40**, 895-907.

Carter, C. G., Houlihan, D. F., Brechin, J. and McCarthy, I. D. (1993). The relationship between protein intake and protein accretion, synthesis, and retention efficiency for individual grass carp, *Ctenopharyngodon idella* (Valenciennes). *Can. J. Zool.* **71**, Pp 392-400.

Carter. C. G., Houlihan, D. F. and Owen, S.F. (1998). Protein synthesis, nitrogen excretioin and long-term growth of juvenile *Pleuronectes flesus*. *Journal of fish biology*. **53**, Pp 272-284.

Chang, E. S. (1993). Comparative endocrinology of malting and reproduction: insects and crustaceans. *Annu. Rev. Entomol.* **38**, Pp 161-180.

Chang, E. S., Bruce, M. J. and Tamone, S. L. (1993). Regulation of crustacean molting: a multi-hormonal system. *Am. Zool.* **33**, Pp 324-329.

Chaffee, C. and Strathmann, R. R. (1984). Constraints on eggs masses. I. Retarded development within thick embryo masses. *J. Exp. Mar. Biol & Ecol.* **84**, Pp 73-83.

Chang, J., Knowlton, A. A. and Wasser J. S. (2000). Expression of heat shock proteins in turtle and mammal hearts: relationship to anoxia tolerance. *Am. J. Physiol. Regulatory Integrative Comp. Physiol.* **278**, Pp R209-R214

Cheek, P. B., Holt, A. B., Hill, D. E. and Talbert, J. L. (1971). Skeletal muscle cell mass and growth: the concept of the deoxyn'ribonucleic acid unit. *Pediator. Res.* **5**, Pp 312-328.

Cheng, S. H., Chan, C. H., So, P. K., Cheng, C. W. and Wu, R. S. S. (2003). Cloning of the Hsp70 gene in barnacle larvae and its expression under hypoxic conditional. *Baseline / Marine Pollution Bulletin*. **46,** Pp 659-676**.**

Cheng, S., So, C., Chan, P., Cheng, C. and Wu, R. (2006). Cloning of the Hsp70 gene in barnacle larvae and its expression under hypoxic conditions. *Baseline Marine Pollution*. **46**, Pp 659-675.

Cheung, T. S. (1966). The development of egg-membranes and egg attachment in the shore crab, *Carcinus Maenas,* and some related decapods. *J. MarBiol. Ass. UK.* **46**, Pp 373-400.

Christy, J. H. (1987). Competitive mating, mate choice and mating associations of brachyuran crabs. *Bull. Mar. Sci.* **41**(2), Pp 177-191.

Chu, K. and Ovsianico-Koulikowsky, N. (1994). Ontogenetic changes in metabolic activity and biochemical composition in the shrimp, Metapenaeus ensis. *J. Exp. Mar. Biol & Ecol.* **183**, Pp 11-26.

Chung J. and Webster, S. (2004). Expression and release patterns of neuropeptides during embryonic development and hatching of the green shore crab, *Carcinus maenas. Development.* **131**, Pp 4751- 4761.

Cimino, E. J., Owens, L., Bromage, E. and Anderson, T. A. (2002). A newly developed ELISA showing the effect of environmental stress levels of hsp86 in *Cherax quadricarinatus* and *Penaeus monodon. Comparative Biochemistry and Physiology Part A.* **132**, Pp 591-598.

Clegg, J. S. (1993). Respiration of *Artemia franciscana embryos* after continuous anoxia over 1-year period. *J. Comp. Physiol.* **163**B, Pp 48-51.

Clegg, J. S., Jackson, S. A. and Warner, A. H. (1994). Extensive intracellular translocations of a major protein accompany anoxia in embryos of *Artemia franciscana. Exp. Cell Res.* **212**, Pp 77-83.

Clegg, J. S., Jackson, S. A., Liang, P. and Macre, T. H. (1995). Nuclear-cytoplasmic translocations of protein p26 during aerobic-anoxic transitioins in embryos of *Artemia franciscana. Experimental cell research.* **219**, Pp 1-7.

Clegg, J. S., Drinkwater, L. E. and Sorgeloos. P. (1996). The metabolic status of diapause embryos of *Artemia franciscana. Physiol. Zool.* **69**, Pp 49-66.

Clegg, J. S. (1997). Embryos of *Artemia franciscana* survive four years of continuous anoxia, the case for complete metabolic rate depression. *J. Exp. Biol.* **200**, Pp 467-475.

Clegg, J. S., Willsie J. K. and Jackson, S. A. (1999). Adaptive significance of a small Heat shock/α-crystallin protein (p26) in encysted embryos of the brine shrimp, *Artemia franciscana. Amer. Zool.* **39,** Pp 836-847.

Cochran, R. E. and Burnett, L. E. (1996). Respiratory respones of the salt marsh animals, *funulus heteroclitus, leiostomus xanthurus,* and *palaemonetes puglo* to environmental hypoxia and hypercapnia and to the organophosphate pesticide, a zinphosmethyl. *J. Exp. Mar. Biol. Ecol.* **195** (1), Pp 125-144.

Cohen, C. S. and Strathmann, R. R. (1996). Embryos at the edge of tolerance: effects of enviroment and structure of egg masses on supply of oxygen to embryos. *Bio. Bull.* **190**, Pp 8-15.

Cohen, J. (1967). Living embryos: An introduction to the study of animal development, **2nd ed.** Pergamon press. *Comp. Biochem. Physiol.* **119**A, Pp 469-476.

Conceição, L. E. C., van der Meeren, T., Verreth, J. A. J., Evjen, M. S., Houlihan, D. F. and Fyhn, H. J. (1997). Amino Acid metabolism and protein turnover in larval turbot (*Scophthalmus maximus*) fed natural zooplankton or *Artemia. Marine Biology.* **129**, Pp 255-265.

Conceição, L. E. C., Houlihan, D. F. and Verreth, J. A. J. (1997). Fast growth, protein turnover and costs of protein metabolism in yolk-sac larvae of the African catfish (*Clarias gariepinus*). *Fish Physiol and Biochem.* **16**, Pp 291-302.

Costa, T. M., Stella, M. J. S. and Negreiros-Fransozo, M. L. (2006). Reproductive pattern comparison os *Uca thayeri* Rathbun, 1900 and *U. uruguayensis Nobili, 1901(*Crustacea, Decapoda, Ocypodidae*). Brazilian archieves of biology and technology.* **49**, Pp 117-123.

Craig, E. A. and Gross, C. A. (1991). Is Hsp70 the cellular thermometer? *Trends in Biochemical Science.* **16,** Pp 135-140.

Dahms, H. U. (2000). Phylogenectic implications of the crustacean nauplins. *Hydrobiologia.* **417** (1), Pp 1573-5117.

Dall, W. and Smith, D.M. (1978). Water uptake at ecdysis in the western rock lobster. *J. Exp. Mar. Biol.* **35**, Pp 165-176.

Dandy, J. W. (1970). Activity responses to oxygen in thr brook trout, *Salvelinius fontinalis*. *Can. J. Zool.* **48**, Pp 1067-1072.

Darken, R. S., Martin, K. L. and Fisher, M. C. (1998). Metabolism during delayed hatching in terrestrial eggs of a marine fish, the grunion *Leuresthes tenuis. Physiol Zool.* **71**, Pp 400-406.

David, E., Tanguy, A., Pichavant, K. and Moraga, D. (2005). Response of the Pacific oyster *Crassostrea gigas* to hypoxia exposure under experimental conditions. *FEBS Journal*. **272**, Pp 5635-5652.

Davis, R. E. and King, M. L. (1989). The developmental expression of the heat-shock response in *Xenopus laevis*. *Development*. **105**, Pp 212-222.

DE Vries, M. C., Rittschof, D. and Forwar, Jr. R. B. (1991). Chemical mediation of larval release behaviours in the crab *Neopanope sayi. Reference. Bio. Bull*. **180**, Pp 1-11.

Desmarais, J., Bordignon, V., Lopes, F., Smith, L. and Murphy, B. (2004). The escape of the mink embryo from obligate diapause. *Biology of reproduction*. **70**, Pp 662- 670.

Diaz, R. and Rosenburg, R. (1995). Marine Benthic Hypoxia: A review of its ecological effects and the behavioural responses of benthic macrofauna. *Oceanography and Marine Biology Annual review*. **33**, Pp 245-303.

Douglas, R. M., Xu, T. and Haddad, G. G. (2001). Cell cycle progression and cell division are sensitive to hypoxia in *Drosophila melanogaster* embryos. *Am. J. Physiol. Regulatory Integrative Comp Physiol*. **280,** Pp R1555-R1563.

Douglas, R. M., Farahani, R., Morcillo, P., Kanaan, xu, A. and Haddad, G. G. (2005). Hypoxia induces major effects on cell cycle kinetics and protein expression in *Drosophila melanoyster* embryos. *AM. J Physiol*. **288**, Pp 511-521.

Durstewitz, G. and Terwilliger, N. B. (1997). Developmental changes in hemocyanin expression in the dungeness crab, *Cancer magister. J. Biol.Chem*. **272**(7), Pp 4347-4350.

Ebert, B. L. and Bunn, H. F. (1998). Regulation of transcription by hypoxia requires a multiprotein complex that includes Hypoxia-Inducible factor 1, and p300/CREB binding protein. *Moll Cell Biol*. **18** (7), Pp 4089-4096.

Edwards, E. (1979). The edible crab and its fishery in British waters. *Fishing News Books Ltd*. Pp 48-82.

Efford, I. E. (1969). Egg size in the sand crab *Emerita* analoga (Decapoda Hippidae). *Crustaceana*. **16**, Pp 15-26.

El Haj, A. J., Govind, C. K. and Houlihan, D. F. (1984). Growth of lobster leg muscle fibres over intermolt and molt. *J. Crus. Biol*. **4**, 536-545.

El Haj, A. J. and Houlihan, D. F. (1987). In vitro and in vivo protein synthesis rates in a crustacean muscle during the moult cycle. *J. Exp. Biol*. **127**, Pp 413-426.

El Haj, A. J., Clarke, S. R., Harrison, P. and Chang, E. S. (1996). In vivo muscle protein synthesis rates in the American lobster *Homarus Americanus* during the moult cycle and in response to 20-Hydroxyecdysone. *J. Exp. Biol*. **199**, Pp 579-585.

Encina, L. and Granado-Lorencio, C. (1997). Seasonal variations in the physiological status and energy content of somatic and reproductive tissues of chub. *J. of Fish Biology*. **50**, Pp 511-522.

English, T. E. and Storey, K. B. (2003). Freezing and anoxia stresses induce expression of metallothionein in the foot muscle and hepatopancreas of the marine gastropod *Littorina littorea*. *J. Exp. Biol*. **206**, Pp 2517-2524.

Epel, D. (1967). Protein synthesis in sea urchin eggs: a 'late' response to fertilisation. Proc. *Natl. Acad. Sci. USA*. **57**, Pp 899-906.

Epel, D., Hemela. K., Shick, M. and Patton, C. (1999). Development in the floating world: Defences of eggs and embryos against damage from UV radiation. *Amer. Zool.* **39**, Pp 271-278.

Epel, D. (2003). Protection of DNA during early development: adaptations and evolutionary consequences. *Evolution and Development*. **5**(1), Pp 83-88.

Eriksson, S. P., Nabbing and Joman, S. (2006). Is brood care in *Nephrops norvegicus* during hypoxia adaptive or a waste of energy? *Functional ecology*. **0**, Pp 1365-2435.

Fauconneau, B. (1984). The measurement of whole body protein synthesis in larval and juvenile carp (*Cyprinus carpio). Comp. Biochem. Physiol.* **78**B, Pp 845-850.

Fauconneau, B. and Arnal, M. (1985). *In vivo* protein synthesis in different tissues and the whole body of rainbow trout (*Salmo gairdnerii* R.). Influence of environmental temperature, *Comp. Biochem. Physiol.* **82**A(1), Pp179-187.

Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annu Rev Physiol*. **61**, Pp 243-282.

Fernández, M., Bock, C. and Portner, H. (2000). The cost of being a caring mother: the ignored factor in the reproduction of marine invertebrates. *Ecology Letters*. **3**(6), Pp 487-494.

Fernández, M., Pardo, L. and Baeza, J. (2002). Patterns of oxygen supply in embryo masses of brachyuran crabs throughout development: the effect of oxygen availability and chemical cues in determining female brooding behaviour. *Mar. Ecol. Prog. Ser*. **245,** Pp 181-190.

Fernández, M. and Brante, A. (2003). Brood care in brachyuran crabs: the effect of oxygen provision on reproductive costs. *Revista Chilena de Historia Natural*. **76**, Pp 157-168.

Fernández, M., Ruiz-Tagle, N., Cifuentes, S., Portner, H. O. and Arntz, W. (2003). Oxygen-dependant asynchrony of embryonic development in embryo masses of brachyuran crabs. *Mar. Biol*. **142,** Pp 559-565.

Fick, A. (1855). Ueber diffusion. *Ann. Phys.* **170**, Pp 59-86.

Finn, R. N., Widdows, J. and Fyhn, J. H. (1995). Calorespirometry of developing embryos and yolk-sac larvae of Turbot (*Scophthalmus-maximus*). *Mar. Biol*. **122**(1), Pp 157-163.

Fraser, K. P. P., Lyndon, A. and Houlihan, D. F. (1998). Protein synthesis and growth in Atlantic juvenile halibut, Hippoglossus hippoglossus (L.): application of ^{15}N stable isotope technique. *Aquaculture Research*. **29**, Pp 289-298.

Fraser, K. P. P., Houlihan, D. F., Lutz, P. L., Kabler, S. L., Manuel, L. and Brechin, J. G. (2001). Complete suppression of protein synthesis during anoxia with no post-anoxia protein synthesis debt in the red-eared slider turtle *Trachemys scripta elegans*. *J. Exp. Biol*. **204**, Pp 4353-4360.

Fraser, K. P. P., Clarke, A. and Peck, L. S. (2002). Low-temperature protein metabolism: seasonal changes in protein synthesis and RNA dynamics in the Antartic limpet *Nacella concinna* Strebel 1908. *J. Exp. Biol.* **205**, Pp 3077-3086.

Fraser, K. P. P., Peck, L. S. and Clarke, A. (2004). Protein synthesis, RNA concentrations, nitrogen excretion, and metabolism vary seasonally in the Antarctic holothurian *Heterocucumis steineni (*Ludwig 1898). *Physiological and Biochemical Zoology*. **77**(4), 556-569.

Frazier, M. R., Woods, H. A. and Harrison, J. F. (2001). Interactive effects of rearing temperature and oxygen on the development of *Drosophila melanogaster. Physiological and Biochemical Zoology*. **74**(5), Pp 641-650.

Freichs, K. U., Smith, C. B., Brenner, M., DeGracia, D. J., Krause, G. S., Marrone L., Dever, T. E., Hallenbeck, J. M. (1998). Supression of Protein synthesis in brain during hibernation involves inhibition of protein initiation and elongation. *Proc. Natl. Acad. Sci.* **95**, Pp14511-14516.

Fujiwara, A. and Yasumasu, I. (1997). Does the respiratory rate in sea urchin embryos increase during early development without proliferation of mitochondria?. *Dev. Growth Differ.* **39**, Pp 179-189.

Garayoa, M., Martinez, A., Lee, S., Pio, R., An, W. G., Neckers, L., Trepel, J., Montuerga, L. M., Ryan, H., Johnson, R., Gassmann, M. and Cuttutta, F. (2000). Hypoxia-Inducible factor-1 (HIF-1) up-regulates adrenomedullin expression in human tumor cell lines during oxygen deprivation: A possible promotion mechanism of carcinogenesis. *Molecular Endocrinology.* **14**(6), Pp 848-862.

Garcia-Guerrero, M. U. and Hendrickx, M. E. (2004). Embryology of Decapod Crustaceans I. Embryonic development of the Mangrove crabs *Goniopsis Pulciira* and *Aratus Pisonii* (Decapoda: Brachyura). *Journal of Crustaceana Biology.* **24**(4), Pp 666-672.

Garcia-Guerrero, M. U. and Hendrickx, M. E. (2006). Embryology of Decapod crustaceans, II: Gross embryonic development of *petrolisthes robsonae* glassell, 1945 and petrolisthes armatus (Gibbes, 1850) (Decapoda, anomura, porcellanidae). *Crustaceana.* **78**(9), Pp 1089-1097.

Garlick, P. J., Mcnurlan, M. A. and Preedy, V. R. (1980). A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of [^{3}H]phenylalanine. *Biochem. J.* **192,** Pp 47-62.

Geraci, F., Pinsino, A., Turturici, G., Savona, R., Giudice, G. and Sconzo, G. (2004). Nickel, lead, and cadmium induce differential cellular responses in sea urchin embryos by activating the synthesis of different Hsp70s. *J. Biochemical and Biophysical Research Communications.* **322**, Pp 873-877.

Gibbs, A. G., Chippindale, A. K. and Rose, M. R. (1997). Physiological mechanisms of evolved desiccation resistance in *Drosophila Melanogaster. J. Ex. Biol.* **200**, Pp 1821-1832.

Giese, A. C. (1959). Comparative physiology: annual reproductive cycles of marine invertebrates. *Annu Rev Physiol.* **21**, Pp 547-576.

Giese, A. C. and Kanatani, M. (1987). *Maturation and spawning.* Pp 251-329. In Giese, A. C., Pearse, J. S., and Pearse, V. B., eds. Reproduction of marine invertebrates. Vol **IX**. *Blackwell Scientific, Palo Alto, Calif., and Boxwood. Pacific Grove, Calif.*

Gilchrist, B. M. (1978). Scanning electron microscope studies of the egg shell in some anostraca (Crustacea: Branchiopoda). *Cell and tissue research.* **193**(2), Pp 337-351.

Gleed, R.D. and Mortola, J.P. (1991). Ventilation in newborn rats after gestation at simulated high altitude. *Journal of applied physiology,* **70**, Pp 1146-1151.

Goda, N., Ryan, H. E., Khadivi, B., McNulty, W., Rickert, R. G. and Johnson, R. S. (2003). Hypoxia-inducible factor 1α is essential for cell cycle arrest during hypoxia. *Moll CellBiol.* **23**, Pp 359-369.

Goldberg, M. A., Dunning, S. P. Bunn, H. F. (1988). Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. *Science* **242**, Pp 1412-1415.

Gonzalez-Baro, M. R., Heras, H., Pollero, R. J. (2000). Enzyme activities indudved in lipid metabolism during embryonic development of *Macrobrachium borellii. The Journal of Experimental Zoology.* **286**(3), Pp 231-237.

Gordon, S., Bharadwaj, S., Hnatov, A., Ali, A. and Nick, O. (1997). Distinct stress-inducible and developmentally regulated heat shock transcription factors in *Xenopus Oocytes. Developmental biology.* **181**, Pp 47-63.

Gorr, T. A., Cahn, J. D., Hideo, Y. and Bunn, F. (2004). Hypoxia-induced Synthesis of Hemoglobin in the Crustacean *Daphnia magna* Is Hypoxia-inducible Factor dependent. *Journal of Biological Chemistry*, **279**, Pp 36035-36047.

Gorr, T. A., Grassmann, M. and Wappner, P. (2006). Sensing and responding to hypoxia via HIF in model invertebrates. *Journal of insect physiology*. **52**, Pp 349-364.

Goudeau, M. (1976). Secretion of embryonic envelope and embryonic moulting cycles in *Hemioniscuc balani* Buchholtz, Isopoda Epicaridea. *J. Morph*. **148,** Pp 427-452.

Goudeau, M. and Lachaise, F (1980b). 'Endogenous yolk' as the precursor of a possible fertilisation envelop *(Carcinus maenas). Tissue and Cell*. **12**, Pp 503-512.

Goudeau, M. and Lachaise, F. (1983). Structure of the egg funiculus and deposition of embryonic envelopes in a crab. *Tissue & cell*. **15,** Pp 719-723

Goudeau, M., Talbot, P. and Harper, R. (1987). Mechanism of egg attachment stalk formation in the lobster, *Homarus*. *Gamete Research*. **18**, Pp 279-289.

Gracey, A. Y., Troll, J. V. and Somero, G. N. (2001). Hypoxia-induced gene expression profiling in the euroxic fish gillichthys mirabilis. *Proc. Natl. Acad. Sci. USA*. **98**, Pp 1993-1998.

Gracey, A. Y. and Cousins, A. R. (2003). Application of microarray technology in environmental and comparative physiology. *Annul. Rev. Physiol.* **65**, Pp 231-259.

Grandin, N. and Charbonneau. (1989). Intracellular pH and the increase in protein synthesis accompanying activation of *Xenopus* eggs. *Biology of the cell*. **67**, Pp 321-330.

Green, B. S. and McCormick, M. I. (2005). O_2 replenishment to fish nests: males adjust brood care to ambient conditions and brood development. *Behavioural ecology*. **16**(2), Pp 389-397.

Gross, P. R., Malkin, L. I. and Moyer, W. A. (1964). Templates for the first proteins of embryonic development. *PNAS*. **51**, Pp 407-414.

Guadagnoli, J. A. and Reiber, C. L. (2005). Changes in caridac output and hemolymph flow during hypoxic exposure in the gravid grass shrimp, *Palaemonetes pugio*. *J Comp Physiol B*. **175**, Pp 313-322.

Gunamalai, V., Kirubagaran, R. and Subramoniam, T. (2003). Sequestration of ecdysteroid hormone into the ovary of the mole crab *Emerita asiatica (*Milne Edwards). *Current science.* **85** (4), Pp 493-496.

Guppy, M. and Withers, P. (1999). Metabolic depression in animals: physiological perspectives and biochemical generalisations. *Biol Rev*. **74**, Pp 1-40.

Gwak, W-S. and Tanaka, M. (2002). Changes in RNA, DNA and protein contents of laboratory-reared Japanese flounder *paralichthys olivaceus* during metamorphosis and settlement. *Fisheries science*. **68**(1), Pp 27-33.

Gwak, W-S. (2003). Nucleic acids and protein content as a measure to evaluate the nutritional condition of Japanese Flounder *Paralichthys olivaceus* Larvae Juveniles. UJNR Technical Report (In Press).

Gyllstrom, M. and Hansson, L. A. (2003). Dormancy in freshwater zooplankton: Induction, termination and the importance of benthic-pelagic coupling. *Aquat. Sci*. **66**, Pp 274-295.

Haas, C., Klein, U. and Kloetzel, P. M. (1991). Developmental expression of *Drosophilla melanogaster* small heat-shock proteins. *Journal of Cell Sciences*. **96**, Pp 413-418.

Hagerman, L. and Szaniawska, A. (1986). Behaviour, tolerance and anaerobic metabolism under hypoxia in the brackish water shrimp *Crangon crangon*. *Mar. Ecol. Prog. Ser.* **34**, Pp 125-132.

Hagermann, L. and Vismann, B. (1995). Anaerobic metabolism in the shrimp *Crangon crangon* exposed to hypoxia, anoxia and hydrogen sulfide. *Mar. Biol.* **123**, Pp 235-240.

Hairston, N. G. Jr. (1996). Zooplankton egg banks as biotic reservoirs in changing enviroments. *Limnol oceanogr.* **41**, Pp 1087-1092

Hand, S. C. and Hardewig, I. (1996). Downregulation of cellular metabolism during environmental stress: Mechanisms and implications. *Annu. Rev. Physiol.* **58**, Pp 539-563.

Hand, S. C. (1997). Oxygen, pH_i and arrest of biosynthesis in brine shrimp embryos. *Acta Physiol Scand.* **161,** Pp 543-551.

Hand, S. C. (1998). Quiescence in *Artemia franciscana* embryos: Reversible arrest of metabolism and gene expression at low oxygen level. *J. Exp. Biol.* **201**, Pp 1233-1242.

Hardewig, I., Anchordoguy, T. J., Crawford, D. L. and Hand, S. C. (1996). Profiles of nuclear and mitochondrial encoded mRNAs in developing and quiescent embryos of *Artemia franciscana*. *Molecular and cellular biochemistry*. **158**, Pp 139-147.

Harms, J., Meyer-Harms, B., Dawirs, R.R. and Anger, K. (1994). Growth and physiology of *Carcinus maenas* (Decapoda, Portunidae) larvae in the field and in laboratory experiments. *Mar. Ecol. Prog. Ser.* **108**, Pp 107-118.

Harper, S. and Reiber, C. (2004). Physiological development of the embryonic and larval crayfish heart. *Biol. Bull.* **206**, Pp 78-86.

Harrison, K. (1988). Seasonal reproduction in deep-sea Crustacea (Isopoda: Asellota). *Journal of Natural History*. **22 (1)**, Pp 175-197.

Hartnoll, R. G. (1968). Morphology of the genital ducts in female crabs. *J. Linn. Soc. (Zool)*. **47**, Pp 279-300.

Hartnoll, R. G. (1969). Mating in Brachyura. *Crustaceana*. **16**, Pp 161-181.

Hartnoll, R. G. and Paul, R. G. K. (1982). The embryonic development of attached and isolated eggs of *Carcinus maenas*. *International Journal of Invertebrate Reproduction*. **5**, Pp 247-252.

Harvey, H. W. (1955). The chemistry and fertility of sea waters. *Cambridge University Press*. **8**, Pp 224.

Harzcsch, S., Miller, J., Benton, J., Dawirs, R. R. and Beltz, B. (1998). Neurogenesis in the thoracic neuromeres of two crustaceans with different types of metamorphic development. *The journal of experimental biology*. **201**, Pp 2465-2479.

Hawkins, A. J. S., Widdows, J. and Bayne, B. L. (1989). The relevance of whole-body protein metabolism to measured costs of maintenance and growth in *Mytilus edulis*. *Physiological zoology*. **62**(3), Pp 745-763.

Hazlett, B. A. (1983). *Parental behavior in decapod crustacean*, Pp171-193. In: Rebach, S. and Dunham, D. W., eds. Studies in adaptation - the behavior of higher Crustacea. John Wiley & Sons, Inc., New York, New York, USA.

Head, S. and Terwilliger, N. (2005). Expression of HIF-1α in the brachyuran Crustacean *Cancer magister*. Society for Integrative and Comparative Biology. Conference abstract.

Heikkila, J. J., Ovsenek, N. and Krone, P. (1987). Examination of heat shock protein mRNA accumulation in early *Xenopus laevis* embryos. Bio. Chem. Cell Biol. **65**, 87-94.

Heikkila, J. J., Ohan, N., Tam, Y. and Ali, A. (1997). Heat shock protein gene expression during Xenopus development. *Cell. Mol. Life Sci.* **53**, Pp 114-121.

Helluy, S. M. and Beltz, B. S. (1990). Stages in the embryonic development of the American of the American lobster *Homarus americanus* with special emphasis on its nervous system. *Frontiers in Crustacean Neurobiology*. Pp 530-536.

Helluy, S. M. and Beltz, B. S. (1991). Embryonic Development of the American Lobster (*Homarus americanus*): Quantitative Staging and Characterisation of an Embryonic Molt Cycle. *Biol. Bull.* **180**, Pp 355-371.

Hensey, C. and Gautier, J. (1998). Programmed cell death during *Xenopus* development: A Spatio-temporal Analysis. *Developmental Biology*. **203**, Pp 36-48.

Hines, A. H. (1982). Allometric constraints and variables of reproductive effort in brachyuran crabs. *Mar. Biol.* **69**, Pp 309-320.

Hines, A. H. (1991). Fecundity and reproductive output in ninespecies of Cancer crabs (Crustacea, Brachyura, Cancridae). *Can. J. Fish. Aq. Sci.* **48**, Pp 267-275.

Hines, A. H. (1992). Constraint on reproductive output in brachyuran crabs: Pinnotherids test the rule. *Amer. Zool.* **32**, Pp 503-511.

Hoback, W., Podrabsky, J., Higley, L., Stanley, D. and Hand, S. (2000). Anoxia tolerance of con-familial tiger beetle larvae is associated with differences in energy flow and anaerobiosis. *J. Comp. Biochem. Physiol. B*. **170**, Pp 307-314.

Hochachka, P. W. (1986). Defense strategies against hypoxia and hypothermia. *Science*. **23**, Pp 234-241.

Hochachka, P. W., Lutz., P. L., Sick, T., Rosenthal, M. and Van den Thillart. (1993). Surviving hypoxia. *CRC Press, Boca Raton, FL*. Pp 127-135.

Hochachka, P. W., Buck, L., Doll, C. and Land, S. C. (1996). Unifying theory of hypoxia tolerance: Molecular / metabolic defence and rescue mechanisms for surviving oxygen lack. *Proc. Natl. Acad. Sci. USA*. **93**, Pp 9493-9498.

Hochachka, P. W., Land, S. C. and Buck, L. (1997). Oxygen sensing and signal transduction in metabolic defence against hypoxia: Lessons from vertebrate facultative anaerobes. *Comp. Biochem. Physiol.* **118**A, Pp 23-29.

Hochachka, P. W. (1997). Oxygen- A key regulatory metabolite in metabolic defence against hypoxia. *Amer. Zool.* **37**, Pp 595-603.

Hochachka, P. W. and Lutz, P. L. (2001). Mechanism, origin, and evolution of anoxia tolerance in animals. *Comparative Biochemistry and Physiology*. **130**B, Pp 435-459.

Hoegh-Guldberg, O. and Pearse, J. S. (1995). Temperature, food, availability, and the development of marine invertebrate larvae. Amer. Zool. **35**(4), Pp 415-425.

Hofmann, G. E. and Hand, S. C. (1992). Comparison of messenger NA pools in *Artemia franciscana* embryos: evidence for translational control. J exp biol. **164**, Pp 103-116.

Hofmann, G. E. and Hand, S. C. (1994). Global arrest of translation during invertebrate quiescence. Proc. Natl. Acad. Sci. USA. **91**, Pp 8492-8496.

Hofmann, G. E., Buckley, B. A., Place, S.P. and Zippay, M. L. (2002). Molecular Chaperones in Ectothermic Marine Animals: Biochemical Function and Gene Expression. *Integ. And Comp. Biol.* **42**, Pp 808-814.

Holland, H. D. (1994). Early proterozoic atmospheric change. In: Early life on earth. *Nobel symposium,* **84**, Pp 237-244.

Houlihan, D. F., McMillan, D. N. and Laurent, P. (1986). Growth rates, protein synthesis, and protein degradation rates in rainbow trout: effects of body size, *Physiol. Zool.* **59**(4), Pp 482-493.

Houlihan, D. F., Hall, S. J., Gray, C. and Noble, B. S. (1988). Growth rates and protein turnover in Atlantic cod, *Gadus morhua*, *Can. J. Fish. Aquat. Sci.* **45**, Pp 951-964.

Houlihan, D. F., Hall, S. J. and Gray, C. (1989). Effects of protein turnover in cod. *Aquaculture.* **79**, Pp 103-10.

Houlihan, D. F., Waring, C. P., Mathers, E. and Gray, C. (1989). Protein synthesis and oxygen consumption of the shore crab *Carcinus maenas* after a meal. *Physiological Zoology*. **63**(4), Pp 735-756.

Houlihan, D. F., McMillan, D. N., Agnisola, C., Genoino, I. T. and Foti, L. (1990). Protein synthesis and growth in *Octopus vulgaris*. *Marine Biology*. **106**, Pp 251-259.

Houlihan, D. F. (1991). Protein turnover in ectotherms and its relationship to energetics. *Advances in Comparative and Environmental Physiology*. **7**, Pp 1-37.

Houlihan, D. F., Wieser, W., Foster, A. and Brechin, J. (1992). In *vivo* protein synthesis rates in larval nase (Chondrostoma nasus L.). *Canadian journal of zoology*. **70**, Pp 2436-2440.

Houlihan, D. F., Pannevis, M. and Heba, H. (1993). Protein synthesis in juvenile tilapia *Oreochromis mossambicus*. *Journal of the World Aquaculture Society*. **24**(2), Pp 145-151.

Houlihan, D. F., Pedersen, B. H., Stefferson, J. F. and Brechin, J. (1995). Protein synthesis, growth and energetics in larval herring (*clupea harengus)* at different feeding regimes. *Fish physiology and biochemistry*. **14**(3), Pp 195-208.

Howard, A. E. (1982). The distribution and behaviour of ovigerous edible crabs (*Cancer pagurus*) and consequent sampling bias. *Journal du Conseil*. **40**, Pp 259-261.

Huang, L. E., Arany, Z., Livingston, D.and Bunn, H. (1996). Activation of hypoxia-inducible transcription factor depends primarily upon redox sensitive stabilization of it's α subunit. *Journal of biological chemistry*. **271**, Pp 32253-32259.

Huang, L. E., Gu, J., Schau, M. and Bunn, H. F. (1998). Regulation of hypoxia-inducible factor 1α is mediated by o_2-dependant degradation domain via the ubiquitin-proteasome pathway. *Proc natl acad sci USA.* **95**, Pp 7987-7992.

Hughes, G. M. (1973). Respiratory Responses to Hypoxia in Fish. *Amer. Zool.* **13**, Pp 477-489.

Huey, R. B. and Bennett, A. F. (1990). Physiological adjustments to fluctulating thermal environments: an ecological and evolutionary perspective. In Stress Proteins in Biology and Medicine (ed. Morimoto, R. I., Tissieres, A. and Georgopoulos). NY: Cold Spring Harbour Lab Press.

Hutter, M. M., Sievers, R. E., Barbosa, V. and Wolfe, C. L. (1994). Heat-shock protein induction in rat hearts: a direct correlation between the amount of heat-shock protein induced and the degree of myocardial protection. *Circulation.* **89**, Pp 355-360.

Irwin, J. T., Bennett, V. A. and Lee Jr, R. E. (2001). Diapause development in frozen larvae of the goldenrod gall fry, *Eurosta solidaginis* fitch (diptera: tephritidae). *J Comp Physiol B*. **171**, Pp 181-188.

Isaacks, R., Harkness, D. and Witham, P. (1978). Relationship between the major phosphorylated metabolic intermediates and oxygen affinity of whole blood in the loggerhead turtle (*Caretta*

caretta) and the green sea turtle (*Chelonia mydas*) during development. *Devel. Biol.* **62**, Pp 344-353.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, W. G. Jr. (2001). HIFa targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. *Science*, **292**, Pp 464-468.

Iyer. N. V., Kotch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., Gassmann, M., Gearhart, J. D., Lawler, A. M., Yu, A. Y. and Semenza, G. L. (1998). Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1α. *Genes and Development I.* **12**, Pp 149-162.

Jackson, S. A. and Clegg, J. S. (1996). Ontogeny of low molecular weight stress protein p26 during early development of the brine shrimp, *Artemia franciscana*. *Develop. Growth Differ.* **38,** Pp 153-160.

Jackson, D. C. (2000). Living without oxygen: lessons from the freshwater turtle. *Comparative biochemistry and physiology. Part A*. **125**, Pp 299-315.

Jaeckle, W. B. and Manahan D. T. (1989). Feeding by a "nonfeeding" larva: uptake of dissolved amino acids from seawater by lecithotrophic larvae of the gastropod H*aliotis rufescens*. *Mar. Biol*. **103**(1), Pp 87-94.

Jiang, B. H., Semenza, G. L., Bauer, C. and Marti, H. H. (1996) Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O_2 tension. Am. J. Physiol. **271**, C1172-C1180.

Jiang, B. H., Zheng, J. Z., Leung, S. W., Roe, R. and Semenza, G. L. (1997). Transactivation and inhibitory domains of hypoxia-inducible factor 1α: modulation of transcriptional activity by oxygen tension. *J Biol. Chem.*. **272**, Pp 19253-19260.

Jiang, B. H., Guo, R., and Powell-Coffman, J.A. (2001). The *Caenorhabditis elegans* hif-1 gene encodes a BHLH-PAS protein that is required for adaptation to hypoxia. PNAS, **98**, Pp 7916-7921.

Jorgenson, D., Bourne, G. B., Burnet, L. E., DeFur, P. L. and McMahon, B. (1982). Circulatory function during hypoxia in the Dugeness crab. *Cancer magister*. *Am. Zool.* **22**, Pp 958A.

Kattner, G., Graeve, M., Calcagno, J. A., Lovrich, G. A., Thatje, S. and Anger, K. (2003). Lipid, fatty acid and protein utilization during lecithotrophic larval development of *Lithodes santolla* (Molina) and *Paralomis granulosa* (Jacquinot). *Journal of experimental marine biology and ecology*. **292**, Pp 61-74.

Keitzmann. T., Schmidt, H., Unthan-Feschner, K., Probst, I. and Jungermann, K. (1993). A ferro-heme protein senses oxygen levels which modulate the glucagon dependent activation of the PEPCK gene in rat hepatocyte cultures. *Biochem. Biophys. Res. Comm*. **195**, Pp 792-798.

Kelly, B. D., Hackett, S. F., Hirota, K., Oshima, Y., Cai, Z., Berg-Dixon, S., Rowan, A., Yan, Z., Campochiaro, P. A. and Semenza, G. L. (2003). Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. *Circ. Res.* **93**, Pp 1074-1081.

Klarman, A. and Daniel, E. (1980). Oxygen binding properties of stripped (Calcium ion and magnesium ion free) hemocyanin from the scorpion *Leirus quinquestriatus*. *Biochemistry NY.* **19**, Pp 5176-5180.

Kline, D. D., Peng, Y. J., Manalo, D. J., Semenza, G. L. and Prabhakar, N. R. (2002). Defective carotid body function and impaired ventilatory responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1a. *Proc natl acad sci USA*. **99**, Pp 821-826.

Kolsch, G. (2001). Anoxia tolerance and anaerobic metabolism in two tropical weevil species (*Coleoptera, Curculionidae*). *J Comp Physiol B*. **171**, Pp 595-602.

Kotch, L. E., Iyer, N.V., Laughner, E. and Semenza, G. L. (1999). Defective vascularisation of Hif 1α null embryos is not associated with VEGF deficiency but with mesenchymal cell death. Developmetal Biology. **209**, Pp 254-267.

Krogh, A. (1941). The comparative physiology of respiratory mechanisms. Philedelphia: University of Pennsylvania Press.

Krug, P. J. (1998). Poecilogony in a esturine opisthobranch: planktotrophy, lecithotrophy and mixed clutches in a population of the ascoglossan *Alderia modesta*. *Marine Biology*. **23**, Pp 933-941.

Kwast, K. E. and Hand, S. C. (1996a). Oxygen and pH regulation of protein synthesis in mitochhondria from *Artemoa franciscana* embryos. *Biochem. J.* **313**, Pp 207-213.

Kwast, K. E. and Hand, S. C. (1996b). Acute Depression of Mitochondrial Protein Synthesis during Anoxia. Contributions of oxygen sensing, matrix acidification, and redox state. *The Journal of Biological Chemistry*. **271**, Pp 7313-7319.

Kwast, K., Shapiro, J. I., Rees, B. B. and Hand, S. C. (1995). Oxidative phosphorylation and the realkalinisation of intracellular pH during recovery from anoxia in *Artemia franciscana* embryos. *Biochem, biophys. Acta*. **1232**, Pp 5-12.

Lachaise, F. Hoffmann, J. A. (1982).Ecddysteroids and embryonic development in the shore crab *Carcinus maenas*. Hoppe-Seyler's Z. Physiol. Chem. **363**, Pp 1059-1067.

Land, S. C., Buck, L. T. and Hochachka, P. W. (1993). Response of protein synthesis to anoxia and recovery in anoxia-tolerant hepatocytes, *Am. J. Physiol.* **265**, R41-R48.

Land, S. C. and Hochachka, P. W. (1995). A heme protein based oxygen sensing mechanism controls the expression and suppression of multiple proteins in anoxia-tolerant turtle hepatocytes. *Proc. Natl. Acad. Sci. USA*. **92**, Pp 7505-7509.

Lando, D., Gorman, J., Whitelaw, M. and Peet, D. (2003). Oxygen-dependant regulation of hypoxia inducible factors by prolyl and asparaginyl hydroxylatio. *Eur. J. Biochem*. **270**, Pp 781-790.

Larade, K., Nimigan, A. and Storey, K. B. (2001). Transcription pattern of ribosomal protein L26 during anoxia exposure in *Littorina littorea*. *J. Exp. Zool*. **290,** Pp 673-790.

Larade, K. and Storey, K. B. (2002). Reversible suppression of protein synthesis in concert with polysome disaggregation during anoxia exposure in *Littorina littorea*. *Mol. Cell. Biochem*. **232**, Pp 121-127.

Lee, C. E. and Strathmann, R. R. (1998). Scaling of Gelatinous Clutches: Effects of Siblings' Competition for Oxygen on Clutch Size and Parental Investment per Offspring. *The American Naturalist*. **151**(4), Pp 293-310.

Legeay, A. and Massabuau, J. C. (1999). Blood oxygen requirments in resting crab *Carcinus maenas* 24 hours after feeding. *Can. J. Zool*. **77**, Pp784-794.

Legeay, A. and Massabuau, J. C. (2000). The ability to feed in hypoxia follows a seasonally dependent pattern in shore crab *Carcinus maenas*. *J. Exp. Mar. Biol. Ecol.* **247**, Pp 113-129.

Lemmens, J. W. T. J. (1995). Nucleic acid levels, cellular activity and growth during the puerulus phase of the western rock lobster (*Panulirus cygnus;* Decapoda: Palinuridae). *J. Exp. Mar. Biol. Ecol*. **194**, Pp 143-156.

Lizarda-Daudt, H. M. and Bond-Buckup, G. (2003). Morphological aspects of the embryonic development of *Aegla platensis* (Decapoda, aeglidae). *Crustaceana*. **76**(1), Pp 13-25.

Loeffler, C. A. and Lovtrup, S. (1970). Water balance in the salmon egg. *J. Exp. Biol*. **52**, Pp 291-298.

Lonsdale, D. J. and Levinton, J. S. (1985). Latitudinal differentration in embryonic duration, egg size, and newborn survival in a harpacticoid copepod. *Bio. Bul*. **168**, Pp 419-431.

Lovrich, G. A. and Vinuesa, J. H. (1993). Reproductive biology of the false southern king crab (*Paralomis granulosa*, lithodidae) in the beagle channel, Argentina. *Fishery bulletin*. **91**, Pp 664-675.

Lovrich, G. A. and Ouellet, P. (1994). Patterns of growth and triacylglycerol content in snow crab *Chionoecetes opilio* (Brachyura: Majidge) zoeal stages reared in the laboratory. *Mar. Biol*. **120**, Pp 585-591.

Lubzens, E., Ravid, T., Khayat, M., Daube, N. and Tietz, A. (1997). Isolation and characterisation of the high-density lipoproteins from the hemolymph and ovary of the penaeid shrimp *Penaeus semisulcatus* (de Haan): apoproteins and lipids. *J. Exp. Zool*. **278**(6), Pp 339-348.

Lucas, M. and Crisp, D. (1987). Energy metabolism of eggs during embryogenesis in *balanus balanoides*. *J. Mar. Biol. Ass. UK*. **67**, Pp 27-54.

Lutz, P. L. and Storey, K. B. (1997). Adaptaions to variations in oxygen tension by vertebrates and invertebrates. In: Handbook of physiology, section 13: Comparative physiology, (ed.) W.H Dantzler, **2**, Pp 1479-1522, *Oxford university press, Oxford.*

Mangum, C. P. (1992). Physiological aspects of molting in the blue crab *Callinectes sapidus*. *Am. Zool*. **32**(3), Pp 459-469.

Mangum, C. P. (1998). Past-presidential address: Major events in the evolution of the oxygen carriers. *Amer. Zool*. **38**, Pp 1-13.

Manahan, D. T. and Crisp, D. J. (1982). The role of dissolved organic material in the nutritional of pelagic larvae: Amino acid uptake by bivalve velligers. *Am. Zool*. **22**(3), Pp 635-646.

Manahan, D. T. and Crisp, D. J. (1983). Autoradlographic studies on the uptake of dissolved amio acids from sea water by bivalve larvae. *J. Mar. Biol. Assoc. U.K*. **63**(3), Pp 673-682.

Mann, K. H. and Lazier, J. R. N. (2006). Dynamics of marine ecosystems: Biological-physical interactions in the oceans. *Blackwell Publishing*. Pp 7-118.

Manwell, C. (1963). Fetal and adult haemoglobins of the spiny dogfish Squalus suckleyi. *Arch. Biochem. Biophys*. **101**, Pp 504-511.

Marois, R. and Croll, R. P. (1991). Hatching asynchrony within the egg mass of the pond snail, *Lymnaea sganalls*. *Invertebr. Reprod. Dev*. **19**(2), Pp 139-146.

Martin, F., Linden, T., Katschinski, D. M., Oehme, F., Flamme, I., Mukhopadhyay, C. K., Eckhardt, K., Troger, J., Barth, S., Camenisch, G. and Wenger, R. H. (2005). Copper-dependant activation of hypoxia factor (HIF)-1: implications for ceruloplasmin reguation. *Blood*. **105**. Pp 4613-4619.

Mathers, E. M., Houlihan, D. F., McCarthy, I. D. and Burren, L. J. (1993). Rates of growth and protein synthesis correlated with nucleic acid content in fry of rainbow trout, *Oncorhynchus mykis*: effects of age and temperature. *Journal of fish biology*. **43**.

Mayrand, E., Dutil, J-D. and Guderley, H. (2000). Changes in muscle of postmoult snow crabs *Chionoecetes opilio* (O. Fabricus) fed different rations. *J. Exp. Marine Biol. Ecol.* **243**, Pp 95-113.

McAllen, R., Taylor, A. C. and Davenport, J. (1999). The effects of temperature and oxygen partial pressure on the rate of oxygen consumption of the high-shore rock pool copepod Tigriopus brevicornis. *Comp Biochem Phys A.* **123**(2), Pp 195-202.

McCarthy, I. D., Moksness, E., Pavlov, D. P. and Houlihan, D. F. (1999). Effects of water temperature on protein synthesis and protein growth in juvenile Atlantic wolffish (*Anarhichas lupus). Canadian Journal of Fisheries and Aquatic Sciences.* **56**, Pp 231-241.

McClintock, J. B. and Baker, B. J. (1997). Palatability and chemical defense of eggs, embryos and larvae of shallow-water Antarctic marine invertebrates. *Mar. Ecol. Prog. Ser.* **154**, Pp 121-131.

McDonald, D., McMahon, B. and Wood, C. (1977). Patterns of heart and scaphognathite activity in the crab *Cancer magister. J. Exp. Zool.* **202**, Pp 33-44.

McEdward, L. R. (1997). Reproductive strategies marine benthic invertebrates revisited: facultative feeding by planktotrophic larvae. *American Naturalist.* **150**, Pp 48-72.

McMahon, B. R. and Wilkens, J. L. (1975). Respiratory and circulatory responses to hypoxia in the lobster *Homarus americanus. J. Exp. Biol.* **62**, Pp 637-655.

McMahon, B. R. (2001). Respiratory and circulation compensation to hypoxia in crustaceans. *Respiration Physiology.* **128**, Pp 349-364.

McMillan, D. N. and Houlihan, D. F. (1988). The effect of refeeding on tissue protein synthesis in rainbow trout. *Physiol. Zool.* **61**(5), Pp 429-441.

Meier, P., Finch, A., and Evan, G. 2000. Apoptosis in development. *Nature.* **407**, Pp 796-801.

Mente, E., Coutteau, P., Houlihan, D., Davidson, I. and Sorgeloos, P. (2002). Protein turnover, amino acid flux in juvenile shrimp *Litopenaeus vannamei:* effects of dietary protein source. *J. Exp. Biol.* **205**, Pp 3107-3122.

Mente, E., Legeay, A., Houlihan, D. and Massabuau, J. (2003). Influence of oxygen partial pressures on protein synthesis in feeding crabs. *Am. J. Physiol. Regul. Comp. Physiol.* **284**, Pp R500-510.

Mestril, R., Chi, S.-H., Sayen, M. R., O'Reilly, K. and Dillmann, W. H. (1994). Expression of inducible stress protein 70 in rat heart myogenic cells confers protection against simulated ischemia-induced injury. *J. Clin. Invest.* **93**, Pp 759-767.

Michaelidis, B. (2002). Studies in the extra- and intracellular acid-base status and its role on metabolic depression in the land snail *Helix lucorum* during estivation. *J Comp Physiol B.* **172**, Pp 347-354.

Millward, D. J., Garlick, P. J., James, P. T., Nnanyelugo, D. O. and Ryatt, J. S. (1973). Relationship between protein synthesis and RNA content in skeleton muscle. *Nature, Lond.* **241**, Pp 204-205.

Millward, D. J., Garlick, P. J., Stewart, R. J. C., Nnanyelugo, D. O. and Waterlow, J. C. (1975). Skeleton muscle growth and muscle turnover. *Biochem. J.* **150**, Pp 235-243.

Minagawa, M., Chiu, J-R., Kudo, F., Ito, F. and Takashima, F. (1993). Female reproductive biology oocyte and development of the red frog crab, *Ranina ranina,* off Hachijojima, Izu islands, Japan. *Mar. Biol.* **115**. Pp 613-623.

Mitchell, N. J. and Seymour, R. S. (2003). The effects of nest temperature, nest substrate, and clutch size on the oxygenation of embryos and larvae of the Australian moss frog, *Bryobatrachus nimbus. Physiological and Biochemical Zoology*. **76**(1), Pp 60-71.

Morange, M., Diu, A., Bensaude, O. and Babinet, C. (1984). Altered expression of heat shock proteins in embryonal carcinoma and mouse early embryonic cells. *M. Cell. Biol.* **4**(4), Pp 730-735.

Morgan ll, R. P., Kramarsky, E. and Sulkin, S. D. (1978). Biochemical changes during larval development of the Xanthid crab *Rhithropanopeus harrisii.* lll. Isozyme changes during ontogeny. *Marine Biology*. **48**, Pp 223-226.

Morgan, I. J., McCarthy, I. D. and Metcalfe, N. B. (2000). Life-history strategies and protein metabolism in over wintering juvenile Atlantic salmon: growth is enhanced in early migrants through lower protein turnover. *Journal of Fish Biology*. **56**, Pp 637-647.

Morgan, S. G. and Christy, J. H. (1995). Adaptive significance of the timing of larval release by crabs. *The American Naturalist*. **145**, Pp 457-479.

Moriyasu, M. and Lanteigne, C. (1998). Embryo development and reproductive cycle in the snow crab, Chionoecetes opilio (Crustacea: Majidae), in the southern Gulf of St. Lawrence, Canada. *Can. J. Zool*. **76**, Pp 2040-2048.

Morris, S. and Bridges, C. R. (1989). Interactive effects of temperature and L-Lactate on the binding of oxygen by the hemocyanin of the two arctic boreal crabs, *Hyas araneus* and *Hyas coarctatus*. *Physiological Zoology*. **62**(1), Pp 62-82.

Morris, S. and Bridges, C. R. (1994). Properties of respiratory pigments in bimodal breathing animals: Air and water breathing by fish and crustaceans. *Amer. Zool*. **34**, Pp 216-228.

Morris, S. and Callaghan, J. (1998). Respiratory and metabolic responses of the Australian Yabby Cherax destructor to progressive and sustained environmental hypoxia. *J. Comp. Physiol. B*. **168**, Pp 377-388.

Mortola, J. P. and Frappell, P. B. (2000). Ventilatory responses to changes in temperature in mammals and other vertebrates. *Annurev. Physiol*. **62**, Pp 847-874.

Muller, Y., Ammar, D. and Nazari, E. (2004). Embryonic development of four species of palaemonld prawns (Crustacea, decapoda): pre-naupilar, naupilar and post-naupilar periods. *Revista Braslleira de Zoologia*. **21**(1), Pp 27-32.

Munro fox, H. (1948). The haemoglobin of *Daphnia*. *Proc. Royal. Soc. London B-Biol. Sci*. **271** (**879**), Pp. 195-212.

Munro, H. N. and Fleck, A. (1969). Analysis of tissues and body fluids for nitrogenous constituents. In *Mammalian Protein Metabolism* (Munro. H. N., ed.). *Academic Press, NY*. **3**, Pp 424-525.

Mykles, D. L. (1980). The mechanism of fluid absorption at ecdysis in the American lobster, *Homarus americanus*. *J, Exp. Biol*. **84**, Pp 89-101.

Naylor, J., Taylor, E. and Bennett, D. (1997). The oxygen uptake of ovigerous edible crabs (*Cancer pagurus*) and their eggs. *Mar. Fresh. Behav. Physiol*. **30**, Pp 29-44.

Naylor, J. and Taylor, E. (1998). Heart rate and gill ventilation in ovigerous and non-ovigerous edible crabs, *Cancer pagurus*: The effects of disturbance, substrate and starvation. *Mar. Fresh. Behav. Physiol*. **00**, Pp 1-17.

Naylor, J., Taylor, E. and Bennett, D. (1999). Oxygen uptake of developing eggs of *Cancer pagurus* (Crustacea: Decapoda: Cancridae) and consequent behaviour of the ovigerous females. *J. Mar. Biol. Ass. UK*. **79**, Pp 305-315.

Nichols, J. H., Thompson, B. M. and Cryer, M. (1982). Production: drift and mortality of the planktonic larvae of the edible crab *Cancer pagurus* of the north-east coast of England. *Netherlands Journal of Sea Research*. **16**, Pp173-184.

Nilsson, G. E. (2001). Surviving anoxia with the brain turned on. *News Phsiol. Sci.* **16**, Pp 217-221.

Nilsson, G. E. and Ostlund-Nilsson, S. (2004).Hypoxia in paradise: widespread hypoxia tolerance in coral reef fishes. *Proc. Royal. Soc. London B-Biol. Sci.* **271**, Pp 530-533.

Nishino, M. (1980). Geographical variations in body size, brood size and egg size of a freshwater shrimp, *Palaemon paucidens* de Haan, with some discussion on brood habit. *Japanese Journal of Limnology*. **41**, Pp 185-202.

Ohan, N. W. and Heikkila, J. J. (1995). Involvement of differential gene expression and mRNA stability in the development regulation of the Hsp 30 gene family in the heat-shocked *Xenopus laevis* embryos. *Development genetics*. **17**, Pp 176-184.

Orensanz, J. M., Parma, A. M., Armstrong, D. A., Armstrong, J. and Wardrup, P. (1995). The breeding ecology of *Cancer gracilis* (Crustacea: Decapoda: Cancridae) and the mating systems of cancrid crabs. *J. Zool. Lond.* **235**, Pp 411-437.

Pace, D. and Manahan, D. (2006). Fixed metabolic costs for highly variable rates of protein synthesis in sea urchin embryos and Larvae. *J. Exp. Biol.* **209,** Pp 158-170.

Packay, J. L., Withers, P. C., Hobbs, A. A. and Guppy, M. (2002). In vivo downregulation of protein synthesis in the snail *Helix apersa* during estivation. *Am. J. Physiol Regulatory Integrative Comp Physiol.* **283**, Pp 197-204.

Padilla P. A. and Roth M. B. (2001). Oxygen deprivation causes suspended animation in the zebrafish embryo. *Proc Natl Acad Sci USA* **98**, Pp 7331-7335,

Padilla, P. A., Nystal, T. G., Zager, R. A., Johnson, A. C. M. and Roth, B. (2002). Dephosphorylation of cell cycle-regulated proteins correlates with anoxia-induced suspended animation in *caenorhabditis elegans*. *Molecular Biology of the Cell*. **13**, Pp 1473-1483.

Pandian, T. (1970). Ecophysiological studies on the developing eggs and embryos of the european lobster *Hommarus gammarus*. *Mar. Biol.* **5**, Pp 154-167.

Pannevis, M. C. and Houlihan, D. F. (1992). The energetic cost of protein synthesis in isolated hepatocytes of rainbow trout (*Oncorhynchus mykiss*). **162**, Pp 393-400.

Patel, B. and Crisp, D. J. (1960). Rates of development of the embryos of several species of barnacles. *Physiol. Zool.* **33**, Pp 104-119.

Pauli, D., Tonka, C. H., Tissieres, A. and Arrigo, A. P. (1990). Tissue-specific Expression of the Heat Shock Protein HSP27 during *Drosophila melanogaster* Development. *The Journal of Cell Biology*. **111**, Pp 817-828.

Pearse, J. S. (1979). *Polyplacophora*. Pp 27-85. In Giese, A. C. and Pearse, J. S., eds. Reproduction of marine invertebrates. *Vol V. Academic Press, NY.*

Pechenik, J. A. (1987). Environmental influences on larval survival and development. In: Giese A. C. Pearse, J. S. Pearse, V. B. (eds). *Reproduction of marine invertebrates*, Vol **IX**. Seeking unity in diversity. Blackwell Scientific, Palo Alto, Calif., and Boxwood., Pacific Grove, Calif.

Petersen, S. and Anger, K. (1997). Chemical and physiological changes during the embryonic development of the spider crab, Hyas araneus L (Decapoda:Majidae). *Comp Biochem Phys B*. **117**(2), Pp 299-306.

Peterson, G. L. (1977). A simplification of the protein assay method of lowry et al. which is more generally applicable. *Annal Biochem.* **83**(2), Pp 346-56.

Pianka, E. R. (1970). On "r" and "K" selection. *American Naturalist.* **104**, Pp 592-597.

Pinder, A.W., Storey, K. B. and Ultsch, G. R. (1992). Estivation and hibernation. Pp 250-274. In: Environmental physiology of the amphibians (M.E Feder and W.W Burggen. eds.). University of Chiccago Press.

Pinheiro, A. M. and Hattori, H. Y. (2003). Embryology of the Mangrove crab, Ucides Cordatus *(Brachyura: Ocypodidae). Journal of Crustacean Biology.* **23**(3), Pp 729-737.

Podolsky, R. D. and Strathmann, R. R. (1996). Evolution of egg size in free-spawners: consequences of the ferilisation-fecundity trade-off. *American Naturalist.* **148**, Pp 160-173.

Podrabsky, J. E. and Hand, S. C. (2000). Depression of protein synthesis during Diapause in Embryos of the annual killifish *Austrofundulus limnaeus. Physiological and Biochemical Zoology.* **73**(6), Pp 799-808.

Portner, H. O., MacLatchy, L. M. and Toews, D. P. (1991). Metabolic responses of the Toad *Bufo marinus* to environmental hypoxia: an analysis of the critical PO2. *Physiology Zoology.* **64** (3), Pp 836-849.

Pough, F., Janis, C. and Heiser, J. (1999). Vertebrate Life, 6th edition. Prentice Hall, Inc.

Prabhakar, N., (2000). Oxygen sensing by the carotid body chemoreceptors. *J. Appl. Physiol.* **88**, Pp 2287-2295.

Preedy, V. R., Smith, D. M. and Sugden, P. H. (1985). The effects of 6 hours on protein synthesis in rat tissues in vivo and in vitro. *Biochem. J.* **228**, Pp 179-185.

Preedy, V. R., Smith, D. M., Kearney, N. and Sugden, P. H. (1985). Regional variation and differential sensitivity of rat heart protein synthesis in vivo and in vitro. *Biochem. J.* **225**, Pp 487-492.

Preedy, V. R., Paska, L., Sugden, P. H., Schofield, P. S. and Sugden, M. C. (1988). The effects of surgical stress and short-term fasting on protein synthesis *in vivo* in diverse tissues of the mature rat. *Biochemical Journal.* **250**.

Preedy, V. R. and Sugden, P. H. (1989). The effects of fasting or hypoxia on rates of protein synthesis in vivo in subcellular fractions or rat heart and gastrocnemius muscle. *Biochem J.* **257**, Pp 519-527.

Purves, W., Orians, G., Heller, H. and Sadava, D. (1998). *Life the science of biology. Sinauer Associates.*

Ramlov, H. and Westh, P. (2001). Cryptobiosis in the eutardigrade *Adorybiotus (Richtersius) coronifer*: tolerance to alcohols, temperature and *de novo* protein synthesis. *Zool. Anz.* **240**, Pp 517-523.

Rees, B. B., Patton, C., Grainger, J. L. and Epel, D. (1995). Protein synthesis increases after fertilisation of sea urchin eggs in the absence of an increase in intracellular pH. *Dev. Biol.* **169**, Pp 683-698.

Reiber, C. L. (1995) Physiological adaptations of crayfish to the hypoxic enviroment. *Am. Zool.* **35**, Pp 1-11.

Reiber, C. L. (1997). Ontogeny of cardiac and ventilatory function in the crayfish *Procambarus clarkii. Amer. Zool.* **37,** Pp 82-91.

Reiber, C. L. and McMahon, B. R. (1998). The effects of progressive hypoxia on the crustacean cardiovascular system: a comparison of the freshwater crayfish. (Procambarus clarkii), and the lobster (*Homarus americanus). J. Comp. Physiol. B.* **168**, Pp 168-176.

Rivers, D. B., Antonelli, A. L. and Yoder, J. A. (2002). Bags of the bagworm thridopteryx ephemeraeformis (Lepidoptera: Psychidae) protect diapausing eggs from water loss and chilling injury. *Annals of the Entomological Society of America.* **95**(4), Pp 481-486.

Robertson, R. F., El-Haj, A. J., Clarke, A. and Taylor, E. W. (2001a). Effects of temperature on specific dynamic action and protein synthesis rates in the Baltic isopod crustacean, *Saduria entomon. J. Exp. Mar. Biol. Ecol.* **262**, Pp 113-129.

Robertson, R. F., El-Haj, A. J., Clarke, A., Peck, L. S. and Taylor, E. W. (2001b). The effects of temperature on metabolic rate and protein synthesis following a meal in the isopod *Glyptonotus antarcticus* Eights (1852), *Polar Biol.* **24**, Pp 677-686.

Rolfe, D. F. S. and Brown, G. C. (1997). Cellular energy utilisation and molecular origin ofstandard metabolic rate in mammals. *Physiol. Rev.* **77**, Pp 731-758.

Routley, M. H., Nilsson G. E. and Renshaw, G. M. C. (2002). Exposure to hypoxia primes the respiratory and metabolic responses of the epaulette shark to progressive hypoxia. *Comp Biochem Phys.* A, **131**(2), Pp 313-321.

Ruppert, E.E. and Barnes, R. D. Invertebrate Zoology 6th Edition. Saunders College Publishing.

Ruiz-Tagle, N., Fernández, M. and Portner, H-O. (2002). Full time mothers: daily rhythms in brooding and non-brooding behaviours of Brachyuran crabs. *J. Exp. Mar. Biol. And Ecol.* **276**, Pp 31-47.

Ryan. H. E., Lo, J. and Johnson, R. S. (1998). HIF-1α is required for solid tumor formation and embryonic vascularisation. *The EMBO Journal.* **17**, Pp 3005-3015.

Sanders, B. M., Hope, C., Pascoe, V. M. and Martin, L. S. (1991). Characterisation of the stress protein response in two species of *Collisella* limpets with different temperature tolerances. *Physiological Zoology.* **64**, Pp 1471-1489.

Saigusa, M., Terajima, M. and Yamamoto, M. (2002). Structure, formation, mechanical properties, and disposal of the embryo attachment system of and estuarine crab, *Sesarma haematocheir. Biol. Bull.* **203**, Pp 289-306.

Schill, R. O., Gunther, H. B., Steinbruck and Kohler, H-R. (2004). Stress gene (hsp70) sequences and quantitative expression in *Milnesium tardiradum* (Tardigrada) during active and cryptobiotic stages. *J. Exp. Biol.* **207**, Pp 1607-1613.

Schmitt, A. S. C. and Uglow, R. F. (1998). Metabolic responses of *Nephrops norvegicus* to progressive hypoxia. *Aquat. Iving Resour.* **11**(2), Pp 87-92.

Schmidt-Nielsen, K. (1997). Animal physiology: *Adaptation and Environment, 5th ed. Cambridge University Press.*

Scott, G. and Crunkilton, R. L. (2000). Acute and chronic toxicity of nitrate to fathead minnows (*Pimephales promelas),ceriodaphnia dubia* and *daphnia magna. Envioron. Toxicol. Chem.* **19**, Pp 2918-2922.

Scott, M. A., Locke, M. and Buck, L. T. (2003). Tissue-specific expression of inducible and constitutive Hsp70 isoforms in the western painted turtle. *J. Exp. Biol.* **206**, Pp 303-311.

Seagroves T. N., Ryan, H. E., Lu, H., Wouters, B. G., Knapp, M., Thibault, P., Laderoute, K. and Johnson, R. S. (2001). Transcription factor HIF-1 is a necessary mediator of the Pasteur effect in mammalian cells. *Mol Cell Biol.* **21**, Pp 3436-3444.

Semenza, G. L. and Wang, G. L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol.* **12**(12), Pp 5447-5454.

Semenza, G. L. (1999). Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. *Annu. Rev. Cell Dev. Biol.* **15**, Pp 551-578.

Semenza, G. L. (2004). Hydroxylation of HIF-1: Oxygen Sensing at the Molecular Level. *Physiology*. **19**, Pp 176-182.

Seneviratna, D. and Taylor, H. H. (2006). Ontogeny of osmoregulation in embryos of intertidal crabs (*Hemigrapsus sexdentatus* and H. *crenulatus,* Grapsidae, Brachyura): putative involvement of the embryonic dorsal organ. *The Journal of Experimental Biology*. **209**, Pp 1487-1501.

Serrano-Pinto, V., Vazquez-Boucard, C. and Villarreal-Colmenares, H. (2002). Yolk proteins during ovary and egg development of mature female freshwater crayfish (*Cherax quadricarinatus*). *Comparative Biochemistry and Physiology Part A*. **134**, Pp 33-43.

Seymour, R. S. and Bradford, D. F. (1987). Gas exchange through the jelly capsule of the terrestrial eggs of the frog, *Psuedo-phryne bibroni. J Comp Physiol B*. **157**, Pp 477-481.

Seymour, R. S. and Roberts, J. D. (1991). Embryonic respiration and oxygen distribution in foamy and nonfoamy egg masses of the frog *Limnodynastes tasmaniensis. Physiol Zool*. **64**, Pp 1322-1340.

Seymour, R. S. and Loveridge, J. P. (1994). Embryonic and larval respiration in the arboreal foam nests of the African frog *Chiromantis xerampelina. J. Exp. Biol*. **197**, Pp 31-46.

Shapiro, J. P., Wasserman, H. A., Greany, P. D. and Nation J. L. (2000). Short communication Vitellin and vitellogenin in the soldier bug, podisus maculiventris: Identification with monoclonal antibodies and reproductive response to diet. *Archieves of Insect Biology and Physiology*. **44**, Pp 130-135.

Sharp, V. A., Miller, D, Bythell J. C. and Brown, B. E. (1994). Expression of low molecular weight Hsp70 related polypeptides from the symbiotic sea anemone *Anemonia viridis* forskal in response to heat shock. *J. Exp. Mar. Biol. Ecol*. **179**, Pp 179-193.

Sharp, V. A., Brown, B. E. and Miller, D. (1997). Heat shock protein (HSP 70) expression in the tropical reef coral *Goniopora djiboutiensis. J. Therm. Biol.* **22**, Pp 11-19.

Sheader, M. (1996). Factors influencing egg size in the gammarid amphipod *Gammarus insensibilis*. *Marine Biology*. **124**, Pp 519-526.

Shelton, G. and Boutilier, R. (1982) Aponea in amphibians and reptiles. *J. Exp. Biol.* **100**, Pp 245-273.

Shields, J. D. and Kuris, A. M. (1988). An in vitro analysis of egg mortality in *cancer anthonyi*: The role of symbionts and temperature. *Biol. Bull*. **174**, Pp 267-275.

Shields, J. D., Okazaki, R. K. and Kuris, A. M. (1991). Fecundity and the reproductive potential of the yellow rock crab cancer *anthonyi. Fishery bulletin, U.S*. **89**, Pp 299-305.

Shilling, F. M. and Manahan, D. T. (1990). Engergetics of early development for sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* and the crustacean artemia sp. *Mar. Biol.* **106**(1), Pp 119-127.

Shine, R. (1978). Propagule size and parental care: the "safe harbor" hypothesis. *J. Theor. Biol.* **75**, Pp417-424.

Sibert, Y., Ouellet, P. and Brethes, J. (2004). Changes in yolk total proteins and lipid components and embryonic growth rates during lobster (*Homarus americanus*) egg development under a simulated seasonal temperature cycle. *Mar. Biol.* **144**, Pp 1075-1086.

Sills, N. S., Gorham, D. A. and Carey, H. V.(1998). Stress protein expression in a mammalian hibernator. *FASEB J.* **12**, A379.

Smith, C. C. and Fretwell, S. D. (1974). The optimal balance between size and number of offspring. *The American Naturalist.* **108**, Pp 499-506.

Smith, R. W. and Houlihan, D. F. (1995). Protein synthesis and oxygen consumption in fish cells. *J. Comp. Physiol. B.* **165**, Pp 93-101.

Smith, R. W, Houlihan, D. F., Nilsson, G. E. and Brechin, J. G. (1996). Tissue specific changes in protein synthesis in vivo during anoxia in crucian carp. *Am. J. Phys.* **271**, Pp R897-R904.

Smith, R. W., Houlihan, D. F., Nilsson, G. E. and Alexandre, J. (1999). Tissue specific changes in RNA synthesis in vivo during anoxia in crucian carp. *Am. J. Phys. Reg.* **277**(46), R690-R697.

Soitamo, A. J., Rabergh, M. I., Gassmann, M, Sistonen, L. and Nikinmaa, M. (2001). Characterisation of a hypoxia-inducible factor (HIF-la) from rainbow trout. *The Journal of Biological Chemistry.* **276**, Pp 19699-19705.

Sollid, J., Angelis, P. D., Gundersen, K. and Nilsson, G. E. (2003). Hypoxia induces adaptive and reversible gross morphological changes in crucian carp gills. *J. Exp. Biol.* **206**, Pp 3667-3673.

Spees, J. L., Chang, S. A., Mykles, D. L., Snyder, M. J. and Chang, E. S. (2003). Molt cycle-dependent molecular chaperone and polyubiquitin gene expression in lobster. *Ell Stress & Chaperones*. **8**(3), Pp 258-264.

Spicer, J. L. (1994). Ontogeny of cardiac function in the brine shrimp *Artemia franciscana* Kellogg *1906 (*Branchiopoda: Anostraca). *J. Exp Zool.* **270**, Pp 508-516.

Spicer, J. I. (1995). Ontogeny of respiratory function in crustaceans exhibiting either direct or indirect development. *J Exp Zool.* **272**, Pp 413-418.

Staples, J. F., Hershkowitz, J. J. and Boutilier, R. G. (2000). Effects of ambient po_2 and temperature on oxygen uptake in *Nautilus pompilius*. *J. Comp. Physiol. B.* **170**, Pp 231-236.

Staver, J. M. and Stathmann, R.R. (2002). Evolution of fast development of planktonic embryos to early swimming. *Biol. Bull.* **203,** Pp 58-69.

Stearns, S.C. (1992). The Evolution of Life Histories. *Oxford University Press, NY.*

Steer, M. A., Moltschaniwskyj, N. A. and Gowland, F. C. (2002). Temporal variability in embryonic development and mortality in the southern calamary *Sepioteuthis australis*: a field assessment. *Mar. Ecol. Prog. Ser.* **243**, Pp 143-150.

Steinhart, M. and Eckmann, R. (1992). Evaluating the nutritional condition of individual whitefish (*Coregonus* spp.*)* larvae by the RNA/DNA ration. *Journal of Fish Biology*. **40**, Pp 791-799.

Stephanini, M., De Martino, C. and Zamboni, L. (1967). Fixation of ejaculated spermatozoa for electron microscopy. *Nature*. **216**, Pp 173-174.

St-Pierre, J., Brand, M. D. and Boutilier, R. G. (2000). Mitochondria as ATP consumers: Cellular treason in anoxia. *Proc. Natl. Acad. Sci. SUA.* **97**. Pp 8670-8674.

Storch, D., Heilmayer, O., Hardewig, I. and Portmer, H. (2003). In vitro protein synthesis capacities in a cold stenothermal and a temperate eurythermal pectinid. *J Comp Physiol B*. **173**, Pp 611-620.

Storey, K. B. and Mosser, D. (Unpublished Results). In: Storey, K. B. (1999). Stress-induced gene expressionin freeze tolerant and anoxia tolerant vertebrates. *Environmental stress and gene regulation*. Bios Scientific Publishers, Oxford. Pp 1-23.

Strathmann, R. R. and Strathmann, M. F. (1982). The relationship between adult size and brooding in marine invertebrates. *The American Naturalist*. **119**, Pp 91-101.

Strathmann, R. R. and Chaffee, C. (1984). Constraints on eggs masses. II.Effects of spacing size and number of eggs on ventilation of masses of embryos in jelly, adherent group, or thin-walled capsules. *J. Exp. Mar. Biol & Ecol*. **84**, Pp 85-93.

Strathmann, R. R. (1985). Feeding and non feeding larval development and life-history evolution in marine invertebrates. *Annu. Rev. Ecol. Syst*. **16**, Pp 339-361.

Strathmann, R. R. and Strathmann, M. F. (1989). Evolutionary opportunities and constraints demonstrated by artificial gelatinous egg masses. In: Ryaland, J. S. and Tyler, P. A. (eds.). *Reproduction, Genetics and Distribution of Marine Organisims*. Olsen and Olsen, Fredensbor.

Strathmann, R. R. and Strathmann, M. F. (1995). Oxygen supply and limits on aggregation of embryos. *J. Mar. Biol. Assoc. U.K*. **75**, Pp 413-428.

Strathmann, R. R. and Hess, H. C. (1999). Two designs of marine egg masses and their divergent consequences for oxygen supply and desiccation in air. *Amer. Zool*. **39**, Pp 253-260.

Strathmann, R. R., Staver, J. M. and Hoffman, J. R. (2002). Risk and the evolution of cell-cycle durations of embryos. *Evolution*. **56**(4), Pp 708-720.

Stroka, D. M., Burkhardt, T., Desbaillets, I., Wenger, R. H., Neil, D. A. H., Bauer, C., Gassmann, M. and Candinas, D. (2001). HIF-1 is expressed in normoxic tissue and displays an organ specific regulation under systemic hypoxia. *The Faseb Journal*. **15**, Pp 2445-2453.

Subramoniam, T. (1977). Aspects of sexual biology of the anomuran crab *Emerita asiatica*. *Marine Biology*. **43**(4), Pp 369-377.

Subramoniam, T. (1991). Yolk utilisation and esterase activity in the mole crab *Emerita asiatica* (Milne Edwards). I: Wenner, A. M., Kuris, A. M. (Eds.). *Crustacean Issues*. **7**, Pp 19-29.

Subramoniam, T. (1993). Spermatophores and sperm transfer in marine crustaceans. *Adv. Mar. Biol*. **29**, Pp 129-214.

Subramoniam, T. and Gunarnalai, V. (2003). Breeding biology of the interidal sand crab, *Emerita* (Decapoda: Anomura). *Mar. Biol*. **46**, Pp 91-182.

Susanto, G. N. and Charmantier, G. (2001). Crayfish freshwater adaptation starts in eggs: ontogeny of osmoregulation in embryos of *Astacus leptodactylus*. *Journal of Experimental Zoology*. **289**(7), Pp 433-440.

Suslick, K. S. (1989). The chemical effects of ultrasound. *Scientific American*. Pp 80-86.

Suslick, K. S. (1998). Applications of ultrasound to materials chemistry. *Annu. Rev. Mater. Sci*. **29**, Pp 295-326.

Suslick, K. S. (1998). In Kirk-othmer encyclopedia of chemical technology. New York: *Wiley and Sons, 4th ed*. **26**, Pp 517-541.

Talbot, P. and Goudeau, M. (1988). A complex cortical reaction leads to formation of the fertilisation envelope in the lobster, *Homarus*. *Gamete Res*. **19**, Pp 1-18.

Taylor, A.C. (1976). The respiratory responses of *Carcinus maenas* to declining oxygen tension. *J. Exp. Biol*. **65**, Pp 309-322.

Taylor, A. C. and Moore, P. G. (1995). The burrows and physiological adaptations to a burrowing lifestyle of *Natatolana borealis* (Isopoda: Cirolanidae). *Marine Biology.* **123,** Pp 805-814.

Taylor, A. C., Astal, C. and Atkinson, R. (2000). A comparative study of the oxygen transporting properties of the hemocyanin of five species of thalassinidean mud-shrimps. *J. Exp. Mar. Biol & Ecol.* **244**, Pp 265-283.

Taylor, E. W. and Wheatly, M. G. (1981). The effect of long-term aerial exposure on heart rate, ventilation, respiratory gas exchange and acid-base status in the crayfish *Austropotamobius pallipes*. *J. Exp. Biol.* **92**, Pp109-124.

Taylor, E. W. (1982). Control and co-ordination of ventilation and circulation in crustaceans: responses to hypoxia and exercise. *J. Exp. Biol.* **100**, Pp 289-319.

Taylor, E. W. and Whitely, N. M. (1989). Oxygen transport and acid-base balance in the haemolymph of the lobster *Homarus gammarus*, during aerial exposure and resubmersion. *J. Exp. Biol.* **144**, Pp 417-436.

Taylor, H. and Leelapiyanart, N. (2001). Oxygen uptake by embryos and ovigerous females of two intertidal crabs, *Heterozius rotundifrons* (Belliidae) and Cyclograpsus lavauxi (Grapsidae): Scaling and the metabolic costs of reproduction. *J. Exp. Biol.* **204**, Pp 1083-1097.

Terwilliger, N. B. (1991). Hemocyanins in crustacean oöcytes and embryos. In A. Wenner and A. Kuris (eds), *Crustacean egg production*. Pp 31-36A. A. Balkema, Rotterdam.

Terwilliger, N. B. and Brown, A. C. (1993). Ontogeny of hemocyanin function in the dungeness crab cancer magister: The interactive effects of developmental stage and divalent cations on hemocyanin oxygenation properties. *J. Exp. Biol.* **183**, Pp 1-13.

Terwilliger, N. B. (1998). Functional adaptations of oxygen-transport proteins. *J. Exp. Biol.* **201**, Pp 1085-1098.

Terwilliger, N. B. and Ryan, M. (2001). Ontogeny of crustacean respiratory proteins. *Amer. Zool.* **41**, Pp 1057-1067.

Thatje, S., Calcagno, J. A., Lovrich., G. A., Sartoris, F. J. and Anger, K. (2003). Extended hatching periods in the subantarctic lithodid crabs *Lithodes santolla and Paralomis granulosa* (Crustacea: Decapoda: Lithodidae). *Helgol Mar Res.* **57**, Pp 110-113.

Thiel, M., Sampson, S., Watling, L. (1997). Extended parental care in two endobenthic amphipods. *Journal of Natural History.* **31**, Pp 713-725.

Thiel, M. (2003). Extended parental care in crustaceans- an update. Revista Chilena de Historia Natural **76**, Pp 205-218.

Tirumalai, R. and Subramoniam, T. (1992). Purification and characterisation of vitellogenin and lipovitellins of the sand crab *Emerita asiatica*: molecular aspects of crab yolk proteins. *Mol. Reprod. Dev.* **33**, Pp 16-22.

Tirumalai, R. and Subramoniam, T. (2001). Carbohydrate components of lipovitellin of the sand crab *Emerita asiatica*. Molecular reproduction and development. **58**, Pp 54-62.

Tomanek. L. (2002). The heat-shock response: Its variation, regulation and ecological importance in intertidal gastrophods (*genus Tegula). Integ. And comp. Biol.* **42**, Pp 797-807.

Tomanek, L. and Somero, G. N. (2002). Interspecific- and acclimation-induced variation in levels of heat-shock proteins 70 (Hsp70) and 90 (Hsp90) and heat-shock transcription factor-1 (HSF1) in congeneric marine snails (genus Tegula): implications for regulation of *hsp* gene expression. *J. Exp. Biol.* **205**, Pp 677-685.

Ton, C., Stamation, D. and Liew, C-C. (2003). Gene expression profile of zebrafish exposed to hypoxia during development. *Physiol. Genomics.* **13**, Pp 97-106.

Tsukimura, B. (2001). Crustacean vitellogenesis: Its role in Oocyte development. *American Zoologist.* **41**(3), Pp 465-476.

Van Pattern, P. (2006). Spreading shell rot disease "eating" new England lobsters. *University of Conneticut Seagrant.*

Varvra, J. and Manahan, D. T. (1999). Protein metabolism in lecithotrophic larvae (Gasstropoda: *Haliotis rufescens). Biol. Bull.* **196**, Pp 177-186.

Vernberg, F. J. and Costlow, Jr. J. D. (1966). Studies on the physiological variation between tropical and temperate-zone fiddler crabs of the genus UCA. IV. Oxygen consumption of larvae and young crabs reared in the laboratory. *Physiol. Zool.* **39,** Pp 36-52.

Vidal, J. (1980). Physioecology of zooplankton. II. Effects of phytopplankton concentration, temperature, and body size on the development and molting rates of *calanus pacipicus* and *pseydocalanus* sp. *Mar. Biol.* **56**(2), Pp 135-146.

Villeneuve, T. S., Ma, X., Sun, Y., Oulton, M. M., Oliver, A. E. and MacRae, T. H. (2006). Inhibition of apoptosis by p26: implications for small heat shock protein function during Artemia development. *Cell Stress and Chaperones.* **11**(1), Pp 71-80.

Vincent, K. A., Shyu, K. G., Luo, Y., Magner, M., Tio, R. A., Jiang, C., Goldberg, M. A., Akita, G. Y., Gregory, R. J. and Isner J. M. (2000). Angiogenesis is induced in a rabbit model of hindlimb ischemia by naked DNA encoding an HIF-1α/VP16 hybrid transcription factor. *Circulation.* **102**, Pp 2255-2261.

Visser, A. W. and Jonasdottir, S. H. (1999). Lipids, buoyancy and the seasonal vertical migration of *Calanus finmarchrcus. Fish, Oceanogr.* **8**, Pp 100-106.

Vogan, C. L., Costa-Ramos, C. and Rowley, A. F. (2001). A histological study of shell disease syndrome in the edible crab Cancer pagurus. *Dis Aquat Organ.* **47**(3), Pp 209-17.

Vogan, C. L., Costa-Ramos, C. and Rowley, A. F. (2002). Shell disease syndrome in the edible crab, Cancer pagurus--isolation characterization and pathogenicity of chitinolytic bacteria. *Dis Aquat Organ.* **146**(3), Pp 743-754.

Vuorinen, I., Hurki, H., Bosma, E. and Kalangali. (1999). Vertical distribution and migration of pelagic copepoda in Lake Tanganyika. *Hydrobiologia.* **407**(0), Pp 115-121.

Wang G. L. and Semenza, G. (1993). General involvement of hypoxia inducible factor 1 in transcriptional response to hypoxia. *Proc. Natl. Acad. Sci. USA.* **90**, Pp 4304-4308.

Wang, G. L., Jiang, B.-H., Rue, E. A. and Semenza, G. L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc. Natl. Acad. Sci. USA, **92**, Pp 5510-5514.

Wang W. and Widdows, J. (1991). Physiological responses of mussel larvae *Mytilus edulis* to environmental hypoxia and anoxia. *Marine Ecology Progress Series.* **70**, Pp 223-236.

Wang, W. and Widdows, J. (1993). Metabolic responses of the common mussel Mytilus edulis to hypoxia and anoxia. *Marine Ecology Progress Series.* **95**, Pp 205-214.

Wang, T., Branco, L. G. S. and Glass, M. L. (1994). Ventrilatory responses to hypoxia in the toad *Bufo parancnemis* before and after a decrease in haemoglobin oxygen-carrying capacity. *Journal of experimental biology.* **186**, Pp 1-8.

Warkentin, K. M. (2002). Hatching timing, oxygen availability, and external gill regression in the treefrog, *Agalychnis callidryas. Physiol. Bio. Zool.* **75**, Pp 155-164.

Warkentin, K. M., Gomez-Mestre, I. and McDaniel, J. G. (2005) Development, surface exposure, and embryo behavior affect oxygen levels in eggs of the red-eyed treefrog, *Agalychnis callidryas. Physiological and Biochemical Zoology.* **78**(6), Pp 956-966.

Warrier, S. R., Tirumalai, R. and Subramoniam, T. (2001) Occurrence of vertebrate steroids, estradiol 17 beta and progesterone in the repreoducing females of the mud crab *scylla serrata. Comp Biochem Physiol A Mol Integr Physiol.* **130**(2), Pp 238-294.

Watson, N. H. F. and Smallman, B. N. (1971). The Physiology of diapause in *Diacyclops naus* Herrick (Crustacea, Copepoda). *Canadian Journal of Zoology.* **57**, Pp 1449-1454.

Wear, R. G. (1967). Embryonic and post-embryonic development of *Pilumnus Novaezealandiae* Filhol, 1886, and of *P. Lumpinus* Bennett, 1964 (Xanthidae, Pilumninae). *N.Z. Journal of Marine and Freshwater Research.* Pp 482- 539.

Wear, R. G. (1974). Incubation in British decapod crustacea, and the effects of temperature on the rate and success of embryonic development. *J. Mar. Biol. Ass. UK.* **54**, Pp 745-762.

Wenner, A. M., Fusaro, C. and Oaten, A. (1974). Size at onset of sexual maturity and growth rate in crustacean populations. *Can. J. Zool.* **52**, Pp 1095-1106.

West, T. and Boutilier, R. (1998). Metabolic suppression in Anoxic frog muscle. *J. Comp Physiol.* **168**, Pp 273-280.

Whatley, K. M., John, P. and Whatley, F. R. (1979). From extracellular to intracellular: the establishment of mitochondria and chloroplasts. *Proc. R. Soc. Lond. B.* **204**, Pp 165-187.

Wheatly, M. and Taylor, E. (1979). Oxygen levels, acid-base status and heart rate during emersion of the shore crab *Carcinus maenas* (L.) into air. *J Comp Physiol B.* **132 (4)**, Pp 305-311.

Wheatly, M. (1981). The provision of oxygen to developing eggs by female shore crabs (*Carcinus maenas*). *J. Mar. Biol. Ass. UK.* **61**, Pp 117-128.

Whiteley, N. M., Taylor E. W. and El Haj, A. J. (1996). A comparison of the metabolic cost of protein synthesis in stenothermal and eurythermal isopod crustaceans. *Am. J. Phys. Reg.* **271**(5), Pp 1295-1303.

Whiteley, N. M. and El Haj, A. J. (1997). Regulation of muscle gene expression over the moult cycle. *Comp. Biochem. Physiol B.* **117**, Pp 323-331.

Whiteley, N. M., Robertson, R. F., Meagor, J., El Haj, A. J. and Taylor, E. W. (2001). Protein synthesis and specific dynamic action in crustaceans: effects of temperature. *Comparative Biochemistry and Physiology Part A.* **128**, Pp 595-606.

Whiteley, J. and Faulkner, L. S. (2005). Temperature influences whole-animal rates of metabolism but not protein synthesis in a temperate intertidal isopod. *Physiological and Biochemical Zoology.* **78**(2), Pp 227-238.

Whitlock, N. A., Argarwal, N., Ma, J.-X. and Crossan, C. E. (2005). Hsp27 upregulation by HIF-1 signaling offers protection against retinal ischemia in rats. *Investigation ophthalmology and visual science.* **46**, Pp 1092-1098.

Whyte, J. N. C., Bourne, N. and Ginther N. G. (1990). Biochemical and energy changes during embryogenesis in the rock scallop *Crassadoma gigantea. Marine Biology.* **106**, Pp 239-244.

Whyte, J. N. C., Bourne, N. and Ginther, N. G. (1991). Depletion of nutrient reserves during embryogenesis in the scallop *Patinopecten yessoensis* (Jay). *J. Exp. Mar. Biol. Ecol.* **149**, Pp 67-79.

Wiklund, A-K. E. and Sundelin, B. (2001). Impaired reproduction in the amphipods monoporeia *affinis* and *Pontoporeia femorata* as a result of moderate hypoxia and increased temperature. *Mar. Ecol, Prog. Ser.* **222**, *Pp 131-141.*

Wilder, M. N., Okumara, T., Aida, K. and Hanyu, I. (1990). Ecdysteroid Fluctuations during Embtyogenesis in the Giant Freshwater Prawn, *Macrobrachium rosenbergii*. **80**, Pp 93-100.

Wilkens, J. L. and Young, R. E. (1992). Regulation of pulmonary blood flow and of blood pressure in a mangrove crab (*Goniopsis cruentata). J. Exp. Biol*. **163**, Pp 297-316.

Williamson, H. C. (1900). Contributions to the life-history of the edible crab (*Cancer pagurus,* Linn.). Report of the Fishery Board for Scotland. **18**, Pp 77-143.

Williamson, H. C. (1904). Contributions to the life-histories of the edible crab (Cancer pagurus) and of other decapod Crustacea: impregnation; spawning; casting; distribution; rate of growth. *Rept. Fish. Board Scotland*. **22**, Pp100-140.

Willsie, J. K. and Clegg, J. S. (2001). Nuclear p26, a small heat shock/a-crystallin protein and its relationship to stress resistance in *Artemia franciscana* embryos. *J Exp. Biol*. **204**, Pp 2339-2350.

Withers, P. (1992). Comparative Animal Physiology. Saunders College Publishers

Wolgemuth, D. J. and Gruppi, C. M. (1991). Heat shock gene expression during mammalian gametogenesis and early embryogenesis. Springer- Verlag Berlin Heidelberg .

Woods, H. A. and Hill, R. I. (2004). Temperature-dependent oxygen limitation in insect eggs. *J Exp Biol*. **207**, Pp 2267-2276

Zeis, B., Neis, A., Bridges, C. R. and Grieshaber, M. K. (1992). Allosteric modulation of haemocyanin oxygen-affinity by l-Lactate and Urate in the Lobster *Homarus vulgaris*. I. Specific and additive effects on Haemocyanin oxygen-affinity. *J. Exp. Biol*. **168**, Pp 93-110.

Zeng, C., and Naylor, E. (1997). Rhythms of larval release in the shore crab *Carcinus maenus* (Decapoda: Brachyura). *J. Mar Biol Assoc UK*. **77**, Pp 451-461.

Zhao, L. and Eghbali-Webb, M. (2001). Release of pro- and anti-angiogenic factors by human cardiac fibroblasts: effects on DNA synthesis and protection under hypoxia in human endothelial cells. *Biochimica et Biophysica Acta 1538.* Pp 273-282.

Zhou, B. S., Wu, R. S. S., Randall, D. J. and Lam, P. K. S. (2001). Bioenergetics and RNA/DNA ratios in the common carp (*Cyprinus carpio*) under hypoxia. *J Comp Physiol B*. **171**, Pp 49-57.