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Effects of Multiple Stressors on the Development and Performance of Decapod Crustaceans

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Effects of Multiple Stressors on the Development and Performance of Decapod Crustaceans

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Abstract

Many marine crustacean larvae develop in a relatively stable pelagic environment; therefore, they are likely to be sensitive to perturbations in their surrounding environmental conditions. Ocean Acidification (OA) is occurring on a globalised scale and may cause disruptions to crustacean larval survival. However, species and/or life history stages are not expected to respond uniformly to these near-future predicted changes. The performance of species that lack a compensatory capacity to cope with the changing conditions may potentially be detrimentally affected, which in turn may impact recruitment. In addition to this, little information exists surrounding the impacts of ocean acidification in conjunction with additional environmental stressors, such as salinity, temperature and food availability, which are predicted to covary with OA, upon brachyuran crustacean larvae.

This research focused on the effects of elevated CO_2, in combination with other environmental stressors, upon rates of larval development, performance and survival of a brachyuran crustacean species common to Europe (*Carcinus maenas*) and two species of shrimp (*Palaemon serratus* and *Palaemon varians*). These species have varying physiological abilities to cope with salinity change and such attributes may influence their capacities to survive elevated CO_2 in combination with other environmental changes.

Exposure of early larval stages to combinations of salinity, temperature and food limitation in *C. maenas* revealed that high temperature ameliorated the effect of low salinity on survival and developmental duration. Limited access to food also affected developmental duration, but exposure to elevated CO_2 alone in a second experiment only affected survival, and low salinity alone had no effect.

Exposure of early juvenile stages of *C. maenas* to CO_2 and salinity, revealed that developmental duration was significantly affected by elevated CO_2 and/or salinity at varying levels, whereas, for survival, such influences were only observed in later juvenile stages. These results suggest the possibility of a physiologically sensitive bottleneck within the life cycle of *C. maenas*.

Exposure of early larval stages of the estuarine species, *P. varians*, to CO_2 and salinity had no effect on either survival or developmental duration. For the predominantly coastal species, *P. serratus*, developmental duration was negatively influenced by the interaction of elevated CO_2 and low salinity, but there was limited observed effect on overall survival at the early stages studied.

Overall, evaluations of the effects of climate driven variables on physiological performance demonstrated that differences can occur among broods. In future, further studies are required to incorporate seasonal (and possibly spatial) variability in responses, due to maternal effects or phenotypic variation, as conclusions based on individuals collected over a short time frame are unlikely to fully represent population level responses.

Key words: larval development; decapod crustaceans; OA; salinity; feeding/nutrition; survival; multiple stressors

Statement of original authorship

I declare that the entirety of the work contained herein is my own, original work and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Amy Elizabeth Curry

Date: 31st October 2019

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FOR MY DAUGHTERS,
AMELIA & EVELYN

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1 Chapter One: General Introduction

1.1 Ocean Acidification: An Introduction

Carbon dioxide (CO_2) levels are increasing in the Earth's atmosphere, predominantly as a result of burning fossil fuels (e.g. coal, oil, gas), cement production and forest felling (Caldeira and Wickett 2003, IPCC 2014). Globally, the atmospheric levels of CO_2 have risen from an average value of around 280 µatm, circa 650,000 years prior to the industrial revolution, to the current level of ~410 µatm (recorded at the Mauna Loa Observatory, Hawaii (NOAA-ESRL) in July 2018) (Caldeira and Wickett 2003, Sabine, Feely et al. 2004, Lüthi, Le Floch et al. 2008, Tans and Keeling 2013, IPCC 2014). If anthropogenic CO_2 emissions remain unabated (i.e. the "*Business as usual*" scenario), then models predict that the levels of CO_2 in the atmosphere will increase to ~700-1000 ppm by the year 2100, and to ~3000 ppm by the year 2300 (Plattner, Joos et al. 2001, Caldeira and Wickett 2003, Sabine, Feely et al. 2004, IPCC 2014).

Human-derived CO_2 has increased by ~80% (21Gt CO_2 per year – 38 Gt CO_2 per year) from 1970-2004 (Gattuso and Hansson 2011). There are three fates of the CO_2 derived from anthropogenic activities. From 2000-2008 ~45% stayed in the atmosphere, 26% was absorbed into surface oceans and 29% by terrestrial ecosystems (Le Quéré, Raupach et al. 2009, Gattuso and Hansson 2011). CO_2 accretion into Earth's atmosphere causes climatic changes to proliferate by increasing 'the greenhouse effect' (Gattuso and Hansson 2011, IPCC 2014). The most important greenhouse gas is CO_2 and the relationship between CO_2 and increasing atmospheric temperatures has been well documented (IPCC 2014), however, as well as being involved in global warming; CO_2 modifies ocean chemistry (Kurihara 2008, IPCC 2014, Gattuso, Magnan et al. 2015).

Earth's atmosphere and oceans exchange huge levels of CO_2 naturally (Gattuso and Hansson 2011). It is estimated that the atmospheric influx into the oceans prior to the industrial revolution amounted to 70 Gt C per year, and the opposite flux amounted to 70.6 Gt C per year (IPCC 2014). Since then, anthropogenic emissions have become

superimposed onto this natural CO_2 equilibrium-exchange (Gattuso, Magnan et al. 2015).

Over one-third of the anthropogenically-derived CO_2 has been absorbed into surface oceans since the onset of the 19th century (Sabine, Feely et al. 2004, Feely, Sabine et al. 2008, Gattuso, Magnan et al. 2015). Together, the Earth's oceans act as a gigantic sink, that aid in the mitigation of climate change, therefore, without this crucial role the atmosphere would hold a larger amount of CO_2, potentially causing further climatic perturbations (Gattuso, Magnan et al. 2015). However, when the rate of CO_2 influx exceeds the natural equilibrium, CO_2 partial pressure (pCO_2) in surface waters increases (Gattuso, Magnan et al. 2015). This then causes a concomitant increase in H^+ ions (thereby a decrease in pH) and changes the dissolved inorganic carbon (DIC) equilibrium (decrease in carbonate ion concentration (CO_3^{2-}), increase in carbonic acid (H_2CO_3) and bicarbonate (HCO_3^-), thus altering seawater chemistry in a collective process termed ocean acidification (OA) (Caldeira and Wickett 2003, Gattuso and Hansson 2011, Gattuso, Magnan et al. 2015) (Figure 1.1).

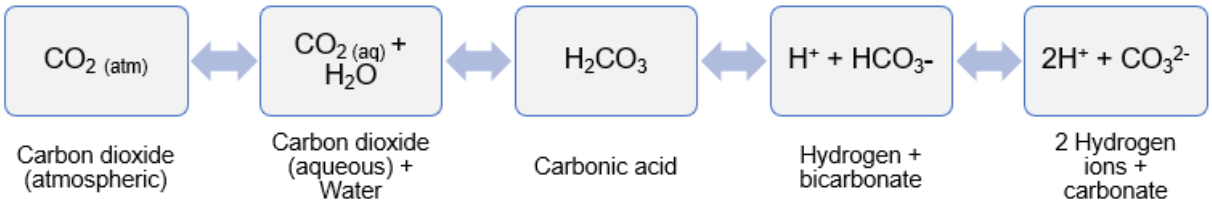


Figure 1.1: Ocean acidification chemistry. As CO_2 dissolves in seawater, three kinds of DIC are produced: carbonic acid (H_2CO_3), bicarbonate (HCO_3-) and carbonate (CO_3^2). Carbonic acid dissociates rapidly forming H^+ and HCO_3-. H^+ then reacts with carbonate ions forming bicarbonate in reverse. The net effect is a decrease in pH due to a decline of carbonate ions and an increase in bicarbonate, carbonic acid and hydrogen ions.

OA is a phenomenon caused by anthropogenically derived CO_2 changing the carbonate chemistry, and thereby reducing pH and altering the bicarbonate buffering system, of the oceans globally (Caldeira and Wickett 2003, Gattuso, Magnan et al. 2015).

Historically over geological time, open-ocean pH has been comparatively stable due to the natural buffering capacity of seawater (due to the extremely slow weathering of alkaline rocks over millennia) at around pH 8.16 (Sabine, Feely et al. 2004, Gattuso and Hansson 2011). However, since the industrial revolution began the oceans' capacity to buffer changes has been overcome due to the rapid rate of change and pH has reduced by approximately 0.1 units (IPCC 2014). This may not appear significant but in fact relates to a 30% rise of H^+ ions and a 16% decline in CO_3^{2-} ions (Feely, Sabine et al. 2004, Riebesell, Fabry et al. 2010). It is predicted that surface ocean pH will decrease to ~pH 7.7 by the end of the century and to ~pH 7.3 by the year 2300 (Caldeira and Wickett 2003, IPCC 2014). Globally, average oceanic pH of surface waters is expected to decrease from the year 2100 to 2300 by 0.4-0.7 pH units respectively, under present day CO_2 emissions (Caldeira and Wickett 2003, IPCC 2014, Sperfeld, Mangor-Jensen et al. 2017).

The result of this is that marine biota may be influenced by changes in seawater carbon chemistry positively, neutrally or negatively, dependent upon phylogeny (Doney, Fabry et al. 2009, Kroeker, Kordas et al. 2013, Wittmann and Portner 2013), life cycle stage (Kroeker, Kordas et al. 2013, Przeslawski, Byrne et al. 2015) and also prior exposure to environmental change (Gunderson, Armstrong et al. 2016). Such changes can therefore influence ecosystem functioning in the oceans (Nagelkerken and Connell 2015, Sperfeld, Mangor-Jensen et al. 2017).

1.2 Ocean Acidification: Biological & Ecological Relevance

Research into the effects of OA is relatively new in the scientific literature (79% of research was only published after 2003 (Caldeira and Wickett 2003, Gattuso and Hansson 2011). Despite this, the rapidly increasing evidence infers that many marine ecosystems are potentially at risk, with possible additional socio-economic repercussions (Prather, Pelini et al. 2013, Gattuso, Magnan et al. 2015).

Throughout geological history Earth's climate has naturally fluctuated, however, in the last 250 years, seawater chemistry has been altered by anthropogenic CO_2 emissions

at a rapidly increasing rate (Caldeira and Wickett 2003, Kurihara 2008, Kurihara, Matsui et al. 2008). Biologically this has crucial implications as many marine organisms will be exposed to changing environmental conditions, which their predecessors would likely not have experienced previously (Pörtner, Langenbuch et al. 2004, Dupont and Thorndyke 2009, Melzner, Gutowska et al. 2009). The overall effect of this on marine ecosystems is unclear, but species, populations and life-history stages are not expected to respond uniformly to the future predicted changes in ocean chemistry (Orr, Fabry et al. 2005, Ries, Cohen et al. 2009, Riebesell, Fabry et al. 2010, Wittmann and Pörtner 2013). Effects will vary depending upon species, life-history, region and habitat (Byrne and Przeslawski 2013, Przeslawski, Byrne et al. 2015). Ocean currents modulate the magnitude of change, but global change is also causing these to shift (Doney, Ruckelshaus et al. 2012, Byrne and Przeslawski 2013). Organisms that have rapid population turnover (short life-cycles/fast reproduction e.g. holoplanktonic copepods) may have a greater potential for evolutionary adaptation than then those that have relatively long generation times (Pörtner, Langenbuch et al. 2004, Kurihara 2008, Widdicombe and Spicer 2008, Melzner, Gutowska et al. 2009, Hu, Zhou et al. 2017, Thor, Bailey et al. 2018). Species that are able to adapt or acclimate to the carbonate chemistry alterations may thrive (e.g. intertidal or errant/active marine species); whereas others may not have this capacity over the timescales that the changes are occurring (e.g. many early-life history stages, sedentary, subtidal, deep sea or polar species) (Melzner, Gutowska et al. 2009, Kroeker, Gambi et al. 2013, Wittmann and Pörtner 2013). This may potentially alter species compositions and ecosystem dynamics and hence impact biodiversity, which is crucial for maintaining ecological, social and economic aspects of the oceans globally (Orr, Fabry et al. 2005).

The possible biological effects of elevated levels of pCO_2 in the marine realm are highly variable (Miles, Widdicobe et al. 2007, Fabry, Seibel et al. 2008, Kurihara 2008, Moya, Tambutté et al. 2008, Kroeker, Kordas et al. 2010, Wittmann and Pörtner 2013, Baumann 2019). Reviews to date describe how calcification processes, feeding, reproductive success (fertilisation and embryonic development), larval development, and a host of physiological processes, such as acid-base balance, ion regulation and metabolism may be impacted. Many calcified organisms such as hard corals, coccolithophores, echinoderms and molluscs are negatively impacted by high pCO_2, often due to their heavily calcified external structures (Kroeker, Kordas et al. 2010,

Wittmann and Pörtner 2013, Sperfeld, Mangor-Jensen et al. 2017). In particular, the elicited responses of early life-history stages are not well known, but data suggest that embryos and/or larvae of many marine invertebrates are likely to be the most sensitive stages (Kurihara and Shirayama 2004, Kurihara 2008, Dupont and Thorndyke 2009, Przeslawski, Byrne et al. 2015). A recent meta-analysis, however, has demonstrated that food supply confers resistance of calcifiers to elevated pCO_2 (Ramajo, Pérez-León et al. 2016).

Many calcifying organisms are only able to biomineralise and hence maintain $CaCO_3$ morphological structures when seawater is sufficiently saturated in regards to CO_3^{2-} (Riebesell, Fabry et al. 2010, Gattuso and Hansson 2011), however other species utilise different calcification pathways (Anger 2001, Arnold, Findlay et al. 2009). As a consequence of OA, saturation-states of the different carbonate minerals may change, with under saturation occurring in surface waters (See Orr et al. 2005) (Caldeira and Wickett 2003, Gattuso and Hansson 2011, Gattuso, Magnan et al. 2015). Calcifying organisms utilise carbonate minerals of aragonite, calcite or magnesium-calcite for exoskeleton formation, therefore this may affect the ability of some calcifying organisms to biomineralise and/or avoid dissolution (Gattuso and Hansson 2011). High latitude areas are thought to become under-saturated first with respect to aragonite (used by echinoderms for exoskeleton formation) (Orr, Fabry et al. 2005, Pörtner 2006, Pörtner, Peck et al. 2007). This is particularly problematic because Polar Regions have high levels of endemic species due to the relatively stable nature of the environment, which could render species here more susceptible to change (Pörtner 2006, Pörtner, Peck et al. 2007, Thor, Bailey et al. 2018).

Studies which have assessed the influence on marine invertebrates (and/or their early life stages) have predominantly focused on calcifying species such as echinoderms (Dupont, Ortega-Martínez et al. 2010, Hu, Casties et al. 2014, Suckling, Clark et al. 2015, Ross, Parker et al. 2016, Rodríguez, Hernández et al. 2017) and bivalve/gastropod molluscs (Michaelidis, Ouzounis et al. 2005, Fabry, Seibel et al. 2008, Ellis, Bersey et al. 2009, Bechmann, Taban et al. 2011, Kapsenberg, Miglioli et al. 2018). This disparity is largely due to the fact that these organisms are thought to be the most susceptible to OA due to their dependence on calcification processes to mineralise their exoskeletons/shells, plus their limited capacity to buffer internal CO_2

increases and their limited ion regulatory ability (Whiteley 2011, Wittmann and Pörtner 2013, Baumann 2019). They have also received greatest attention in part due to the larval and adult stages of these organisms being sufficient model organisms to assess biological effects of OA, with relative ease in laboratory spawning and rearing (Hörstadius 1973, Dupont, Ortega-Martínez et al. 2010, Byrne and Przeslawski 2013, Przeslawski, Byrne et al. 2015). Larvae have a greater body surface-area to volume-ratio than adult forms (Bechmann, Taban et al. 2011). They also often have more specific requirements and potentially a limited capacity to cope with metabolic disturbances compared to their adult counterparts, however, elicited responses are dependent upon population and species in question (Ries, Cohen et al. 2009, Bechmann, Taban et al. 2011, Whiteley 2011, Thor, Bailey et al. 2018).

As a consequence of biotic and abiotic stressors, natural mortality for early life stages of marine invertebrates in the oceans is high (Anger 2001, Anger 2003, Bechmann, Taban et al. 2011, Torres, Giménez et al. 2011). Therefore, any additional confounding factors such as OA may potentially add further constraints on certain larval species and/or populations. In turn this could impact recruitment and hence maintenance of species' populations (Bechmann, Taban et al. 2011); and also it may affect species at higher trophic levels which rely upon zooplankton for food energy (Whiteley 2011, Baumann 2019).

The previously mentioned factors highlight the fundamental need for research into tolerances and susceptibilities of early life stages of marine invertebrates to the future projected increases in ocean pCO_2 (Dupont and Thorndyke 2009, Todgham and Stillman 2013, Gunderson, Armstrong et al. 2016). In addition to this, comparatively few studies have considered a holistic environmental approach to how OA may interact with additional environmental conditions (e.g. reduced salinity, increased temperatures, food limitations and hypoxia, for example) (Findlay, Kendall et al. 2010, Gunderson, Armstrong et al. 2016, Baumann 2019, Spitzner, Giménez et al. 2019).

Crustaceans are found in a variety of habitats, with broad environmental variability, from terrestrial to aquatic (fresh water, brackish and marine) (Anger 2001, Whiteley 2011, Rivera Ingraham and Lignot 2017). However, most described species are predominantly marine to various degrees (Anger 2001, Whiteley 2011). Therefore,

they are valuable organisms to study in respect to OA in order to better understand the mechanisms behind tolerances or vulnerabilities to future predicted environmental changes (Whiteley 2011). Marine decapod crustacean responses to CO_2 induced OA will be analysed here, with particular emphasis on early life stages. In addition, the potential synergistic impacts of CO_2 induced OA with reduced salinity will be assessed.

1.3 Multiple Stressors in the Marine Environment

The marine environment experiences a multitude of anthropogenic stressors. More recently, attention has been focused on understanding the effects of simultaneous changes in environmental parameters, which are more realistic than studying the effects of changes of a single factor in isolation. Changing a single factor on its own has limitations in terms of addressing the response of organisms to climate change (Todgham and Stillman 2013, Gunderson, Armstrong et al. 2016, Boyd, Collins et al. 2018). From the small number of studies carried out to date, researchers are beginning to show that environmental factors interact and can either be synergistic, antagonistic or additive (e.g. Harvey, Gwynn-Jones et al. 2013, Kroeker, Micheli et al. 2013). Most studies have focused on OA and warming (e.g. Schram, Schoenrock et al. 2016), with a relatively recent meta-analysis on data collected from crustaceans demonstrating that the effects of high CO_2 and high temperature on early life survival were additive i.e. there was no interaction between the factors (Harvey, Gwynn-Jones et al. 2013). However, the same meta-analysis revealed that high CO_2 and warming were negatively synergetic when it came to growth rates in crustaceans. Such responses are concerning because it indicates that the combination of high CO_2 and warming can have disproportionally adverse effects (Baumann 2019). Other taxa, however, show different responses, and variability is also observed among life stages and trophic levels, whereas a more recent meta-analysis shows that synergistic effects are more common than additive or antagonistic effects between multistressors (Przeslawski, Byrne et al. 2015). Clearly, additional multistressor experiments are required, especially in the case of determining the combined effects of reduced salinity and elevated CO_2, as such studies are limited but relevant to estuarine, shallow coastal water situations under the influence of increased freshening.

1.4 Crustacean Larvae

A biphasic life-history involving a pelagic larval phase followed by benthic juvenile and adult forms is common for many marine invertebrates (Thorson 1950). Meroplanktonic larval biology is usually vastly different compared to that of the adult counterparts in terms of trophic position, behaviour, feeding habit, habitat, physiology and morphology (Anger 2006). An enhanced dispersal potential is enabled by these planktonic phases of development. This phenomenon may also indirectly benefit a given species' fitness levels, by facilitating genetic diversity and stabilising the densities of benthic adult populations (Anger 2006). By studying benthic invertebrate larvae, important information about the ecology of species or ecosystems can be deciphered. This also has important implications for species of commercial importance in fisheries and aquaculture.

Decapod crustacean larvae have been extensively studied in terms of their complex development (Rice 1993). Exogenous environmental factors can often demonstrate species-specific, and sometimes family specific differences in growth and development (Hartnoll 1983). Due to their ectothermic nature, crustacean larvae are sensitive to temperature fluctuations (Anger 2001). For planktotrophic larvae, as opposed to lecithotrophic, larval food resources, or adequate nutrition is paramount for development and survival (Anger 2001).

Metabolic reactions are influenced by nutrition and environmental factors. Temperature, salinity and pH can therefore influence growth, development, behaviour and survival to various degrees (Anger 2006). General trends have been the focus of scientific study in recent years across crustacean larval phyla, for example, in terms of developmental duration, body size and mortality (Angilletta, Steury et al. 2004, O'Connor, Bruno et al. 2007, Gunderson, Armstrong et al. 2016).

1.5 Growth in Decapod Crustacean Larvae

Development of decapod crustaceans after hatching proceeds via a series of moults, as is common with all the Arthropoda in post-embryonic ontogeny (Anger 2001). Nomenclature of larval developmental stages varies greatly depending on the author

of the study. Terminology used in this PhD thesis will be explained for each species in the subsequent sections.

Crustaceans have often been considered to be more tolerant of OA than other marine invertebrate taxa (Kroeker, Kordas et al. 2013, Wittmann and Pörtner 2013). However, some studies ascertain that early life stages are more vulnerable to changes in pCO_2 (as reviewed in Kurihara 2008, Whiteley 2011, Sperfeld et al 2017). Specific groups of crustaceans have received the most attention in terms of OA research to date, such as crustaceans with commercial value (e.g. crabs, shrimps or lobsters) but these studies are dominated by focusing on adult life history stages (Kurihara, Matsui et al. 2008, Long, Swiney et al. 2013, Small, Calosi et al. 2015). Copepods have also dominated the available crustacean literature, due to their rapid generation time in a laboratory setting, their abundance and their predominant roles within pelagic trophic webs (Whiteley 2011, Lewis, Brown et al. 2013, Sperfeld, Mangor-Jensen et al. 2017).

There is a relative paucity of data compared to other taxonomic groups regarding the effects of pCO_2 on crustaceans, especially early life stages. This means that forming robust generalised explanations into the effects on crustaceans to elevated pCO_2 is often difficult (Kroeker, Kordas et al. 2013). Research is needed spanning entire life history stages, multiple generations and on neglected taxa (Sperfeld, Mangor-Jensen et al. 2017). Researchers are only just beginning to understand the complex interaction between co-varying environmental factors on growth and survival in a range of marine crustacean species. These include limited studies on the effects of changes in elevated pCO_2 plus elevations in temperature (Harvey, Gwynn-Jones et al. 2013), and one study on elevated pCO_2 plus salinity reductions (Egilsdottir, Spicer et al. 2009).

1.6 Ocean Acidification: Crustacea

The phylum Arthropoda is the most diverse taxon of described extant metazoans on Earth, with the subphylum Crustacea making up the major marine constituent (Ruppert and Barnes 1994). Crustaceans are a hugely diverse group in terms of their biology and are important for many ecological functions due to their abundance, extensive roles as primary and secondary consumers, and their importance as a food source for other marine species over various trophic levels (Schram and von Vaupel Klein 1999).

The Decapoda contains the crabs, shrimps and lobsters, which are important groups from an ecological point of view and also for commercial purposes (Bondad-Reantaso, Subasinghe et al. 2012).

Despite the critical role that arthropods play in optimum marine ecosystem functioning, the biological implications of ocean acidification (OA) on members of this phylum are not well understood (Whiteley 2018). Organisms living in what could be considered more stable marine environments, such as the deep ocean, may be particularly vulnerable to OA and other environmental stressors (Aronson, Thatje et al. 2007, Fabry, Seibel et al. 2008, Whiteley 2011, Kelly and Hofmann 2013).

Research to date indicates that crustaceans may be affected by OA over the coming century (Whiteley 2011, Amundsen, Anderson et al. 2013, Whiteley 2018), but the responses of the different life cycle stages, species and populations are not expected to be uniform (Dupont, Havenhand et al. 2008, Kroeker, Kordas et al. 2013). To date, crustacean OA research reports diverse effects (i.e. positive, negative and/or neutral) on embryonic, larval developmental stages with studies focused on the Arctic spider crab, *Hyas araneus*, the European and American lobsters (*Homarus gammarus* and *H. americanus*) and the Florida stone crab (e.g. Egilsdottir, Spicer et al. 2009, Schiffer, Harms et al. 2014, Small, Calosi et al. 2015, Gravinese, Kronstadt et al. 2018).

1.6.1 Calcification in Aquatic Crustaceans

Most adult and juvenile crustaceans have an exoskeleton comprising mineralised chitin which is often reinforced with biomineralised calcium and/or magnesium salts (Anger 2001, Whiteley 2011). Exoskeletons of crustaceans contain $CaCO_3$ but largely consisting of calcite/magnesium calcite (which is more stable than aragonite) (Arnold, Findlay et al. 2009, Whiteley 2011). With current knowledge, it is therefore generally thought that calcified exterior components of adult crustaceans are largely resistant to OA (Orr, Fabry et al. 2005, Ries, Cohen et al. 2009, Whiteley 2011, Wittmann and Pörtner 2013).

Ries et al. (2009) exposed the blue crab (*Callinectes sapidus*), the American lobster (*Homarus americanus*) and the Eastern king prawn (*Penaeus plebejus*), in their adult forms, to pCO_2 conditions ranging from 409 ppm to 2,856 ppm at 25°C for 2 months (Ries, Cohen et al. 2009). It was found that at 2,856 ppm calcification increased, therefore, it may appear that OA could benefit these organisms (in reducing predation risks for example). A similar effect has also been shown in an earlier study with *Penaeus monodon* exposed to pH 6.4 for 36 days, whereby calcification increased (Wickins 1984). A confounding study found that *Palaemon pacificus* (Pacific grass shrimp) exhibited antennae shortening after a 30 week exposure to 0.01kPa pCO_2 (Kurihara, Matsui et al. 2008). This was thought to be due to acid-base balance disrupting the $CaCO_3$ structures over the extended exposure period.

Consequently, even if species seem to show tolerance/resilience, as seen in the above examples, it is not clear that they will be able to compensate OA induced changes in longer terms than experienced in the study exposure period (Whiteley 2011). Similarly, species which exhibit a seemingly detrimental response may be able to acclimate/adapt over longer time periods (Kelly and Hofmann 2013). In addition to this, energy is required for increasing calcification and exoskeleton mass; therefore, it is likely that energy is redirected from another function in order to compensate for calcification increases (i.e. energetically costly trade-off): for example, reproduction or growth may eventually be affected (Beniash, Ivanina et al. 2010, Whiteley 2011, Wittmann and Pörtner 2013). In the study by Ries et al. (2009), energy and mineral partitioning were not assessed; therefore, it is not clear how the organisms' overall longevity and health were influenced by OA.

Arnold et al. (2009) looked at larval development of *Homarus gammarus* (European lobster) and found that development and carapace length was unaffected when exposed to 1,200 ppm pCO_2 (Arnold, Findlay et al. 2009). However, exoskeleton mass and calcification were reduced in late zoea stages. This was purported to be due to a reduced ability to precipitate $CaCO_3$, and hence increase shell mass, potentially due to homeostatic disturbances due to hypercapnia, as there was no undersaturation of $CaCO_3$ in the exposure treatments, and no evidence of dissolution (Arnold, Findlay et al. 2009). This phenomenon is highlighted in a study looking at echinoderms by Wood et al. (2008) whereby the brittlestar, *Amphiura filiformis* was found to increase

calcification at low pH levels (pH 7.7, 7.3 and 6.8), which suggests some species may not be as adversely affected as others (Wood, Spicer et al. 2008). The seemingly beneficial metabolism enhancement due to low pH, however, occurred at the expense of arm muscle in the brittlestars (Wood, Spicer et al. 2008). Further direct issues could then arise from this, for example, in affecting respiration, feeding or survival (Wood, Spicer et al. 2008). It can therefore be concluded that even if an organism appears to benefit or be unaffected by low pH, a host of biological processes that may have been overlooked could be impacted, many of which remain unidentified (Wood, Spicer et al. 2008, Ries, Cohen et al. 2009). Therefore, it is important to study a variety of physiological and morphological parameters and responses (Wood, Spicer et al. 2008). In addition, under high pCO_2, even if a species enhances calcification they could be indirectly affected if less tolerant species that they rely upon for food for example are affected (Wood, Spicer et al. 2008, Ries, Cohen et al. 2009). Similarly, higher trophic levels may be indirectly impacted, for example in the study by Wood et al. (2008), *Limanda limanda* (dab) predates upon *A. filiformis*; so if musculature content is compromised then nutritional value for the predators may also be impacted (Wood, Spicer et al. 2008). An additional confounding factor is that species at higher trophic levels are often important commercially, therefore, socio-economical issues may arise (Wood, Spicer et al. 2008).

In contrast to other marine calcifiers, crustaceans are thought to use HCO_3^- or CO_2 and not CO_3^{2-} as their primary carbon source for $CaCO_3$ exoskeleton formation (Cameron 1989, Arnold, Findlay et al. 2009). Regulating pH at the calcification site is thought to be able to aid $CaCO_3$ precipitation by converting HCO_3^- into CO_3^{2-}; carbonic anhydrase (CA) enzymes have a significant role in the calcification process (Cameron 1986, Cameron 1989). Organisms which have the ability to elevate pH (increase/maintain alkalinity) at the calcification site regardless of surrounding seawater pH, via proton regulatory mechanisms, may be able to effectively tolerate OA (Cameron 1986, Cameron 1989, Ries, Cohen et al. 2009, Whiteley 2011). This ability potentially explains why some species can maintain or increase calcification with increasing pCO_2 (Ries, Cohen et al. 2009). Consequently CO_3^{2-} reduction due to OA may not directly inhibit crustaceans' capability to calcify or cause dissolution as with many other species; however more research is needed as the calcification process is

not well understood (Arnold, Findlay et al. 2009, Ries, Cohen et al. 2009, Whiteley 2011).

An additional factor which may contribute to crustacean protection against OA, is an external epicuticle which acts as a barrier protecting their exoskeleton from the surrounding environment (Ries, Cohen et al. 2009). The three crustaceans studied by Ries et al. (2009) possess exoskeletons that remain entirely covered by a protective layer; therefore, species like these in general often exhibit more resilience to high pCO_2, than species which possess an exoskeleton that upon deposition is predominantly exposed to the surrounding seawater (Ries, Cohen et al. 2009). Species that utilise aragonite and magnesium calcite (which are more soluble polymorphs of $CaCO_3$) are predicted to be more susceptible to OA, however Ries et al. (2009) only found a relationship between solubility of polymorphs and the vulnerability of an organism to occur at the highest pCO_2 value used (2856ppm) (Ries, Cohen et al. 2009). Conversely, planktonic larval decapods generally possess a much thinner and un-calcified exoskeleton, which may potentially increase or decrease their susceptibility compared to adult counterparts (Anger 2001, Whiteley 2011). In addition, for megalopa and benthic juvenile instars, a partially calcified and more rigid exoskeleton exists (Anger 2001).

Another way in which OA may impact crustaceans is during the moulting process (Whiteley 2011). Unlike calcifying species such as echinoderms, which continually grow their tests, exoskeletons in crustaceans are periodically shed and minerals from the older exoskeleton are believed to be incorporated into their new moult (Anger 2001, Arnold, Findlay et al. 2009). This may be particularly pertinent during larval development where moulting is a key process allowing progression onto the next stage.

Following moulting, HCO_3^- and Ca^{2+} is obtained and utilised from surrounding waters via the gills, and the deposition of calcium at these stages continues into the intermoult stage (Anger 2001, Whiteley 2011). Increasing H^+ can cause the influx of HCO_3^- to decline and consequently impact calcification and thereby the moulting process (Cameron 1986, Whiteley 2011). In turn OA could indirectly affect crustaceans by increasing predation risks during the vulnerable moulting process (Ries, Cohen et al.

2009, Whiteley 2011). Crustacean larvae moult more frequently (and have less developed, or an absence of gills, using epipodites/brachiostegites) than adults and the physiological challenges surrounding the moulting process may further render them more susceptible to environmental changes associated with OA (Anger 2001, Bechmann, Taban et al. 2011, Whiteley 2011). If the moulting process duration is delayed for larvae, then they may spend longer periods suspended in the water column and therefore increase the potential for predation, therefore potentially reducing survival (Anger 2001, Anger 2003, Whiteley 2011).

1.6.2 Acid-Base Balance and Ion Regulation in Aquatic Crustaceans

Crustaceans have varying capacities to physiologically cope with carbonate chemistry changes as a result of OA, largely depending upon species and life-history stage (Byrne and Przeslawski 2013). These mechanisms are fundamental because elevated pCO_2 in the surrounding environment can cause haemolymph pH to drop. This can then impede oxygen supply and the function of crucial proteins (Melzner, Gutowska et al. 2009, Whiteley 2011). In order to maintain homeostasis of physiological processes, compensatory acid-base-balance mechanisms work to keep body fluids working within efficient limits (Melzner, Gutowska et al. 2009, Wittmann and Pörtner 2013, Sperfeld, Mangor-Jensen et al. 2017).

Acid base balance is closely linked with iono- and osmo-regulation because both processes share the same physiological mechanisms in decapod crustaceans (Truchot 1981, Whiteley, Scott et al. 2001). Species which are efficient iono-osmo-regulators have strong well-developed mechanisms for ion exchange, and therefore are more efficient at compensating haemolymph pH fluctuations. Species capable of ion regulation are therefore more likely to be more adapted to cope with OA (i.e. increasing pCO_2) (Melzner, Gutowska et al. 2009, Whiteley 2011, Sperfeld, Mangor-Jensen et al. 2017).

Continual adjustments to acid-base balance are deemed to be metabolically costly, suggesting that compensation for OA results in a reduction in processes such as reproduction or growth, which are also energetically costly (Seibel and Walsh 2003,

Pörtner and Farrell 2008). An adequate food supply, however, may help to counteract the adverse effects of increased pCO_2 by supplementing energetic demands, maintaining the energetically costly processes of reproduction, growth and calcification, for example (Thomsen, Casties et al. 2013, Towle, Enochs et al. 2015, Ramajo, Pérez-León et al. 2016, Sperfeld, Mangor-Jensen et al. 2017).

Acid base balance is linked to the excretion of CO_2 and is a crucial metabolic mechanism in all animals, for maintaining or restoring internal homeostasis during normal respiratory acidosis (Truchot 1987, Michaelidis, Ouzounis et al. 2005, Gilmour and Perry 2009, Melzner, Gutowska et al. 2009). Acid base balance involves active ion-transportation, producing and utilising metabolically-derived protons and the buffering of body fluids (Michaelidis, Ouzounis et al. 2005). This process predominantly occurs at the gills in adult marine decapod crustaceans (and in equivalent structures in larvae before gills develop) (Charmantier 1998, Anger 2003, Michaelidis, Ouzounis et al. 2005, Melzner, Gutowska et al. 2009). Aquatic crustaceans use gills for many purposes including acid base balance, such as ion regulation, which shares the same mechanistic-pathway (Cameron 1986, Henry 2001, Whiteley 2011).

Studies that have previously observed species' physiological responses to hypercapnia, due to metabolic CO_2 produced during exercise, or due to hypoxic conditions, are now important in OA research in order to understand the mechanisms behind an organisms responses to elevated pCO_2 (Lindinger, Lauren et al. 1984, Somero 1985, Cameron 1986, Truchot 1987, Heisler 1989, Walsh and Milligan 1989). Species with poor compensatory-mechanisms to increased CO_2 levels (e.g. organisms with limited ion-transport capabilities for example sub-tidal or sedentary species/early life-history stages), may not be able to effectively control internal H^+ ion regulation and may be more susceptible to homeostasis disruptions due to OA (Pörtner, Langenbuch et al. 2004, Miles, Widdicobe et al. 2007, Sokolova, Matoo et al. 2016).

When levels of CO_2 increase due to OA, CO_2 excretion at the gills may be hindered causing an accumulation of haemolymph-CO_2 (hypercapnia) (Whiteley 2011). Haemolymph is important for supplying O_2 to crustacean-tissues and this process can be disrupted by pH changes (Whiteley 2011). For larvae in particular, without efficiently developed ventilation/circulation systems, excretion can often depend on favourable

CO_2 gradients between environmental conditions and body tissues (Michaelidis, Ouzounis et al. 2005). Similarly, more CO_2 could potentially diffuse through body membranes, entering tissues and fluids and causing an increase in intra and extracellular acidity (Michaelidis, Ouzounis et al. 2005).

Changes in extracellular pH can potentially be buffered by various means, such as the dissolution of $CaCO_3$ structures, which could compensate temporarily for acidic conditions, but this may be detrimental if exoskeleton maintenance or formation is hindered (Michaelidis, Ouzounis et al. 2005, Fabry, Seibel et al. 2008). Buffering can also occur by proteins in the haemolymph such as haemocyanin and HCO_3^- (predominantly obtained from surrounding seawater) (Michaelidis, Ouzounis et al. 2005, Whiteley 2011). Carbonic anhydrase (CA) enzymes facilitate CO_2 hydration, Cl^- is exchanged for the surrounding HCO_3-, and H^+ for Na^+; a process thought to be driven by Na^+/K^+-ATPase activities (Whiteley 2011, Henry, Lucu et al. 2012, Whiteley 2018). Buffering is particularly important intracellularly as cell functions such as ion regulation, metabolism, synthesis of proteins and control of cell volume may be disrupted by changes in pH (Whiteley 2011).

Species that have high metabolic rates may possess highly specialised organs for respiration and efficient ionic and osmoregulation and are more likely to be able to effectively tolerate acid-base disruptions, potentially rendering them less likely to be susceptible to OA (Pörtner, Langenbuch et al. 2004, Melzner, Gutowska et al. 2009, Whiteley 2011). Gills of the intertidal hypercapnia-tolerant common shore crab, *Carcinus maenas*, have Na^+ /K^+-ATPase activities that are an order of magnitude higher than *Mytilus edulis* (blue mussel) which is sessile and hypometabolic (Melzner, Gutowska et al. 2009). This may be because the gills serve as a feeding mechanism in the species, therefore, the blue mussel may lack efficient ion regulatory abilities (Melzner, Gutowska et al. 2009). Nevertheless, even taxa which may be able to compensate efficiently could be negatively impacted eventually, because the maintenance of homeostasis is energetically costly in the longer term (Pörtner, Langenbuch et al. 2004, Spicer, Raffo et al. 2007, Whiteley 2011). For example, *Palaemon serratus* and *P. elegans* which are effective ion regulators maintained ionic homeostasis at 0.3 kPa pCO_2 (3,000 ppm) after 30 days, however acid-base balance was hindered (Dissanayake, Clough et al. 2010). Similarly, *Necora puber* (velvet

swimming crab) that has comparatively low ion-regulatory abilities, exposed to pH 7.9, pH 7.3, pH 6.7 and pH 6.05 for 24 hours could effectively compensate by bicarbonate buffering of the haemolymph in the short-term (Pane and Barry 2007, Spicer, Raffo et al. 2007). However, after 16 days, compensation began to decline and when exposed to pH 6.05, mortalities occurred after 4-5days of exposure, indicative of a buffering threshold (Spicer, Raffo et al. 2007). While these pH levels are beyond those predicted for the next 300 years, results may still be relevant if for example carbon capture and storage methodologies malfunction (Spicer, Raffo et al. 2007).

1.7 Salinity and Crustaceans

In addition to the increasing levels of pCO_2, a significant ecological influence in the lives of marine crustaceans is salinity; as a multitude of physiological processes depend upon stable ionic and osmotic conditions. Salinity fluctuations may arise due to many factors such as: the melting and formation of sea-ice, freshwater run-off from land, precipitation and evaporation. Salinity in open-ocean surface waters is comparatively consistent (albeit due to the previously mentioned factors), with levels commonly between salinities of 32-36 (Anger 2003). Conversely, intertidal and estuarine salinity is often highly variable due to a range of abiotic factors such as daily changes in tidal-exposure (Henry 2001, Anger 2003). Many crustacean species also inhabit shallow coastal regions, where the input of freshwater, predominantly from rivers, upwelling and biological activity, can cause reductions in seawater pH and associated changes in carbonate chemistry (Hofmann, Peltzer et al. 2011, Sperfeld, Mangor-Jensen et al. 2017). Fluctuations in salinity and seawater carbon chemistry therefore co-exist in the natural environment.

A longitudinal gradient of salinity commonly exists where freshwater enters an estuary to where estuarine waters enter the full-strength seawater. This salinity gradient can range from a salinity of 0-40 and, on the basis of yearly variation in salinity, can be separated into distinct zones which appear to influence estuarine species' ecological distributions (Henry 2001, Henry, Lucu et al. 2012).

Osmotic stress may be induced by salinity fluctuations when the changes exceed the tolerance limits of a species, population or life-history stage (Anger 2003). The osmoregulatory-capacity of an organism determines species or population's tolerance to changes in salinity (Anger 2003, Rivera Ingraham and Lignot 2017). Species and/or life history stage which are stenohaline are usually pre-adapted to live in relatively stable marine environments and many of which are generally osmoconformers (i.e. where salinity variation is low e.g. subtidal/benthic habitats) (Charmantier and Charmantier-Daures 2001, Anger 2003, Pörtner 2008, Melzner, Gutowska et al. 2009). Species that occupy estuarine or brackish habitats are often euryhaline and hence evolutionary adapted physiologically to be able to tolerate or regulate salinity levels to various degrees i.e. osmoregulators (Anger 2003, Larsen, Deaton et al. 2014, Lignot and Charmantier 2015).

Primary osmotic-stress adaptations in adult crustaceans often consists of reducing permeability of cellular membranes and controlling intracellular and extracellular ion and osmo-regulation (Torres, Gimenez et al. 2011). Larvae however, are typically small in size (<1mm) and slow-swimming and may, therefore, potentially be more vulnerable physiologically than their adult counterparts, if for example compensatory mechanisms are not well developed (Anger 2003, Torres, Gimenez et al. 2011). For example meroplanktonic larvae of decapod crustaceans may be influenced by salinity stress potentially causing changes in developmental duration, increased mortalities, moulting cycle duration changes, morphological changes, metabolic changes to energy partitioning, reduced growth, feeding rate changes and behavioural changes (Charmantier 1998, Charmantier and Charmantier-Daures 2001, Charmantier, Giménez et al. 2002, Anger 2003, Torres, Gimenez et al. 2011).

A common strategy to avoid osmotic stress in estuarine decapod crustacean species is to export larvae towards the open-ocean, where conditions are generally more stable (Charmantier, Giménez et al. 2002, Anger 2003, Torres, Gimenez et al. 2011). However, in some species, larvae hatch in brackish conditions and are therefore exposed to unfavourable conditions as they migrate to more stable marine regions (Anger 2003). Similarly, larvae which develop in the offshore plankton are largely passive, and may temporarily become entrained in estuarine or coastal environments for instance and become exposed to fluctuating or low salinities (Anger 2003, Torres,

Gimenez et al. 2011). Larvae may also experience changes in environmental conditions through diel vertical migrations (Garrison 1999). Larvae may temporarily avoid unfavourably low salinities by moving vertically down through the water column (Torres, Gimenez et al. 2011). A trade-off as a result of this behaviour, however, is that food limitations may occur in deeper waters (Gimenez and Anger 2005). Therefore even brief salinity changes may hinder successful development of larval decapod crustaceans (Anger 2003, Torres, Gimenez et al. 2011).

Salinity may also have an impact upon ovigerous females that are in variable intertidal and/or estuarine environments, with their developing embryos (Charmantier and Charmantier-Daures 2001, Anger 2003). Some studies suggest that tolerance levels could be transferred to offspring as a result of adult exposure to low salinity (Giménez and Anger 2001, Giménez and Anger 2003, Spitzner, Giménez et al. 2019). The environmental biotic and abiotic parameters act as selection pressures for the evolutionary development of physiological strategies and adaptations, for early larval stages to salinity (Charmantier 1998, Charmantier and Charmantier-Daures 2001, Charmantier, Giménez et al. 2002, Anger 2003). This highlights the fact that different life history stages of different species and populations may have varying abilities to tolerate salinity stress; coastal/estuarine species in particular may be exposed to unfavourable salinity conditions at some part of their embryonic development (Charmantier 1998, Charmantier and Charmantier-Daures 2001, Charmantier, Giménez et al. 2002, Anger 2003, Spitzner, Giménez et al. 2019). *Carcinus maenas* (the European Green Crab), is a species which is extremely successful globally (Anger 2003, Cieluch, Anger et al. 2004). Salinity levels as low as 4 are known to be tolerated by adult crabs because of effective osmoregulatory capacities, in their natural distribution range. However, the early larvae are not recognised as being able to survive in water salinities below 20 (Anger 2003, Cieluch, Anger et al. 2004). Nevertheless, megalopa/juvenile stages of this species migrate back to coastal/estuarine waters and will therefore be subjected to hypo-osmotic conditions again (Torres, Gimenez et al. 2011, Spitzner, Giménez et al. 2019).

1.7.1 Elevated CO_2: Interactions with Temperature and Salinity

Harvey et al. (2013) reports that single stressor studies upon marine organisms with respect to OA are often not informative in terms of organismal responses that may occur in the natural marine environment, therefore proposes that a more holistic approach to studying OA in conjunction with other environmental stressors should be taken (Harvey, Gwynn-Jones et al. 2013). For example, some studies suggest that CO_2 impacts only become present when combined with elevated temperature, although data is limited (Walther, Anger et al. 2010, Whiteley 2011). However, studies into the effects of salinity and OA together are even rarer and, considering the significant role acid-base and ionic homeostasis plays in influencing species' tolerances to OA, this is surprising (Whiteley 2018).

One of the few studies which has looked at the impacts of both CO_2 induced OA and reduced salinity upon crustaceans to date is a study by Egilsdottir et al. (2009) looking at the marine calcifying intertidal amphipod *Echinogammarus marinus* (Egilsdottir, Spicer et al. 2009). The response of this organism (mainly adults) to low salinity levels is well understood. Many amphipods are intertidal, shore-dwelling or freshwater species and are therefore likely to be generally tolerant to environmental changes (Egilsdottir, Spicer et al. 2009). In particular, freshwater amphipods are known to be strong iono-regulators and osmo-regulators, which helps to explain their survival in acidified freshwater conditions (Felten, Charmantier et al. 2008). Females carrying eggs were subjected to salinities of 35, 22 and 11; and a pCO_2 level of 1,900 ppm (Egilsdottir, Spicer et al. 2009). Embryo developmental duration, number of hatches and the calcification success of hatchlings were measured (Egilsdottir, Spicer et al. 2009). Some detrimental impacts were identified in embryonic development, when both variables were combined. However, low salinity seemed to have more of an impact than acidity, affecting hatch success and calcium content of hatchlings, whereas acidity had no impact (Egilsdottir, Spicer et al. 2009).

The result of salinity being more influential than OA could potentially be explained in the case of the moderate osmoregulator, *C. maenas* which experiences a three to four fold increase in Na^+/K^+ ATPase activity and an eight fold increase in the activities of

carbonic anhydrase (CA) in the gills when transferred from ambient to low salinities (32 to 12) (Henry, Lucu et al. 2012, Whiteley 2018). CA is important in facilitating the breakdown of CO_2 so therefore, the combination of OA with low salinity may be beneficial to some species, but research into this is needed (Henry, Lucu et al. 2012).

When compared with other studies on embryonic crustacea exposed to similar conditions, it is clear that when present, pCO_2 induced impacts were narrowly measurable (Egilsdottir, Spicer et al. 2009). For example, for the marine copepod *Acartia tsuensis*, CO_2 acidified water at 2,380 ppm/pH 7.3 had no influence on hatches or development (Egilsdottir, Spicer et al. 2009). Similarly, in a closely related copepod, *A. erythraea* hatch success declined as acidity increased but was not significant at pH 7.31 (~2,000 ppm) (Kurihara, Shimode et al. 2004). In addition to this, Hauton et al. (2009) found that at pH 7.8 and pH 7.6 growth rates of juvenile *Gammarus locusta* through to adults exhibited no detrimental impacts (Hauton, Tyrrell et al. 2009). More recently, however, studies have shown that elevated pCO_2 levels do influence larval survival. For example, elevated pCO_2 reduced the rate of embryonic development in the commercially important Florida stone crab, *Menippe mercenaria*, and reduced hatching success (Gravinese, Kronstadt et al. 2018). Hatching success was also variable, suggesting that some broods are more tolerant to changes in seawater carbon chemistry. Similar brood-specific differences were observed in the intertidal porcelain crab, *Petrolisthes cinctipes* (Ceballos-Osuna, Carter et al. 2013). Hatching success in *P. cinctipes*, however, was unaffected by elevated pCO_2, but varied among broods, attributed to the variable nature of their intertidal environment. In the European lobster, *Homarus gammarus*, larvae exposed to the pCO_2 level predicted for 2100, experienced oxidative stress, and developmental effects in that intermoult period increased, and growth decreased (Rato, Novais et al. 2017). Larvae from the strong osmoregulating blue crab, *Callinestes sapidus*, were 10 times smaller when reared at a pH of 7.8 compared with controls at a pH of 8.2 (Giltz and Taylor 2017). Survival was reduced by 23%. When exposed to elevated pCO_2 during brooding, subsequent larval survival was reduced in the Arctic spider crab, *Hyas araneus,* and development of the larvae moulting into zoea II was delayed (Schiffer, Harms et al. 2014). Feeding rates were also reduced in zoea I. Collectively these data suggest maternal effects, which have a carry-over effect on the developing larvae.

When crustacean larvae are exposed to a combination of elevated pCO_2 and elevated temperature, temperature appears to be the dominant factor. In *M. mercennia* exposure to elevated temperature had a greater effect on survival than elevated pCO_2 (Gravinese, Kronstadt et al. 2018). Larvae also had shorter moult intervals at higher temperatures, whereas elevated pCO_2 delayed metamorphosis to post-larvae. Exposure to the combined effects of elevated pCO_2 and temperature reduced survival to 20% compared with controls. Larval lobsters (stages I-IV), *Homarus americanus*, reared at 19°C had lower survival rates but developed twice as fast as controls at 16°C (Waller, Wahle et al. 2017). Larvae (stages I-III) from ambient temperature but elevated pCO_2 (750 ppm) had longer carapace lengths and greater dry mass than controls. Stage IV larvae reared in the combined treatment were characterised by higher feeding rates and swimming activities than larvae in all other treatments. The authors concluded that the effects of elevated pCO_2 and temperature on metabolism and behaviour were complex (Waller, Wahle et al. 2017). Another study looking at the combined effects of OA (pH 7.6, 7.3, 7.0) with salinity (35, 25 and 15) on the hatch-rate of the brine shrimp *Artemia franciscana*, found that the less alkaline pH conditions led to a reduction of hatch success, irrespective of salinity (Salma, Uddowla et al. 2012). Salma et al. (2012) argue that this supports the hypothesis that OA elicits negative impacts to hatch success and that salinity has no synergistic relationship with pH in this species (Salma, Uddowla et al. 2012).

1.8 Study Species: *Carcinus maenas* (Linnaeus, 1758)

1.8.1 Taxonomy

Carcinus maenas (Linnaeus, 1758) (Table 1.1), was chosen for this study because it is generally tolerant to environmental change, being a moderate osmoregulator and being able to tolerate a range of temperatures, between 3 and 26°C (Kern, Grosholz et al. 2002). They prefer salinities between 10 to 30 but may survive fluctuations and they normally inhabit intertidal estuarine habitats. They favour a varied diet of fish, crustaceans and molluscs (Klassen and Locke 2007).

Table 1.1: Taxonomic classification of *Carcinus maenas* (Linnaeus, 1758)

Phylum	Arthropoda
Subphylum	Crustacea
Class	Malacostraca
Subclass	Eumalacostraca
Order	Decapoda
Infraorder	Brachyura
Family	Portunidae
Genus	*Carcinus*
Species	*maenas*

Figure 1.2: Ovigerous females of Carcinus maenas showing the developing egg mass, of which there may be ~185,000 developing embryos.

Figure 1.3: *Carcinus maenas* larvae photographed upon hatching at the zoea I stage, with a magnified image of one of the large compound eyes.

Figure 1.4: A sample of *Carcinus maenas* zoea I larvae photographed immediately after hatching, in a collection bowl, prior to selection for use in experiments.

1.8.2 Life Cycle

C. maenas has a life-cycle comprised of four distinct stages: embryos, larvae, juveniles and adults (Figure 1.5) (with zoea and megalopa stages described by Rice and Ingle 1975) (Klassen and Locke 2007). The average longevity of this species varies between three-six years (although this factor varies depending upon geographical location). The reproductive season duration also depends upon geographical location, for example, in regions around southern England, mating processes occur all year round, and conversely, in northern Scotland for example, it is restricted to spring. Copulation is initiated when adults reach sexual maturity (usually 25-30mm in males, 15-31mm in females) and begins by female selection by male crabs (typically those that are about to moult/going through the process of ecdysis, and with a carapace diameter 10mm below their own) (Broekhuysen 1936). Males carry (and therefore protect) their

selected female once she has moulted (amplexus) beneath their carapace and they then copulate (Klassen and Locke 2007).

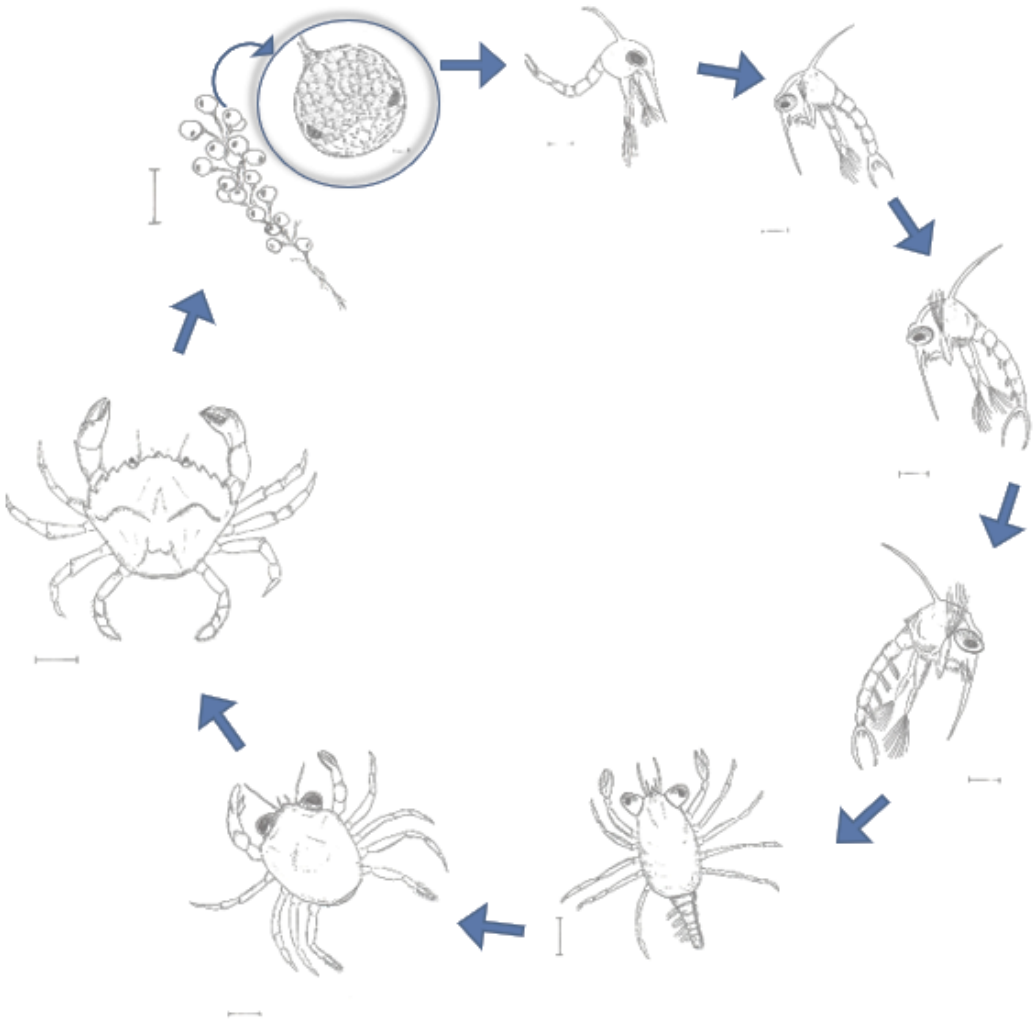


Figure 1.5: The life cycle of *C. maenas* showing development from embryos, zoea I, zoea II, zoea III, zoea IV, megalopa, juvenile one and finally the stereotypical form for later juveniles to adults.

After egg (oocyte) fertilisation has occurred, the female expands her range to deeper waters, where conditions are more stable than in the intertidal zone, the female then attaches the oocytes to the long endopodites (setae) of her pleopods; a cohesive mass is formed (Figure 1.2) (Broekhuysen 1936). For a duration of up to four months (embryonic developmental duration is temperature dependant), the oocyte mass (of

up to 185,000 eggs) is maintained and cared for/brooded in the abdomen of the female (Klassen and Locke 2007).

Upon complete development of the embryos, females move to suitable regions in order for the ebb tides to aid in expelling larvae as they hatch, transporting them towards coastal or offshore marine regions at night (Klassen and Locke 2007). The larvae hatch as zoea I stages/instars (Figure 1.3 and Figure 1.4) exhibiting rostral spines (a characteristic trait of zoea stages) for protection against predation by juvenile fish for example. Zoea larvae follow a circatidal rhythm (aiding in the offshore dispersal where the subsequent zoea stages develop, zoea II-IV) and exhibit diel vertical migratory behaviours. Upon successful completion of the final zoea IV stage, larvae undergo moulting/ecdysis to the megalopa; a stage resembling adult crabs in terms of the morphology of the chelipeds and walking legs. The megalopae are able to swim like the zoea larval instars, however this ability is no longer facilitated by the maxillipeds, but by the pleopods. Megalopa migrate in circatidal rhythms to surface waters during flood tides, whereby they are transported landwards, often with the aid of seaweed floats. Upon recruitment to inshore and to estuaries, megalopae, in their later stages, aim to locate and settle upon structurally complex habitats (such as mussel beds, seagrass patches or filamentous green algae niches) (Queiroga 1998). The process of developing from the first zoeal stage up until the final megalopa stages may take ~50 days, or in laboratory settings ~82 days (Klassen and Locke 2007). The duration of development through each zoeal instar is approximately 5-7 days, with a megalopal developmental period of ~8days (Webster and Dircksen 1991). However, duration of development is temperature dependant (Dawirs 1982) and has been observed to range from anywhere between 2.5-13.5 days for zoea stages and from 5.5-26 days at the megalopal stage (Dawirs and Dietrich 1986), although absence of food/starvation conditions can double duration of zoeal development (Dawirs 1984). At 18°C, with normal strength seawater, zoeal development takes ~4-5 days, and 12 days for the megalopa (Dawirs, Puschel et al. 1986). The megalopae undergo ecdysis to the first juvenile instar. Mortality is high during the settlement period, with predation being particularly problematic for the early settlers. Those that do survive, however, usually moult around 18 times on average (depending on a variety of factors such as temperature and food availability) until they become adult crabs.

1.8.3 Tolerances to Environmental Change

C. maenas (adults) are described as efficient osmoregulators (McGaw, Reiber et al. 1999). As adults, *C. maenas* is euryhaline, which means it can tolerate a wide range of salinities from 4 - 52 (Klassen and Locke 2007). However, mesohaline to polyhaline salinity levels of 10 - 30 are preferred, as physiological mechanisms such as the ability to cope with hypoxic conditions are hindered below a salinity of 10 (Broekhuysen 1936, Legeay and Massabuau 2000, Grosholz and Ruiz 2002, Kern, Grosholz et al. 2002). In response to reduced salinity, adults increased locomotory activity ((Taylor and Naylor 1977). This behaviour, or escape response, was usually seen at a salinity of ~9 - 10 (McGaw, Reiber et al. 1999). Adult *C. maenas* are also able to reduce H_2O permeability across the gills in response to lower salinities (Rainbow and Black 2001). Compared to the red counterparts, green colour morphs are generally more tolerant to environmental stressors such as salinity fluctuations (McKnight, Mathews et al. 2000).

Compared to adults, *C. maenas* larvae are less tolerant to salinity (and other environmental variables) (Klassen and Locke 2007). Newly emerged zoea larvae can survive at a salinity <15, however this hinders development to subsequent instars (Anger, Spivak et al. 1998). A salinity of ≥20 is required for metamorphosis to the megalopa instar (Anger, Spivak et al. 1998). Even fleeting exposure to <20 salinities has been shown to delay development in later stages in addition to increasing mortality in later moulting periods. Compared to salinities of 25 and 32, Anger et al. (1998) found that at salinity 20, developmental duration was delayed significantly and with an increased mortality. At salinities ≤25, Anger et al. (1998) found that respiration and growth rates decreased. Development rates were found to be unaffected by salinities encompassing 20 - 35 (Nagaraj 1993, See Table 1.2). The upper salinity tolerance for successful larval development is a salinity of ~>40, however this is temperature limited and at ~10°C the upper salinity limit for development decreased to 26 (Broekhuysen 1936).

In order to ensure pelagic larvae have a rapid export to coastal or offshore marine regions (i.e. higher salinity than estuaries where many adult populations reside), females move to suitable regions in order for the ebb tides to aid in expelling larvae as they hatch (Queiroga, Costlow et al. 1997, Klassen and Locke 2007). These salinity

tolerance changes are reflective of a progression in the ontogeny of osmoregulation in *C. maenas* with early larval stages, the zoea, being osmoconformers and unable to regulate body fluid osmolality separately from the external environment, to the megalopa, which are weak osmoregulators (Torres, Giménez et al. 2002, Cieluch, Anger et al. 2004). Adults are even better at osmoregulation and are able to hyper-osmoregulate. The capacity to osmoregulate may have a genetic basis, as illustrated by Anger et al. (1998), whereby *C. maenas* from the Baltic Sea tolerated low salinities (more effective at hyper-osmoregulation) than their North Sea conspecifics. This difference was not able to be reversed fully by adaptation (Klassen and Locke 2007).

1.8.3.1 Temperature

C. maenas are ectotherms and therefore their behaviour and physiology is influenced by temperature variations, on a daily and seasonal basis (Klassen and Locke 2007). *C. maenas* is eurythermic which means it is able reproduce at temperatures ranging from 18-26°C, and it is able to survive temperatures ranging from 0 - 35°C, but preferentially 3-26°C (Klassen and Locke 2007, Compton, Leathwick et al. 2010). It has been shown that *C. maenas* adults could tolerate a temperature of 21°C, with 60% relative humidity, through evaporative cooling, causing heat to dissipate from the gills maintaining a body temperature several degrees lower than the surrounding conditions, increasing temperature tolerance of *C. maenas* when exposed (Ahsanullah and Newell 1977, Klassen and Locke 2007).

Reproduction is governed by thermal range, and this in turn limits distribution. *C. maenas* can produce eggs successfully up to 26°C (Klassen and Locke 2007). However, a more confined thermal range is necessary for successful larval development. Larvae have been reared successfully in laboratories from hatching up to the first juvenile crab stage at temperatures encompassing 9-22.5°C (Dawirs, Puschel et al. 1986, Derivera, Hitchcock et al. 2007). Larvae are found in the wild at lower temperatures than this (5°C) but it is not clear if they are undergoing successful moulting cycles (Roff, Fanning et al. 1984). The rate of larval development has been suggested to be determined by the temperature experienced during the initial 30 days of life (Derivera, Hitchcock et al. 2007). For the zoeal phases (I-IV) of larval

development, the average duration of development ranged from 52 days (10°C) to 25 days (25°C); these larvae were all adequately fed (Table 1.2) (Nagaraj 1993).

Table 1.2: Effect of temperature and salinity on the development time of *C. maenas* larvae (zoeal stages). Summarised from (Nagaraj 1993).

Temperature (C)	Salinity	Larval duration (days)	Mean duration (days) at temperature
10	20	54.9	51.9
	25	50.5	
	30	49.1	
	35	53.2	
15	20	46.5	40.1
	25	39.4	
	30	38.2	
	35	36.4	
20	20	37.2	30.5
	25	32.1	
	30	25.4	
	35	27.4	
25	20	24.3	25.4
	25	25	
	30	24.8	
	35	27.3	

1.8.3.2 Food Supply

Under starvation conditions, duration of development can double for *C. maenas* (Dawirs 1984). *C. maenas* can exhibit delayed developmental duration under starvation conditions during zoeal stages (Dawirs 1984). Through zoea I, limited access to prey had limited impact on survival, suggesting larvae are fairly well adapted to cope with natural food shortages (Giménez and Anger 2005). However, some feeding (i.e. not starvation) is required during zoea I in order to allow development to stage II, for example Dawirs 1984 showed that zoea I could not moult to zoea II if they were starved during the first part of their zoea I development, even if feeding resumed towards the end (Dawirs 1984). Zoea I larvae require a threshold of feeding for a minimum of 20% of the initial zoea I development duration (Dawirs 1984).

Alongside developmental duration, metabolic efficiency is influenced by temperature. Compared to 12°C, at 18°C zoeal stages of development accumulated biomass and

energy more efficiently, however during the megalopa stage of development the opposite was the case (Dawirs 1986).

1.8.4 Distribution and Habitat Preference/Ecology

C. maenas is located ubiquitously around all coastal regions of the British Isles, including the Republic of Ireland – its native regions. It is also found in many other regions, being highly invasive and now inhabits coasts in North and Southern America, Asia, Australia and Africa (Compton, Leathwick et al. 2010). *C. maenas* inhabits marine rocky shores and estuarine regions such as salt marsh tributaries. Berried female crabs can typically be found during the winter months, however they can also be encountered in spring and early summer, depending upon geographical location.

1.9 Study Species: *Palaemon serratus* (Pennant, 1777)

1.9.1 Taxonomy

Palaemon serratus (Pennant, 1777) (Table 1.3), known as the common prawn, is morphologically similar to various other Palaemonid shrimps of the northern temperate regions (e.g. *Palaemon elegans*, *Palaemon adspersus* and *Palaemon longirostris*) (Haig, Ryan et al. 2014). However, it has an absence of dorsal teeth present on the anterior end of the upturned rostrum; a characteristic trait of this species (Figure 1.6) (González-Ortegón and Cuesta 2006).

The shrimp has a relatively short life span, thought to be between two to five years depending upon study region/geographical location of the study (Cole 1958, Haig, Ryan et al. 2014). Cole (1958) found that the species has a lifespan of four years in Holyhead, UK (near to where the study species of this thesis were collected). Longevity variation in this species could be explained by different environmental influences throughout their distributions e.g. temperature. Sexual dimorphism exists in this species, with females usually being bigger in length and weight than the males (Forster 1951, Guerao and Ribera 2000). Females mature slower than males, at between nine to ten months, as opposed to six to seven months for males (Forster 1951). At the start of reproduction, female growth slows due to shifts in energy allocation towards producing oocytes, instead of increasing in size during ecdysis (Hartnoll 1985). For populations in the Irish Sea, highest growth (in terms of weight gain) occur from July to September, due to increased sea temperatures throughout this period (Fahy and Gleeson 1996).

Table 1.3: Taxonomic classification of *Palaemon serratus* (Pennant, 1777)

Phylum	Arthropoda
Subphylum	Crustacea
Class	Malacostraca
Subclass	Eumalacostraca
Order	Decapoda
Infraorder	Caridea
Family	Palaemonidae
Genus	*Palaemon*
Species	*serratus*

Figure 1.6: Ovigerous *Palaemon serratus* with eggs distinctly visible

Figure 1.7: *Palaemon serratus* larvae (June 2015)

1.9.2 Life Cycle

Mating happens after the female moults and fertilisation occurs internally. Fertilised eggs are attached to the pleopods and carried externally for approximately four months (at 9-11˚C) (Figure 1.6), but this is largely governed by environmental factors. Egg development is hindered at cooler temperatures, such as inshore coastal regions during winter months (Forster 1951, Fahy and Gleeson 1996). Offshore deeper waters are favoured for larvae to hatch (Figure 1.7), as berried females have a lower tolerance to fluctuating salinities (Panikkar 1941, Forster 1951). Shrimps will shed the eggs in regions where they are likely to disperse and survive, until post larval settlement (Strathmann 1985).

Reproductive patterns in shrimp are largely shaped/governed by temperature (Forster 1959, Haig, Ryan et al. 2014). Maturation age, frequency and timing of spawning, varies among the Palaemonid genus (Haig, Ryan et al. 2014). A close link exists between moulting and mating, with many crustaceans' mating directly after a moult

has occurred (Hartnoll 1985). Genotypic variations in moulting hormones of populations in Irish and French waters are thought to be responsible for differences in the intermoult period, however, these data are qualitative in nature and further molecular studies would be required to verify these observations (Carlisle 1955).

There are four main developmental stages in the life cycle of *P. serratus*. Larvae are adapted to swimming among the nekton. There are usually around 8-9 instars (Fincham and Figueras 1986), however this is largely governed by environmental variables such as salinity, temperature (Kelly, Tully et al. 2012) and availability of food (Reeve 1969). The final instar metamorphoses and moults into a stage similar to adults, a post larval instar (Reeve 1969, Kelly, Tully et al. 2012). During juvenile and adult stages, *P. serratus* are demersal. It was found that the number of larval instars before metamorphosis to the post-larval instar was 'positively influenced' by temperature (Kelly, Tully et al. 2012). Nevertheless, nine instars have been reported for populations in the Irish Sea (Kelly, Tully et al. 2012), and six instars for warmer Mediterranean populations (Fincham & Figueras 1986). Therefore, we would infer that Mediterranean populations would have more instars, if temperature was the primary contributory factor governing moulting frequency, highlighting the complexity surrounding larval development (Haig, Ryan et al. 2014). Stressful conditions can give rise to additional larval instars (often with reduced moult increments/time between moults).

Around 1 month after hatching, shrimps metamorphose to the post-larval (or juvenile) stage (~10mm in length), which typically settles between July-August. This usually occurs in the lower intertidal rocky shore regions (Guerao and Ribera 1996). *P. serratus* migrate further offshore to deeper waters by the middle of October, by which time they are of a size that can be caught by fishing gears (Kelly, Tully et al. 2012).

1.9.3 Tolerances to Environmental Change

P. serratus inhabits both estuaries and in open water (Kirkpatrick and Jones 1985). Compared to other Palaemonid species, *P. serratus* has a narrower salinity tolerance with a minimum of a salinity of 16 and a maximum of 39. Distribution of the different

Palaemonids in estuaries are reflective of the tolerance levels to temperature, salinity and available food resources (Kirkpatrick and Jones 1985, Rowe 2002, Gonzalez-Ortegon, Pascual et al. 2006).

P. serratus will osmoregulate in order to survive salinity variations (Spaargaren 1972, González-Ortegón and Cuesta 2006, González-Ortegón, Subida et al. 2010). The capacity to osmoregulate varies with sex, size and reproductive state (Kirkpatrick & Jones 1985; Panikkar 1941). Berried or ovigerous females are less tolerant to salinity changes than non-ovigerous females and also males (Panikkar 1941), hence they are found in deeper waters, offshore where larvae hatch (Forster 1951).

At extreme salinity levels, low temperature limits osmoregulation (Spaargaren 1972). Juvenile (post-larval) *P. serratus* are thought to be more tolerant to salinity and temperature changes than larvae, which is similar for the Palmonids *P. xiphas* and *P. adspersus* (Kelly, Tully et al. 2012). Precipitation and the resultant influx of riverine water determines much of the temperature and salinity conditions experiences in coastal and estuarine regions (Haig, Ryan et al. 2014). Optimal conditions for both survival and development of *P. serratus* are hindered by the yearly rainfall variation in Irish waters. Local adaptations may be occurring between populations in Irish waters and the Mediterranean due to environmental conditions experienced, with survival and development differences observed (Kelly, Tully et al. 2012). More research is needed to determine at the molecular level, the influence of phenotypic plasticity and genotypes and their variance with populations from different geographical regions (Haig, Ryan et al. 2014).

An increase in metabolic demand arises with the regulation of physiological processes, therefore adaptive tolerances to environmental changes may be restricted by metabolic capacities. Metabolic rate (or oxygen consumption) is size variable in small marine invertebrates. Upon exposure to 5-20°C temperature changes, shrimp that were larger in size (1090-1140mg) were found to have a lower metabolic rate than shrimp that were smaller in size (80-260mg). This is indicative of smaller invertebrates not being as effective in coping with temperature changes. *P. serratus* are thought to be better adapted to warmer conditions than cooler ones, for example Mediterranean

P. serratus were found to have a lower metabolic rate (at 15°C and 20°C), than that of populations from cooler regions (Dalla Via 1985).

1.9.3.1 Food Supply

This species does not exhibit facultative lecithotrophy beyond the first larval instar, i.e. it is predominantly planktotrophic, meaning it feeds from the plankton.

1.9.4 Distribution and Habitat Preference

P. serratus is located ubiquitously around coastal regions of the UK and the Republic of Ireland. It also has a wider geographical distribution spanning from these regions to areas as far south as the Black Sea, Mauritius and Mediterranean (Guerao and Ribera 2000, Haig, Ryan et al. 2014).

P. serratus inhabits both shallow intertidal zones e.g. on the rocky shore in rock pools and also deeper offshore subtidal areas (Forster 1951, Gonzalez-Ortegon, Pascual et al. 2006, Haig, Ryan et al. 2014). The species is thought to exhibit migratory behaviours, diurnally and tidally, whereby they occur predominantly in deeper subtidal regions in the winter months (to 40m), and in summer months are found in shallower waters (Haig, Ryan et al. 2014). This is considered to be due to unfavourable cooler temperatures and reduced salinity levels in winter, with high rain influx in coastal regions (Forster 1951, Reeve 1969, Rodriguez and Naylor 1972, Guerao and Ribera 2000).

For post-larvae and juvenile *P. serratus*, estuarine regions are important nursery habitats. For adults, they are also important feeding areas. As estuaries may experience temperature and salinity fluctuations daily, the distribution of shrimp in these areas has been found to be reflective of their inherent tolerance to these conditions (Kirkpatrick and Jones 1985, Gonzalez-Ortegon, Pascual et al. 2006).

1.10 Study Species: *Palaemon (Palaemonetes) varians* (Leach, 1813-1814)

1.10.1 Taxonomy

Palaemon varians (or formally known as *Palaemonetes varians*) (Leach, 1813-1814) (Table 1.4), is the (Atlantic) ditch shrimp or variable shrimp. There is some disparity in the literature about the naming of this species. It has previously been in the *Palaemonetes* sp. group, and as such compared physiologically with similar species in the group. However, it has been reclassified into the genus *Palaemon* in the literature fairly recently.

Table 1.4: Taxonomic classification of *Palaemon varians* (Leach, 1813-1814)

Phylum	Arthropoda
Subphylum	Crustacea
Class	Malacostraca
Subclass	Eumalacostraca
Order	Decapoda
Infraorder	Caridea
Family	Palaemonidae
Genus	*Palaemon*
Species	*varians*

Figure 1.8: Ovigerous *Palaemon varians* with eggs distinctly visible

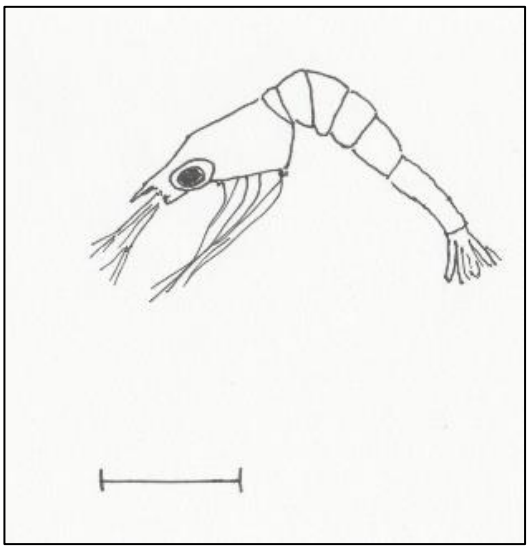


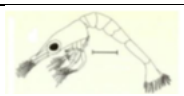
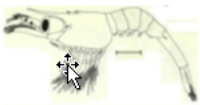
Figure 1.9: *Palaemon varians* diagram of the first larval stage upon hatching

1.10.2 Life Cycle

Breeding of *P. varians* is during late spring or early summer (Figure 1.8, 1.9) (Oliphant 2013). The age of which *P. varians* matures and breeds remains unreported, in

addition the number of broods that females may carry each season are unknown in the literature (Oliphant 2013). The developmental process (duration and number of stages) is fairly shortened in *P. varians* compared to other caridean shrimp species, consisting of 5 distinct instars before reaching the juvenile stage (table 1.5) (Gurney 1924, Fincham 1979, Oliphant 2013). In comparison to other shrimps in the Palaemonidae family, the early zoeal development of the pereiopods (legs), is more advanced compared to other Palaemonidae shrimps (Gurney 1924, Fincham 1979). There are key morphological differences between larval stages of *P. varians*, as described by Fincham (1979) and Oliphant (2013).

Table 1.5: *P. varians* larval developmental morphology, as described by Fincham (1979), adapted from Oliphant 2013. Nomenclature used by Fincham is on the right, and that used by Oliphant is on the left. Scale bars = 0.5mm. NB*. Oliphant (2013) reported that following D1 or ZIII, *P. varians* larvae could moult occasionally but the resultant instar morphology was not able to be assigned to D2 (ZIV) or D3 (ZV) so was called a 'decapodid' of which there could be several before the final juvenile instar arose.

Nomenclature: Oliphant 2013	Instar morphology of *Palaemon varians*	Nomenclature: Fincham 1979	Information
ZI		ZI	Sessile eyes. Straight rostrum. Carapace without spines.
ZII		ZII	Stalked eyes.
D1		ZIII	Larger pleopods – non-functional
D2		ZIV	Pleopod non-functional but movement possible
D3		ZV	Pleopods fully functional and well developed
Juv		Juv	Identical in morphology, miniature form of the adult *P. varians*

1.10.3 Tolerances to Environmental Change

P. varians is a species that predominantly occurs in brackish regions. The broad salinity and temperature tolerance of both the larvae and adults of *P. varians*, is likely a primary factor in determining the success of this species in the highly changeable environment of salt marshes (Oliphant 2013).

Larval development of *P. varians* can occur from salinities ranging ~5-42, therefore, development can occur under salt marsh/estuarine conditions (unlike in *P. serratus*) (Antonopoulou and Emson 1989), however wild-captured samples of *P. varians* larvae have rarely been obtained from either estuarine or marine waters (except for zoea I, II and juveniles from Portuguese waters of lower Ria de Aveiro (Pereira, Pereira et al. 2000). This may be attributed to their rapid development time, locomotory ability or perhaps ineffective capture methods. No study has mapped the entire wild life-cycle of *P. varians* populations (Oliphant 2013). *P. varians* have been demonstrated to survive salinities of 1.7-66 for several days (Lofts 1956).

P. varians is generally highly tolerant to environmental perturbations and is considered to be strongly eurythermal (Oliphant, Thatje et al. 2011, Oliphant 2013). *P. varians* can tolerate temperatures of ~0°C-33°C (Oliphant, Thatje et al. 2011, Ravaux, Leger et al. 2012). Successful larval development occurs at ≥10°C-30°C (Oliphant 2013). Oliphant (2013) demonstrated this wide thermal tolerance range in larvae, for example at 5°C larvae survived several days, at 10°C larvae survived several weeks and at 15-30°C larvae developed fully. The number of larval stages is influenced by temperature. At higher temperatures, a temperature-mediated developmental process was apparent with four instars being common, at lower temperatures five instars were common (Oliphant 2013). At these higher temperatures, there was an increase in larval growth (carbon mass and dry weight) and developmental rates. At lower temperatures, the development of larvae was more constrained with a rise in respiratory energy loss. The constrained growth in the lower temperatures could explain the increased larval instar stages observed (Oliphant 2013).

1.10.3.1 Food Supply

Oliphant (2013) demonstrated that *P. varians* larvae are extremely resistant to starvation. During the first and second larval stages, *P. varians* larvae are considered to be facultative lecithotrophic i.e. upon hatching, having visible yolk/energy reserves but can also feed freely if desired. From the third larval instar, the larvae are considered planktotrophic i.e. feeding on animal or plant matter in the plankton. The factors contributing to the starvation resistance seen in *P. varians* are: large size, high carbon content at ~45% and a C:N ratio of ~4.2, with yolk reserves available upon hatching (Oliphant 2013). These evolutionary adaptations to starvation in the species aid in the larval export success from salt marsh regions to more favourable areas such as lower estuaries and coastal marine regions. The conditions in these areas are less extreme than in salt marshes where the adults reside, so growth, development and the survivorship of larvae is favoured (Oliphant 2013).

Oliphant (2013) observed that rates of respiration were similar in both fed and starved larvae of *P. varians*, although values varied during the moult cycle. This indicates that *P. varians* larvae can maintain metabolism under low food conditions (i.e. low energy) without being energetically costly.

1.10.4 Distribution and Habitat Preference

P. varians is a marine/brackish species and often occurs, in abundance, in almost freshwater or brackish conditions (salinity = 2->45) in regions such as salt marshes, estuarine peripheries, estuarine drainage channels and in coastal pools (Gurney 1924, Lofts 1956). The regions where it resides can be extremely changeable with high turbidity, hypoxic events, frequent (daily tidal/seasonal basis) fluctuations in salinity, temperature and oxygen levels. It is common on all coasts of the British Isles and Ireland, except for northern Scotland where it is scarce (Hindley 2001, Dolmen, Hindley et al. 2004). Further afield it is found on the NE Atlantic coast in West Europe, South Norway to Morocco, with some populations in the Mediterranean Sea (Gurney 1924, Hindley 2001, González-Ortegón and Cuesta 2006, Oliphant 2013). Adults are benthic

in brackish habitats, often closely associated with aquatic algae, plants and vegetational overhangs, the shrimp rarely swims freely in midwater (Hindley 2001).

1.11 Aims of the Study and Structure of the Thesis

The primary aim of this thesis was to examine the effects of elevated seawater pCO_2, reduced salinity, elevated temperature and food limitations on the early life stages of three decapod crustacean species common to the European coastline. The focus of each study was the effects of multiple stressors on survival rates and on rates of development, two important considerations when investigating larval recruitment and subsequent population effects. The study consisted of investigations on one crab species (*Carcinus maenas)* and two shrimp species (*Palaemon serratus* and *Palaemon varians*). The purpose was to compare responses in crustaceans inhabiting a range of different habitats, with differing exposures to salinity, pCO_2 and temperature fluctuations. This work was performed alongside two 9-12 month exposure studies looking at more advanced life stages of *Cancer pagurus* and *C. maenas*, in order to determine the most vulnerable stages, or species, to combined exposures to 'business as usual' pCO_2 levels predicted for 2100, and a reduction in salinity to 25.

Main hypotheses:

- H1: Elevated pCO_2 levels will reduce survival of the early life stages and prolong developmental duration of the survivors.
- H2: Effects of elevated pCO_2 on survival and development will be stronger under osmotic stress, demonstrating multiple stressor synergistic effects.
- H3: The magnitude of the effect will vary depending upon species, in particular, fully marine species will be more sensitive to changes than estuarine species, even at early stages of development.
- H4: Individual variability will occur at the level of broods produced by different females and at the level of individual larvae and juveniles.

This thesis is divided into six chapters. Chapter One is a general introduction that incorporates an independent, original review of existing work in the field, and contextualises the research in relation to the present state of knowledge on larval responses to environmental change. Chapter Two is a methods chapter describing the development of the methodology and the resulting analysis. Chapters Three to Five represent three self-contained experimental chapters. Chapter Three deals with the

effects of salinity, elevated pCO_2, food limitation and temperature on survival and developmental duration in *Carcinus maenas* larvae. Chapter Four considers the effects of salinity and elevated pCO_2 on the early juvenile stages of *C*. *maenas*, exposed to either seasonal changes in temperature, or held at constant temperature. Chapter Five considers the responses of two species of shrimps (*Palaemon serratus* and *Palaemon varians*) with differing experiences of salinity change in their natural environments, after exposure to salinity and elevated pCO_2. Chapter Six is a general discussion that draws together the main findings of the thesis in the context of their original contribution to current knowledge, establishes the significance of the work and outlines the needs and prospects for future research.

2 Chapter Two: Method Development: Experiments using *Artemia sp*. and Larval Rearing of *Carcinus maenas*

The following chapter gives an account of the methodological aspects used to rear larvae under controlled conditions, in order to determine the effect of elevated pCO_2 in crustacean larvae and early juveniles. The first section covers methodological aspects associated with the production of water, with the appropriate chemical conditions. The second concerns the control of the water quality in the larval rearing chambers. The third section covers feeding and handling of *Artemia* sp. nauplii as a food source for rearing crustacean larvae.

2.1 Ocean Acidification/Salinity System

The studies described within this thesis relied on the availability of an aquarium system specifically built to maintain 4 water treatments, in a 2-way factorial design, to include elevated pCO_2 to match the 'business as usual' scenario for 2100 of 1000 µatm and a reduction in salinity to 25. Each treatment consisted of a mixing tank (350 L), a header tank (100 L) and five holding tanks (48 L), as described by Whiteley et al. (2018). Mixing tanks were supplied with natural filtered, UV treated seawater, which was diluted to salinity 25 by adding dechlorinated freshwater until the desired salinity of 25 was detected via conductivity sensors. Elevated pCO_2 levels were applied as outlined in Whiteley et al. (2018). Temperature varied according to season, but rapid, daily fluctuations were avoided, as the whole system was housed in a cooled room with further temperature control provided via individual heaters and chillers on each header tank. The seawater ran from each header tank into the holding tanks by gravity and then ran to waste at a flow rate of 68±6 L h^{-1}. Seawater temperature, salinity and pH were recorded daily, and seawater samples were removed every month for the determination of total alkalinity (AT), Dissolved Inorganic Carbon (DIC) and nutrients, as explained by Whiteley et al. (2018). This system was used in a separate set of experiments on juvenile and adult crabs, but seawater from the system was used in the larval experiments described in this thesis.

- **Flow Rates (Relevant to Chapter 4, Part A only)**

  High flow rates within the experimental system pictured were such that treatment water within each individual holding tank was ensured to be replaced at a rate of approximately every 44 minutes (Whiteley 2018). This enabled non-treatment effects to be minimized e.g. fluctuations in temperature, in addition to maintaining the carbonate chemistry at the chosen levels in the treatment seawater, for holding tanks and in the individual rearing containers.

- **Schematics of the OA/Salinity System**

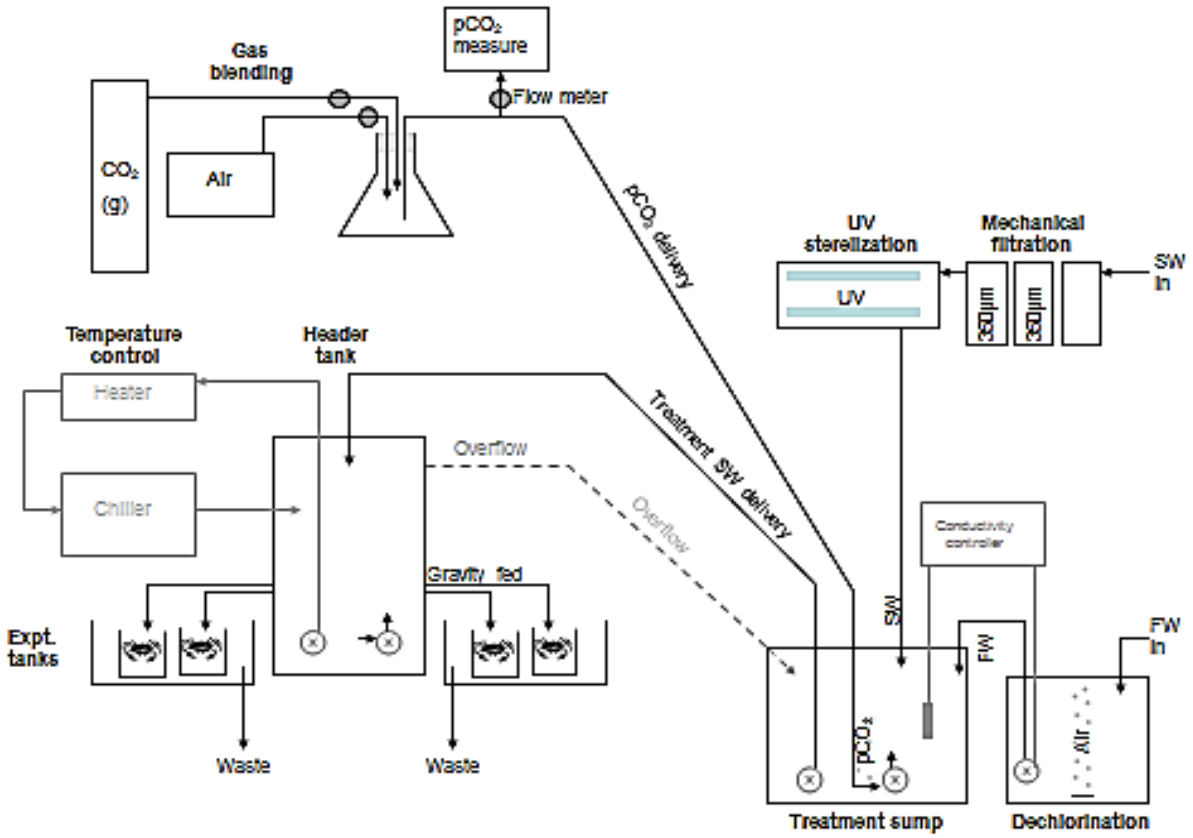


Figure 2.1: Schematics of the aquarium system used to provide 4 treatments representing combinations of ambient and elevated pCO_2, and full strength and dilute seawater. Diagram represents a single unit responsible for one treatment.

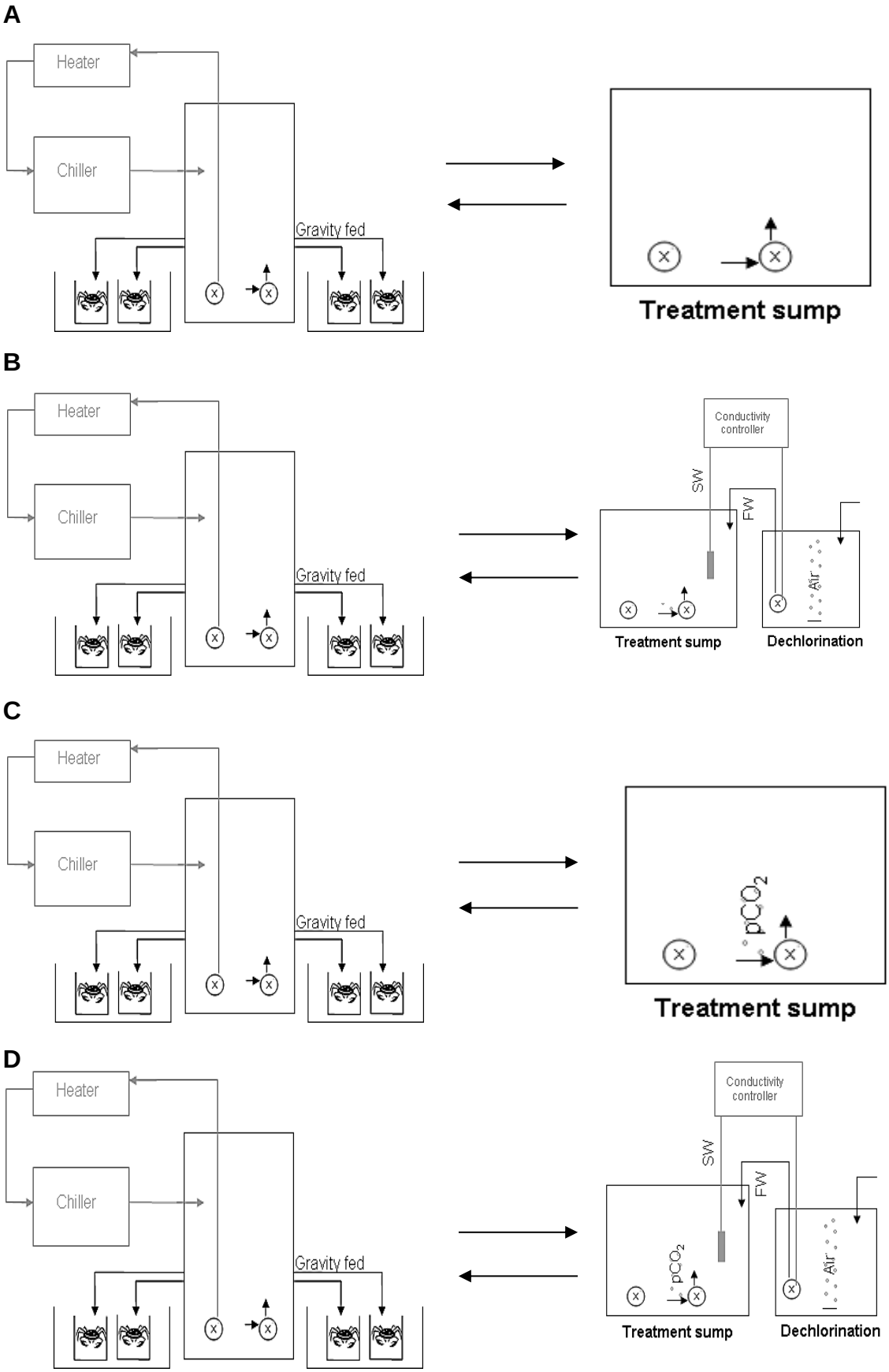


Figure 2.2: Schematics of all 4 treatments. A: Ambient salinity Ambient pCO_2 B: Low salinity, Ambient pCO_2 C: Ambient salinity, High pCO_2 D: Low salinity High pCO_2. Treatment sump = Mixing tank; SW = seawater; FW = freshwater.

Figure 2.3: A panoramic photograph of the aquarium system providing the 4 treatments. Green bins are the mixing tanks, and the blue bins are the header tanks.

Table 2.1: Measured and estimated seawater treatment parameters for the aquarium system (adapted from: Whiteley et al. 2018)

	Parameter	SW + CCO_2 (control)	SW + HCO_2	DW + CCO_2	DW + HCO_2
2013-2014	**pH_{NIST}**	**8.03 ± 0.01^a**	**7.72 ± 0.01^b**	**8.09 ± 0.01^c**	**7.78 ± 0.01^d**
	Salinity	**33.22 ± 0.04^a**	**33.17 ± 0.05^a**	**25.47 ± 0.09^b**	**25.39 ± 0.07^b**
	Temp (˚C)	**12.53 ± 0.06^a**	**12.71 ± 0.06^a**	**12.58 ± 0.05^a**	**12.64 ± 0.06^a**
	TA (µmol kg^{-1})	**2,272 ± 4^a**	**2,272 ± 4^a**	**1,813 ± 5^b**	**1,825 ± 5^b**
	DIC (µmol kg^{-1})	**2,120 ± 4^a**	**2,222 ± 5^b**	**1,716 ± 7^c**	**1,795 ± 4^d**
	pCO_2 (µatm)	527 ± 13^a	1125 ± 56^b	508 ± 49^a	930 ± 27^b
	Ω calcite	2.80 ± 0.06^a	1.54 ± 0.05^b	1.88 ± 0.07^b	1.06 ± 0.03^c
	Ω aragonite	1.78 ± 0.04^a	0.98 ± 0.03^b	1.16 ± 0.05^b	0.66 ± 0.02^c
2014-2015	**pH_{NIST}**	**7.99 ± 0.01^a**	**7.67 ± 0.01^b**	**8.03 ± 0.001^c**	**7.77 ±0.01^d**
	Salinity	**33.17 ± 0.05^a**	**33.27 ± 0.05^a**	**25.60 ± 0.09^b**	**25.32 ± 0.08^b**
	Temp (˚C)	**12.66 ± 0.09^a**	**12.78 ± 0.09^a**	**12.75 ± 0.08^a**	**12.68 ± 0.09^a**
	A_T (µmol kg^{-1})	**2,303 ± 16^a**	**2,297 ± 10^a**	**1,821 ± 13^b**	**1,826 ± 6^b**
	DIC (µmol kg^{-1})	**2,143 ± 8^a**	**2,243 ± 10^b**	**1,721 ± 13^c**	**1,787 ± 9^d**
	pCO_2 (µatm)	570 ± 45^a	1138 ± 77^b	481 ± 30^a	874 ± 56^b
	Ω calcite	3.01 ± 0.28^a	1.61 ± 0.08^b	1.91 ± 0.08^b	1.17 ± 0.05^c
	Ω aragonite	1.92 ± 0.18^a	1.03 ± 0.05^b	1.19 ± 0.05^b	0.73 ± 0.03^c

A_T = total alkalinity, SW = full strength seawater, DW = diluted seawater, CCO_2 = ambient CO_2, HCO_2 = elevated pCO_2.
Values are means ±SEM.
Data represent daily values for pH, salinity and temperature and monthly values for remaining parameters.
Measured values shown in bold used to calculate pCO_2, calcite and aragonite saturation states (Ω calcite and Ω aragonite).
Different letters indicate significant differences among treatments (P <0.05) using one-way ANOVA or Kruskal-Wallis test, and appropriate post hoc tests.

2.2 Controlling Water Quality in Larval Rearing Containers

Studies into the effects of OA (pH and elevated pCO_2) on crustacean larvae have been based on both open and closed containers. Initially, I considered using open containers receiving water through a flow-through system, but I found important logistical challenges associated to keeping larvae in these containers. While open containers may be a solution for large crustacean larvae (e.g. European lobster, *Homarus gammarus*), such containers would not be appropriate for zoeal stages of *Carcinus maenas*, or indeed other zoea with long spines. For such stages, the use of containing nets or mesh, results in larvae being tangled and in the breakage of rostral spines.

There were, in addition, limitations associated to the specific OA system, located in the Nuffield Fish Laboratory at The School of Ocean Sciences, Bangor University. In this system, only one temperature ~12°C could be used, but larval rearing of the species in this thesis is difficult at such temperatures. There was, in addition, limited space to implement experiments onto the main OA system, due to the large numbers of adult crabs in the main system, however, lower bench space was allocated, and additional seawater lines put into the system for these experiments to take place.

I, therefore, considered using closed containers. There has been a previous study on crustacean larvae (Walther, Anger et al. 2010), using closed Kautex bottles. Such experiments were, however, carried out at much lower temperatures than those to be used in this thesis. At the temperatures needed to rear larvae of *Carcinus maenas*, as well as Palaemonid shrimps, increased ammonia excretion and respiration rates may change the chemical conditions and pH in particular, beyond acceptable levels.

Initially, I hoped to emulate the successful larval rearing methodologies used by Walther et al. (2010) studying the effects of OA and warming upon the spider crab *Hyas araneus*. I contacted both Kathleen Walther and Hans Otto Pörtner about the exact specifications of the larval rearing methodology used (i.e. how pH was maintained in a closed system over 24-hour periods), including the type of larval rearing bottle and material used (500ml Polyethylene terephthalate, clear Kautex Textron bottles with screw-top lids). If this method based on Kautex bottles was deemed not appropriate for our own experiments, I wanted to develop a methodology

that would not result in important changes in pH, within a period of 24 hours of larval rearing. This is because the procedure of larval rearing is based on changing water and food every day.

In order to determine whether the method based on Kautex bottles would be appropriate, larval rearing and monitoring of pH levels was carried out under similar conditions to those of Walther et al. 2010, but at 18°C. I therefore set up experiments to monitor changes in pH in response to *Artemia* sp. (larval food) using the Kautex bottles (either sealed, containing an air chamber or open) (Table 2.2). Another group of experiments with glass containers was also proposed. It was decided that a series of experiments using these different types of containers and different food densities would be carried out in order to find the appropriate rearing technique. I began initially by experimenting with different densities of *Artemia* (10/ml and 5/ml) incubated at two different temperatures and salinities (12°C and 15°C, Salinity 25 and Salinity 33) to explore how pH changed over a 24-hour period, within the Kautex bottles, both with and without an air pocket.

Initial experiments were carried out with a density of 10 *Artemia* sp. per ml. These experiments resulted in a strong reduction in pH within 24 hours (>0.8). Further experiments were carried out with 5 *Artemia* sp. per ml. It was found that pH fell less than under the higher density of *Artemia* sp., but it still declined considerably over 24 hours when the lids were closed (dropping by 0.8-0.10 pH units). It was found that by using 250ml of treatment water, with an open lid, maintained pH more effectively (declining by ~0.09pH units).

One particular rearing method (using 300ml open glass containers) led to small reductions in pH (lower than 0.07). I then explored using 300ml open-glass containers. These were found to maintain pH over a 24-hour period at similar levels to the open Kautex bottles. After this I then began experiments using 4 treatment waters (Control (0.04kPa) and 2100 predicted pCO_2 levels (0.08kPa), salinity 25 and control salinity 33, at two different temperatures (12°C and 15°C). Soon after starting, using the high pCO_2 treatment water, I determined that pH was no longer staying stable over 24 hours and subsequently found that by covering the glasses with cling-film (to ensure no air/water gas equilibrium could occur) effectively maintained pH over a 24-hour period.

The main point raised regarding these experiments was the concern surrounding the maintenance of stable pH levels over 24-hour periods, prior to performing water changes. In order to be able to produce publishable science in the field of ocean acidification research, carbonate chemistry tables must be included in any research submitted to a journal, showing parameters such as pH levels over the duration of the experiment. As pH levels could not be reliably maintained, it is likely that results would not be able to be published using the methodology of static glasses in incubators.

I therefore considered the option of rearing larvae under limited access to food, with larvae being fed for four to six hours per day, depending on species. This treatment has been used before as it is thought to better reflect the natural conditions exhibited by crustacean larvae (Sulkin, Blanco et al. 1998, Giménez and Anger 2005). Observations made in the natural environment indicate that larvae persist in surface waters for brief periods of 4 – 6 hours (Gonzalez-Ortegon and Giménez 2014). Because of prey patchiness and the fact that larvae of many crustacean species perform daily vertical migrations, it is likely that feeding is restricted to when darkness occurs, when they are in surface waters, while feeding may not occur at times when larvae are in deeper water e.g. during daylight hours. Sulkin et al. (1998) and Giménez and Anger (2005) showed that zoea I larvae were able to develop under access to prey that was limited to 4-6 hours; Sulkin et al. (1998) showed in particular that photoperiod (i.e. whether access to prey occurred during the night or day) was not relevant for larval survival. Subsequently, it was shown that the capacity to develop under access to prey limited to 4-6 hours occurred in the shrimp *Palaemon serratus* (Gonzalez-Ortegon and Giménez 2014) and the European lobster *Homarus gammarus* (Jackson, Torres et al. 2014) at a wide range of temperatures (15°C to 24°C). There was, however, no information available about the effects of limited access to prey on larvae of the shore crab *Carcinus maenas*.

Preliminary experiments under elevated pCO_2 conditions showed that the reduction in pH were minimal under scenarios of limited access to prey (Figure 2.4). In other words, in situations when *Artemia* sp. nauplii were added to the cultures for only 4-6 hours a day, I was able to maintain stable pH levels (Figure 2.5, 2.6). Given that survival patterns in *P. serratus* was not affected under limited access to prey, I decided to use

such a feeding regime to run the experiments with larvae of the species. In addition, larvae of another shrimp, *P. varians*, are known to be highly tolerant to food limitation (Oliphant 2013) and I assumed that it was unlikely that limited access to prey would affect survival significantly; this assumption proved to be correct (see Chapter 5). The main question was about the potential effect of limited access to prey on the larval development and survival of *Carcinus maenas*. Because I did not have any information available, I decided to run a multiple factorial experiment aimed to determine the effect of limited access to prey on *C. maenas* larvae under different salinities and temperatures. These experiments are part of Chapter 3 and show that larval survival of *C. maenas* can be reduced under limited access to prey, but also that survival to the megalopa is successful. Overall, the experiments carried out here, as well as those published in the literature, suggest that by rearing larvae under limited access to prey, one can evaluate the effect of ocean acidification on larvae with a minimum increase in the level of stress responses. Furthermore, because such conditions are more realistic than those based on feeding larvae ad libitum and on a permanent basis, our experiments would give a more realistic picture about how larvae would respond to ocean acidification in the field.

The only exception where I did not use the static system was the study on the effects of ocean acidification on megalopa and juveniles of *Carcinus maenas*. In that case, I did not expect issues associated to entanglement of larvae on meshes (due to a lack of spines at these stages) required to enable water flow, while keeping the organisms in their containers. I developed two groups of experiments (Chapter 4). The first group was based *C. maenas* megalopa reared in the flow through system and exposed to freshly hatched *Artemia* sp. nauplii for 24 hours per day, until moulting to juvenile crabs. Juvenile crabs were fed with squid for 24 hours per day. NB. This was an automated system capable of maintaining pH due to continuous flowing water at treatment conditions. The second experiment was based on the static approach. Here, *C. maenas* megalopa were exposed to freshly hatched *Artemia* sp. nauplii for 4 hours per day, until moulting to juvenile crabs. Juvenile crabs were fed with small pieces of squid.

2.3 Microscopy

Photomicrographs were taken using a Nikon Labophot light microscope fitted with a condenser capable of producing bright field, dark field or phase contrast images. Both phase contrast and bright field objective lenses were available for viewing. In addition to the built-in light source, the microscope was fitted with a through-the-microscope electronic flash gun. A Canon EOS 60D digital camera with remote shutter release was used to record images. This camera body has an articulated viewing screen allowing image viewing before shutter release.

2.4 Method Development Results

Table 2.2: Effects of open vs closed bottles and *Artemia nauplii* on pH over a period of 24 hours.

Treatment	Container	Volume	Time (Hours)	pH	Salinity
5/ ml	Closed Bottle	250ml	0	8.12	33
	Closed Bottle	250ml	24	8.02	33
5/ ml	Open Bottle	250ml	0	8.10	33
	Open Bottle	250ml	24	8.01	33
Control	Open Bottle	250ml	0	8.11	33
	Open Bottle	250ml	24	8.08	33

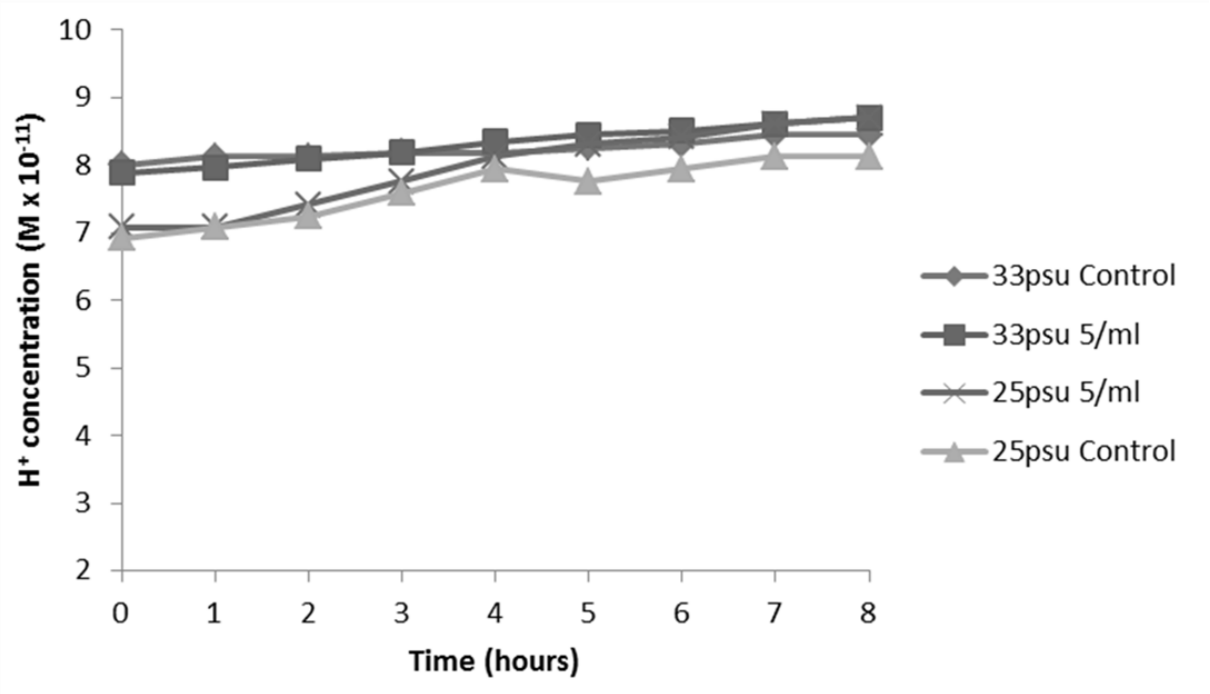


Figure 2.4: Calibration Curve. Change in H^+ concentration over an 8-hour time period in 250ml FSW (salinity 33) and DW (salinity 25) (*Artemia* sp. nauplii density 5/ml in glass containers).

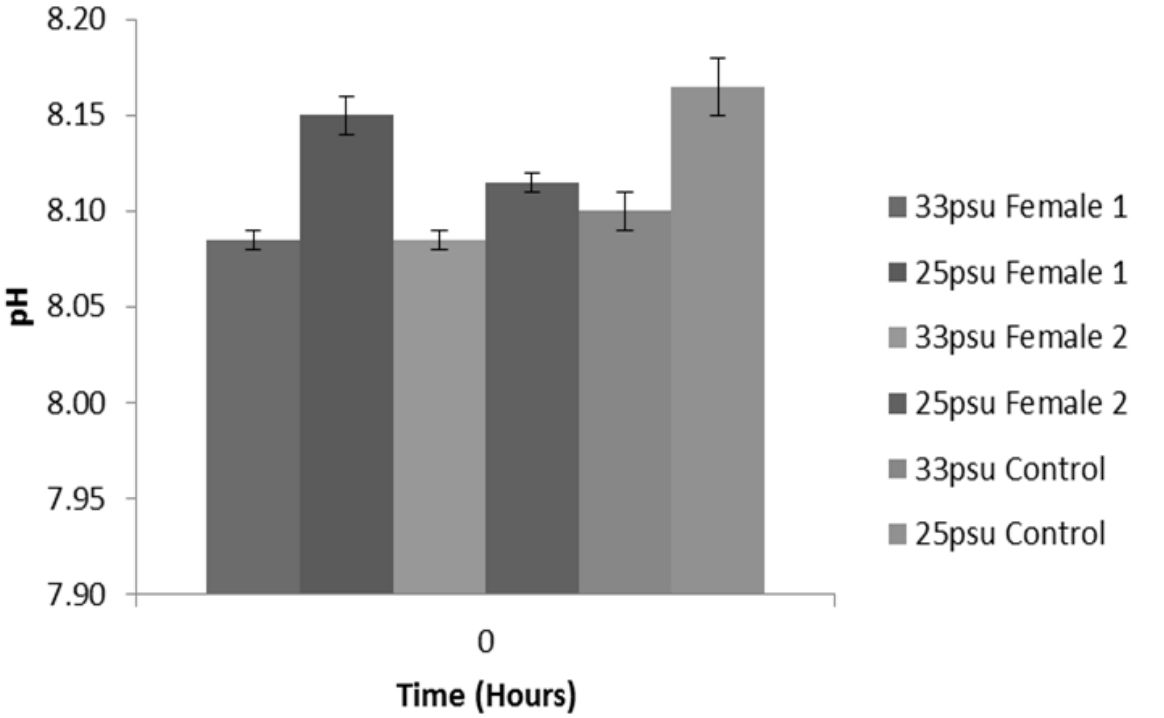


Figure 2.5: Initial pH in the experiment observing the change in pH over 24 hours with newly hatched larvae from two female *C. maenas* (see Figure 2.6 for change after 24 hours) (Treatment: FSW (salinity 33) and DW (salinity 25), 250mls, 5/ml *Artemia* sp. nauplii density, glass containers).

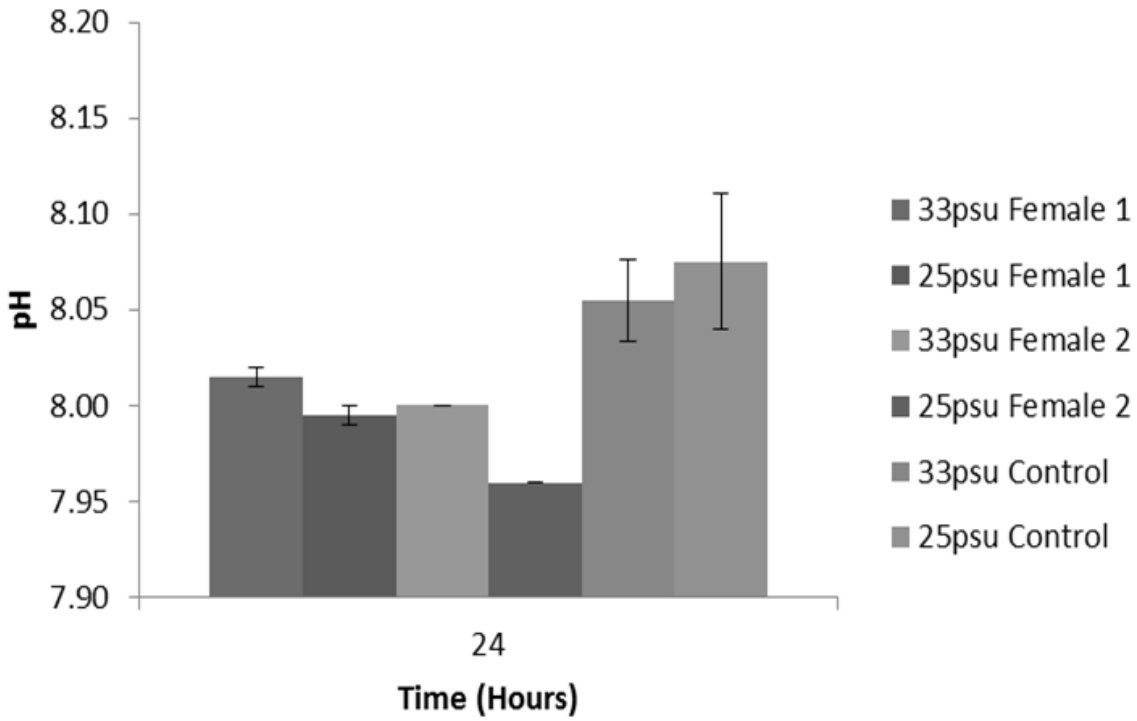


Figure 2.6: Change in pH after 24 hours (following on from Figure 2.5) with newly hatched larvae from two female *C. maenas* (Treatment: FSW (salinity 33) and DW (salinity 25), 250mls, 5/ml *Artemia* sp. nauplii density, glass containers).

2.5 Food Conditions and Animal Husbandry

Larvae were fed with one day old *Artemia* sp. Nauplii on a daily basis. *Artemia* sp. were hatched in spherical glass conical flasks (see Figure 2.7) containing seawater, reared at 24°C, under illumination and under strong bubbling aeration. *Artemia* sp. nauplii were collected by siphoning water out of the cultures; nauplii were then passed through a fine mesh and rinsed in filtered seawater (Figure 2.8).

During preliminary experiments, I developed the procedures to ensure that the process of adding *Artemia* sp. nauplii to the larval cultures would not affect pH values. I found initially that the pH of the water contained in the glass bowls where *Artemia* sp. hatch had a reduced pH despite the consistent bubbling. Preliminary trials determined the number of rinsing and filtering of nauplii through the fine mesh needed to achieve pH levels that were the same as those used in the experiments.

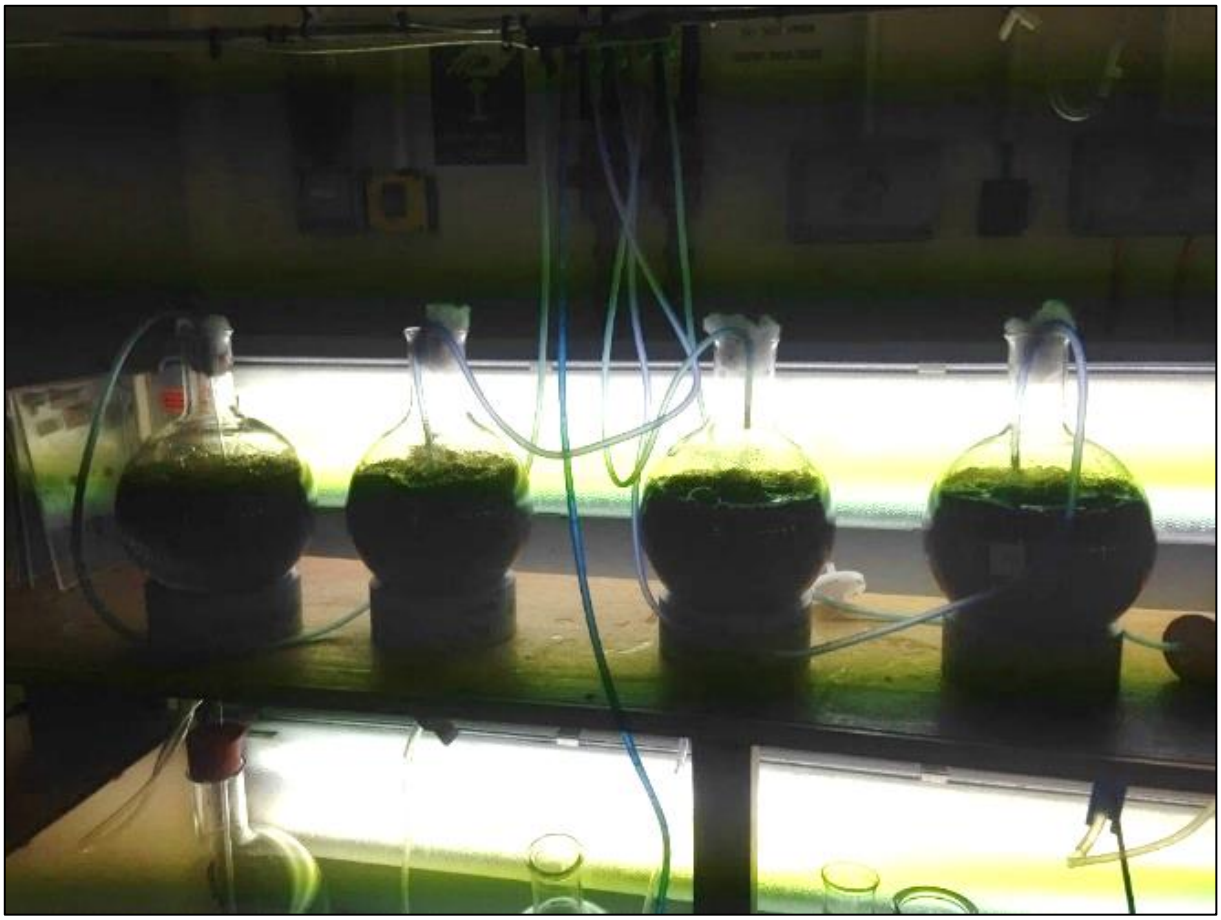


Figure 2.7: Larvae were fed with one day old *Artemia* sp. nauplii. *Artemia* sp. were hatched in spherical glass conical flasks containing seawater, reared at 24°C under illumination and under strong bubbling of air.

Figure 2.8: *Artemia* sp. nauplii after preparation for use in feeding of the experimental larval crustaceans.

3 Chapter Three: An Examination of the Responses of *Carcinus maenas* Larvae to Multiple Stressors

3.1 Introduction

As human populations increase globally, anthropogenic stressors in the aquatic environment increase in both their range and intensity (Crain, Kroeker et al. 2008). Natural marine ecosystems are impacted by anthropogenic activities in many, often simultaneous, ways in terms of factors such as over-exploitation, over-fishing and habitat loss (Jackson et al. 2001; Breitburg et al. 1998; Venter et al. 2006; Halpern et al. 2007a, 2008b). During the last 100 years, this has increased to include factors such as pollution (e.g. excessive nitrogen, heavy metals, pharmaceuticals, plastics), non-native alien invasive species and climatic-induced changes, to name but a few (Gattuso, Magnan et al. 2015, Boyd, Collins et al. 2018).

Natural disturbances in the marine environment may include changes in temperature (e.g. Cuculescu, Hyde et al. 1998, Pörtner 2009), salinity (e.g. Giménez and Anger 2001, Rivera Ingraham and Lignot 2017), oxygen (e.g Diaz and Rosenberg 2008), food availability (e.g. Giménez and Anger 2005, Pansch, Schaub et al. 2014), and CO_2 (e.g. Whiteley 2011, Sokolova, Matoo et al. 2016). The cumulative effects of these multiple environmental factors surpass the range of natural variation and usually elicit a stress response on organisms; referred to in the literature as “drivers” or “stressors” (e.g. Gunderson, Armstrong et al. 2016, Boyd, Collins et al. 2018). The actual effect of multiple stressors on marine organisms is complex and still not well understood (Gunderson, Armstrong et al. 2016, Boyd, Collins et al. 2018). There are many studies providing information on the individual impacts that many stressors have on both species and ecosystems. However, multiple stressor research considering cumulative and/or interactive effects is less common but gathering interest (Gunderson and Leal 2016). Multiple stressor studies are arguably more environmentally relevant than single stressor studies, as they encompass more factors, which may simultaneously occur in the natural environment (Todgham and Stillman 2013). Understanding

multiple stressors is therefore considered to be a core issue in marine science (Rudd 2014, Baumann 2019).

Recent studies have shown that marine invertebrates exposed to elevated pCO_2 and associated reductions in pH, known as 'ocean acidification' (OA), alone may give rise to different responses in conjunction with other stressors (e.g. Darling and Cote 2008, Przeslawski, Byrne et al. 2015). For example, a meta-analysis (Kroeker, Kordas et al. 2013) on the effects of OA and warming shows an increasing trend of sensitivity to OA when temperatures are elevated, but responses vary depending on the approach used, taxonomic group and the life-history stage (Swiney, Long et al. 2017). The main issue with the effect of OA and temperature, as in other cases, is that such stressors may interact instead of acting in an additive way. They may interact synergistically or antagonistically (Crain, Kroeker et al. 2008, Piggott, Townsend et al. 2015). Additive effects refer to situations where the combined effect of two stressors results from the sum of the effect of each stressor acting alone. Synergistic effects refer to situations where the combined effect is higher than the expected from the additive action, while antagonistic effects are defined as those effects that are less than the sum of the individual effects (Folt, Chen et al. 1999, Crain, Kroeker et al. 2008, Gunderson and Leal 2016, Swiney, Long et al. 2017). Both additive and antagonistic effects are reported in a meta-analysis of OA research by both Harvey et al. (2013) and Kroeker et al. (2013) as the most frequent responses, with some instances of synergistic impacts (Swiney, Long et al. 2017). In contrast, synergistic effects were reported to be the most frequent response by Przeslawski et al (2015).

Environmental variables such as temperature and pCO_2 can affect many physiological processes, with the magnitude depending upon taxa and life-history stage (Byrne and Przeslawski 2013). For example, juvenile and adult intertidal marine invertebrates may experience pH and temperature changes that naturally occur with daily tidal movements, the levels of which may exceed any ocean changes predicted by 2100 (Byrne and Przeslawski 2013, Kelly and Hofmann 2013). In contrast, planktonic stages may experience comparatively stable conditions in the subtidal water column. Juvenile and adult benthic invertebrates that reside in intertidal regions, therefore, may have enhanced resilience to stressors such as temperature and/or OA, due to their adaption or acclimation to the natural environmental variability (Melzner, Gutowska et al. 2009, Byrne and Przeslawski 2013). By contrast, planktonic larvae can be more sensitive to

environmental stressors than juveniles or adult counterparts (Byrne and Przeslawski 2013). Therefore, with expected pH/elevated pCO_2 changes associated with OA, larval stages may be a bottleneck, or weak-link (Kurihara 2008, Walther, Anger et al. 2010, Byrne and Przeslawski 2013).

Many previous studies (e.g. Dupont, Ortega-Martínez et al. 2010, Parker, Ross et al. 2012, Parker, O'Connor et al. 2017) have researched the effect of OA on marine larvae in isolation. This information provides important and valuable insights into the mechanisms underlying the ability to cope with OA. However, for the reasons explained above, effects of OA on the performance of organisms needs to be evaluated in the light of changes in additional environmental variables, more in keeping with the changes that are liklely to happen in the natural environment (Todgham and Stillman 2013). Most studies considering OA and an additional variable, focus on elevated temperature. Temperature is clearly a key factor because it is changing in surface waters on a global scale and because it impacts a diverse number of physiological and developmental processes (Calosi, Bilton et al. 2008, Pörtner 2009). There is, however, comparatively little information on the combined role of OA and salinity on marine organisms. Salinity is an important factor for coastal-estuarine species representing one of the major challenges to invertebrates living in estuarine and shallow coastal habitats (Rivera Ingraham and Lignot 2017). As an environmental variable, gradients in salinity lead to strong differences in estuarine benthic diversity (Anger 2003, Spitzner, Giménez et al. 2019). The osmoregulatory-capacity of an organism determines the tolerance of a species or population to changes in salinity (Anger 2003, Whiteley 2018). Species and/or life history stages which are stenohaline are usually pre-adapted to live in relatively stable marine environments and many of which are generally osmoconformers (i.e. where salinity variation is low e.g. subtidal/benthic habitats) (Charmantier and Charmantier-Daures 2001, Anger 2003, Pörtner 2008, Melzner, Gutowska et al. 2009). Species that occupy estuarine or brackish habitats are often euryhaline and hence are physiologically adapted to be able to adjust the osmolality of their body fluids to cope with fluctuations in external salinity i.e. osmoregulators (Charmantier and Charmantier-Daures 2001, Charmantier, Giménez et al. 2002, Anger 2003). The ion exchange mechanisms involved in osmoregulation are also involved in acid-base regulation, which is key to survival in OA (Whiteley 2011, Wittmann and Pörtner 2013). As osmoregulation is sensitive to

temperature, water temperature is also likely to affect survival under elevated pCO_2, as previously shown in larval crustaceans (e.g. Gravinese, Kronstadt et al. 2018).

3.2 Aims and Objectives

The main aim of this study was to evaluate the combined effect of OA and salinity on the survival and development of a marine crustacean, the shore crab *Carcinus maenas*. Adult *C. maenas* are generally tolerant of salinity fluctuations and are considered moderate osmoregulators, being able to hyper-regulate down to a salinity of 8 (Henry et al 2012). *C. maenas* develops through a number of osmoregulatory stages so that the megalopae, which inhabit estuaries and the intertidal, are weak osmoregulators and earlier stages are osmoconformers (Cieluch, Anger et al. 2004). Osmoregulatory stages are likely to be more tolerant to ocean acidification than osmo-conforming stages, because they have the physiological mechanisms to compensate for disruptions in acid-base and ion homeostasis (Whiteley 2011). Although investigations have considered the effects of OA on crustacean larvae, there is an important gap in our knowledge of the combined effect of OA and salinity for coastal species. This is surprising given that shallow coastal areas and estuaries are important nursery grounds, they could be areas where the effects of OA are more variable and fluctuate in combination with changes in salinity caused by freshwater input (Przeslawski, Ahyong et al. 2008). In addition, this study provides information on the intraspecific variation of the combined responses to OA and salinity, as studies are beginning to show intraspecific differences caused by maternal effects and genetic variation (e.g. Carter, Ceballos-Osuna et al. 2013, Uller, Nakagawa et al. 2013, Applebaum, Pan et al. 2014, Parker, O'Connor et al. 2017). Maternal effects, for instance, can influence offspring size or body mass and therefore, performance (Marshall, Bonduriansky et al. 2008). Because larvae of many planktonic species, including *C. maenas,* perform diurnal vertical migrations that restrict feeding for a few hours every day, realistic simulation of feeding regimes need to consider scenarios of limited access to prey i.e. food (Giménez and Anger 2005). Feeding is known to reduce the effects of elevated pCO_2 on juvenile mussels (Thomsen, Casties et al. 2013) and is also likely to affect responses to salinity because of the energetic costs associated with osmoregulation (Sokolova, Frederich et al. 2012, Rivera Ingraham and Lignot

2017). The focus of the current chapter is on the effect of factor combinations on development and survival of *C. maenas* larvae. The investigation involved two experiments carried out on berried crabs caught in the field and returned to the laboratory: one to include the effects of food limitation, as well as combinations of salinity and temperature: and the other to investigate the combined effects of salinity and elevated pCO_2 under restricted feed conditions. The latter involved restricted feed, as continuous supply of live *Artemia* sp. nauplii affected seawater pH levels. Throughout the studies, attention was given to variability in responses among broods in berried crabs caught in the field.

Chapter 3 had the following specific objectives:

- **Objective 1:** To determine the effects of temperature, naturally relevant salinity and limited access to food on the development and survival of *C. maenas* larvae. Applies to experiment A.

- **Objective 2:** To determine the effects of near-future (the year 2100) predicted pCO_2 levels and a physiologically relevant low salinity on the development and survival of the early life stages of *C. maenas* larvae. Applies to experiment B.

- **Objective 3:** To examine any variability in larval performance among broods to explore intraspecific variation in crabs caught in the field. This objective will be addressed in both experiments (A & B).

3.3 Materials and Methods

3.3.1 Collection of Ovigerous Females of *Carcinus maenas*

Ovigerous females of *Carcinus maenas* (Figure 3.1) were obtained sub-tidally by a local fisherman (Trevor Jones) dredging from mussel beds (*Mytilus edulis*) within the Menai Strait, North Wales, UK, during January-Early March 2014 for experiment A, and during late winter months (December-January) of 2014-2015 for experiment B. Females were kept in individually aerated aquaria of full strength seawater (volume =

2 litres) at 15˚C, throughout embryonic development, until larvae hatched (Figure 3.2). They were fed on one half of a mussel (*Mytilus edulis*) every other day, which was removed after a few hours to maintain water quality. Seawater was replaced daily to ensure removal of waste material.

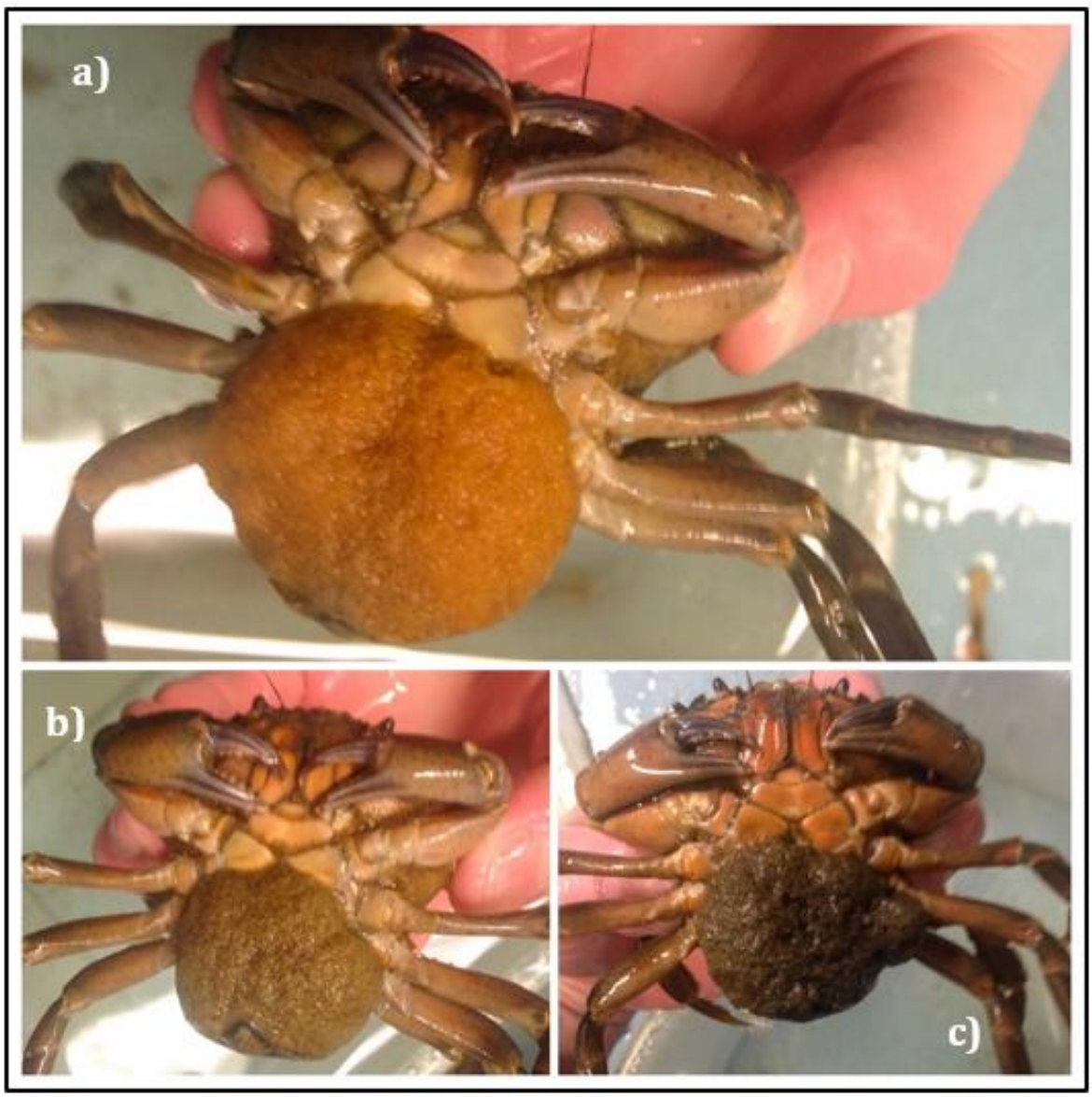


Figure 3.1: Ovigerous *Carcinus maenas* females, showing: a) early embryos in their stereotypical bright orange form (due to high yolk reserves), b) intermediate embryos with yolk reserves starting to deplete as larvae develop and consume more, c) late embryos with yolk reserves depleted and two big compound eyes making up much of the dark colouring.

3.3.2 **Handling and Rearing of Larvae**

Figure 3.2: *Carcinus maenas* larvae at Zoea I stage of development.

3.3.2.1 Experiment A: Effect of Temperature, Salinity and Food Regime on Larval Development

In this experiment, larvae (Figure 3.2) were assigned to a factorial combination of salinities (33 and 25), temperatures (15°C, 18°C, 21°C) and food regimes (food available for 6 hours or 24 hours). The experiment was repeated with larvae that hatched from 3 individual ovigerous females throughout Spring 2014. A total of 480 larvae per female (40 larvae per treatment combination × 3 × 2 x 2 = 480 larvae) were used to start each experimental, resulting in a total of 1440 larvae. In each case, freshly hatched larvae were collected from each brood and distributed among the 7 treatments. Larvae were reared in 3 fully programmable automatic incubators at one of 3 temperatures, and a 12:12 hour light: dark photoperiod.

Larvae were reared individually, in 50ml open containers. Water initially used was the same temperature as that used for embryonic development (15°C). Larvae were then placed into the respective incubator at 15°C, 18°C or 21°C, and allowed to gradually equilibrate temperature over six hours (Gonzalez-Ortegon and Giménez 2014). For subsequent daily seawater changes, seawater was pre-heated to 15°C, 18°C or 21°C using carboys of water at the correct treatment condition housed within the respective incubator. These temperatures were chosen because they are environmentally realistic in the study area and larval development has been shown previously to be successful at these temperatures (Nagaraj 1993). The salinity values are chosen to mimic conditions that are likely to be experienced by larvae that are released in coastal areas or near estuaries.

Larvae were fed daily with either 24-hour or 6-hour exposures to freshly hatched *Artemia* sp. nauplii (with an ad libitum concentration of around 10 nauplii ml-1, see Chapter 2). Individual culture dishes were left open, this helped to maintain stable pH levels until the daily water change took place (see Chapter 2). Seawater changes were performed daily across all treatments, whereby *Artemia* sp. was fully removed after 24 or 6 hours of food exposure by replacing the water in each container with the required treatment condition. The 6-hour feeding regime simulates what could be expected of temporal patterns of access to prey on a daily basis due to patchiness in plankton and due to the diel vertical migration of larvae (see: Sulkin, Blanco et al. 1998, Giménez and Anger 2005, see also in Chapter 2). The control group consisted of larvae fed ad libitum for 24 hours (i.e. permanent access to food). Treatment seawater was prepared a day in advance ready for the next day's experiments (as pH was not being studied, this was not an issue). Water was prepared at a salinity of 25 by mixing filtered seawater with vigorously aerated (in order to blow off the chlorine) fresh tap water. Seawater at full salinity (33) was directly obtained via a seawater filtration system (1 µm UV-filtered seawater). A multi-field handheld salinity meter was used to determine temperature and salinity levels (WTW Cond 315i; ± 0.1).

Response variables were the mean duration of development through to megalopa stage and percentage survival through to megalopa stage. Larvae were checked at daily intervals, for moults and mortalities. Newly hatched fresh *Artemia* sp. nauplii were added for a period of 6 hours and then subsequently removed when all the culture

water was replaced. Experiments continued until all the larvae had either reached the megalopa stage or died.

3.3.2.2 Experiment B: Effect of Elevated pCO_2 and Salinity on Larval Development

In this experiment, larvae were exposed to combinations of salinity (33 and 25), and pCO_2 (400 and ~1,000 µatm) at 15°C. The experiment used larvae that hatched from 3 individual ovigerous females throughout Spring 2015, consisting of 80 larvae per female (20 larvae per treatment combination × 2 × 2 = 80 larvae) and therefore resulting in a total of 240 larvae. Larvae were reared individually as in Experiment 1 but in closed containers in order to minimise variations in pH (see Chapter 2). General procedures of larval rearing were as described in the previous section. Larvae were reared individually, in 100ml open containers. Water initially used was the same temperature as that used for embryonic development (15°C). Larvae were then placed into the incubator at 15°C. For subsequent daily water changes (using water obtained from the Salinity/OA system, Chapter 2), water was pre-heated to 15°C using carboys of water at the correct treatment condition within the incubator. The temperature was chosen because it is environmentally realistic and larval development is successful at this temperature (Nagaraj 1993). The salinity values chosen mimic conditions experienced by larvae released in and along shallow coastal regions. The pCO_2 levels match the 'business as usual' CO_2 predictions for 2100 (IPCC 2014).

3.3.3 Data Analysis

The effect of environmental variables on survival was examined using a generalised linear modelling approach based on the binomial distribution and logit link function. Generalised linear model was used for survival (based on the binomial distribution) because each individual was reared in a separate container and thus each replicate unit can give only two values (1= alive, 0= dead). The inference was carried out based on model selection using Akaike information criteria (Zuur, Tuck et al. 2003, Zuur, Leno et al. 2009). Models were run in R (Team 2013). Model selection was as follows: when the AIC of two models differed by >3 the model with lower AIC was selected; if the difference was < 3 and the simpler model had the lowest AIC, that model was

selected; if by contrast, the more complex model had the lower AIC a log-likelihood test was used to check for significance. For Experiment A, the factors were temperature, salinity and access to prey; for Experiment B, the factors were salinity and pCO_2.

The effects of environmental variables on the duration of development were tested using analysis of variance (ANOVA). In Experiment A the factors considered were temperature, salinity, access to food (all fixed) and female of origin (= “female”; random). In Experiment B, the factors were salinity, pCO_2 (Fixed) and female (random). In the initial test, I considered all the above-named factors; if in the test, the factor “female” (or any interaction with it) was not significant, a new test with only the fixed factors in the model was used. Tests were carried out by stage in order to reduce the effects associated with variance heterogeneity and the potential individual effects. Where a significant three-way interaction was found (ZII to ZIV), a post-hoc test (Bonferroni) was carried out on triplicate variables (temperature), compared against food and salinity (Tables 3.5, 3.6, 3.7, 3.8); using MASS package in R Studio 3.4.4. In the absence of a significant three-way interaction (Megalopa), two-way interactions were investigated and a posthoc test was carried out on variables with significant interactions.

3.4 Results

3.4.1 Experiment A: Effect of Temperature, Salinity and Food Regime on Larval Development

3.4.1.1 Survival

Larval survival responded to the interaction between temperature, salinity and access to prey (Tables 3.1 - 3.4). At all stages studied (Zoea II to Megalopa), the highest survival rates were observed in larvae reared at 15°C in seawater (salinity 33) (Figure 3.3), and both low salinity and high temperature resulted in reductions in larval survival. There was a strong effect of low salinity on survival at 15°C, especially in larvae under limited access to prey and more evident in the survival to zoea II and III. However, such an effect was weaker at 18°C and disappeared in larvae reared at 21°C (Figure

3.3). Overall, high temperatures appeared to ameliorate the effect of low salinity on survival.

The effect of limited access to food on survival was restricted to the early zoeal stages and was not consistent across temperatures and salinities. For instance, at 15°C in seawater, restricted access to food resulted in consistently higher survival; by contrast, in seawater at 18°C and at a salinity of 25 and 21°C, survival was lower under limited access to food. The effect of food limitation on survival to the megalopa was restricted to larvae reared at 15°C in seawater. At other conditions, either low salinity or high temperature reduced survival in both larvae reared under limited or unlimited access to prey (Figure 3.3).

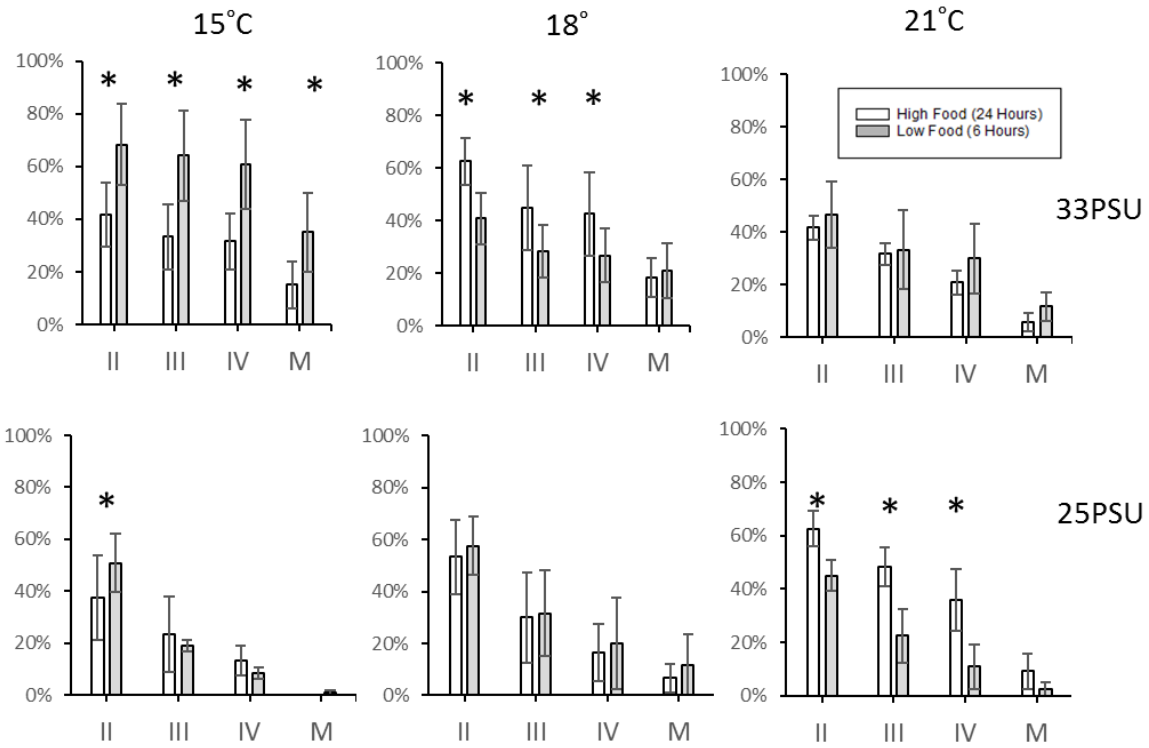


Figure 3.3: *Carcinus maenas*. Effects of temperature, salinity and food limitation on cumulative survival (from hatching through to megalopa). Error bars show SD among groups of larvae hatching from different females (n=3). Asterisks indicate significant differences between food levels after post-hoc tests (Appendix tables). Zoea II, III, IV represented by II, III, IV, and M represents megalopae.

3.4.1.2 Duration of Development

The effect of temperature, salinity and food limitation depended on developmental stage (Figure 3.4). Duration to zoea II was driven by salinity and temperature, but their effects depended on the food condition (Table 3.9. salinity*food and food*temperature), when female was not significant it was removed from the analyses (Table 3.9). Duration of development decreased at high temperature and increased at low salinity or after food limitation (Figure 3.4). These patterns were more evident in the duration of development to zoeal stages III and IV (Figure 3.4, Table 3.10 (ZIII) and Table 3.11 (ZIV): with significant three-way interactions), and also in the megalopa (Table 3.12). At these stages, food limitation had little effect on duration of development when larvae were reared at 15°C in seawater, while the strongest effect occurred at the same temperature but when larvae were reared at a salinity of 25. At 18°C and 21°C, duration of development was consistently longer under limited access to prey than under permanent access. Hence, high temperatures appeared to ameliorate the combined effect of food limitation and salinity on duration of development. By the megalopa, the duration of development was unaffected by any factor, but the statistical test was based on only a few individuals reaching the megalopal stage; the trends observed, however, were similar to those found in the zoea IV. (Figure. 3.5).

Duration of development to the zoea II varied significantly among berried females due to variations in the magnitude of the effect of low salinity or food limitation on development. For zoea III and IV, these differences were not significant (Tables 3.10, 3.11).

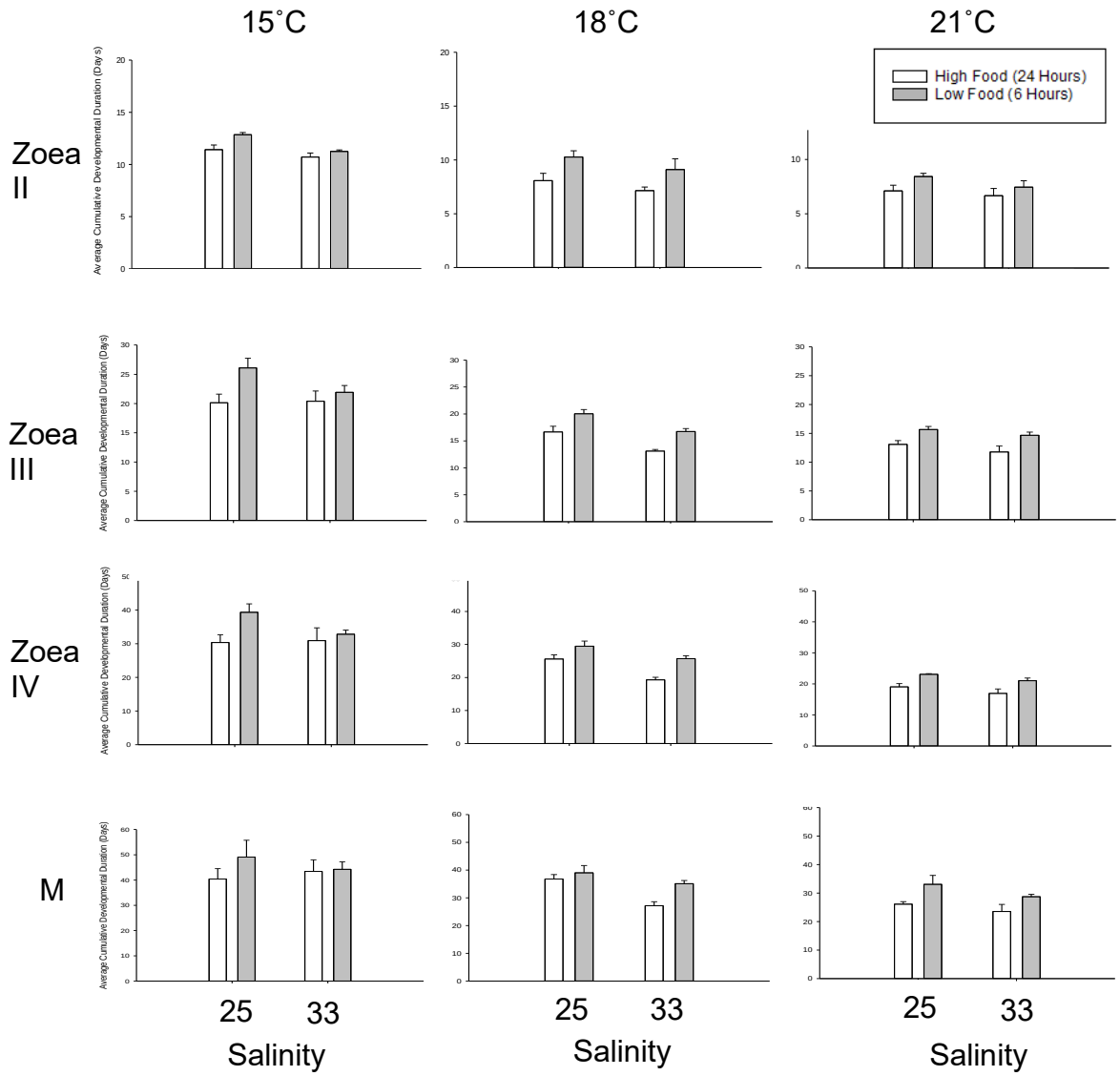


Figure 3.4: *Carcinus maenas*. Effects of temperature, salinity and food limitation on the cumulative duration of development (from hatching through to megalopa). Error bars show SD among groups of larvae hatching from different females (n=3). M represents megalopae.

3.4.2 Experiment B: Effects of Elevated CO_2 and Salinity

3.4.2.1 Survival

Effects of salinity and pCO_2 were tested through generalised linear models because larvae were reared individually. Survival to the zoea II was not affected by either salinity or pCO_2. For survival to zoea III and IV, the best model (based on the logistic link) included pH (Table 3.13). In those stages, survival was consistently lower in larvae reared under low pH (Figure 3.5).

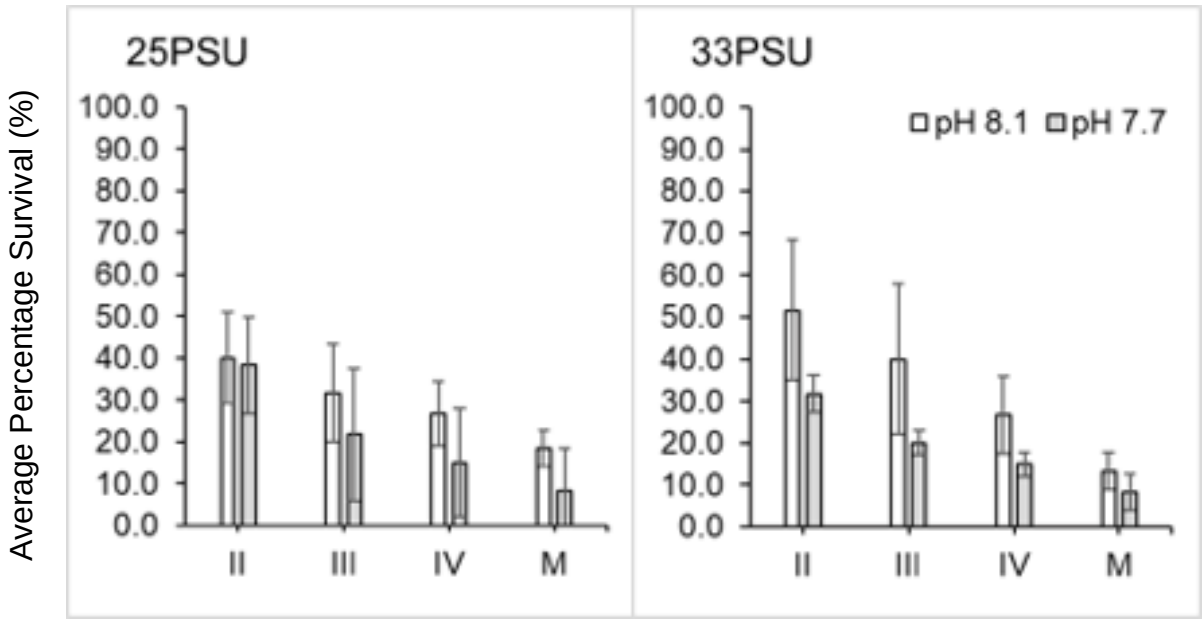


Figure 3.5: *Carcinus maenas*. Effect of pCO_2 and salinity on average percentage survival from hatching to the megalopa.

3.4.2.2 Developmental Duration

Duration of development was not significantly affected by salinity or elevated pCO_2 at any stage studied (Table 3.14 (zoea II), Table 3.15 and Table 3.16 (zoea III), Table 3.17 (zoea IV) and Table 3.18 (megalopa), irrespective of larval stage (Figure 3.6). Differences among treatments in the average duration of development were small amounting to ~ 1 day up to the zoea IV (range 27-28 days). At the megalopa stage, there was significant variation in development duration in larvae hatched from different

females, but neither salinity nor pCO_2 significantly affected the duration of development.

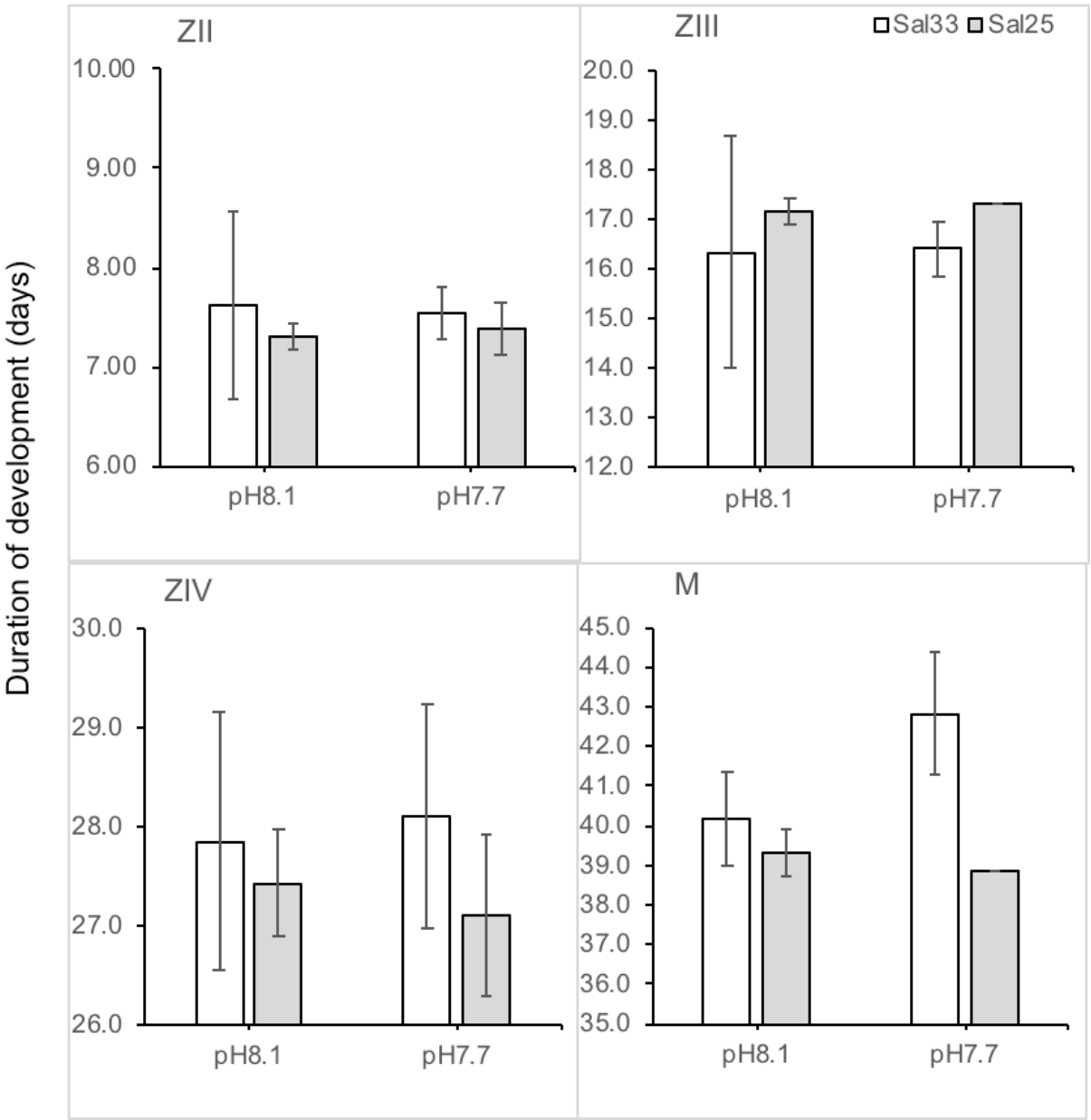


Figure 3.6: *Carcinus maenas*. Effect of pH and salinity on the duration of larval development from hatching to the megalopa.

3.5 Discussion

Two different responses were observed in terms of survival and duration of development, depending on whether *C. maenas* larvae were exposed to different salinities in combination with changes in temperature or with changes in pCO_2. Experiment A indicated interactive responses between salinity and temperature, whereby high temperature ameliorated the effect of low salinity on survival and developmental duration. The effect of limited access to food was inconsistent in terms of survival, as it varied with temperature and salinity, and there was some effect on the duration of development. Experiment B demonstrated that elevated pCO_2 had a consistent effect on survival of *C. maenas* larvae, but had no effect on duration of development. In contrast, salinity had no effect either alone or in combination on survival or developmental duration.

3.5.1 Experiment A: The Effect of Temperature and Salinity on Survival and Developmental Duration

Survival of larvae is dependent on both environmental conditions and also on energy reserves, to ensure growth and continued development. If the environmental conditions are unfavourable or sub-optimal, then larvae can be negatively impacted, especially if energy budgets decline. In Experiment A, the optimal temperature for survival was 15°C. This corresponds with optimal temperatures recorded for *C. maenas* and other temperate marine decapod crustaceans. At this optimal temperature for larval survival, both salinity and food access had an effect. First, survival rates were reduced in low salinity probably because *C. maenas* larvae are stenohaline in the early stages of development and unable to cope with salinity fluctuations. Second, reduced access to food actually increased survival rates in larvae reared in seawater. Such an effect is difficult to explain as continuous access to food would appear to be the most favourable condition. However, it is possible that there were sufficient energy reserves in larvae, despite exposure to limited food. The capture of live food is likely to be energetically costly, and there will be a balance between the

energy gained from prey capture and that used to catch and digest the prey. In the time scale used in Experiment A, it is possible that capture of prey for 24 h has a negative effect on survival. Such a response has not been previously observed even in *C. maenas* larvae in the first zoeal stage and warrants further study (Giménez and Anger 2005).

Larvae reached the megalopa stage in all treatments, although numbers were greatly reduced by the final stage, indicating a wide tolerance level of *C. maenas* larvae to the temperatures and salinities examined. This is expected as the species occurs ubiquitously around global oceans both in its native range and as an invasive species, the only area where it is not found is the Antarctic region (Leignel, Stillman et al. 2014). The temperatures used here were chosen because of their similarity to the conditions normally experienced by *C. maenas* in the intertidal zone. The two salinities represent full strength seawater and a physiological relevant salinity, which was chosen as it presents the salinity at which adult *C. maena*s start to osmoregulate (Whiteley et al. 2018). In the case of the larvae, early stages are stenohaline and therefore it is not surprising that survival rates were reduced in low salinity and were also dependent on developmental stage. An earlier study on temperature/salinity interactions by Nagaraj (1993), also showed that the treatment with the highest survival levels of zoea was at the lowest temperature (10ºC) and highest salinities (30 and 35) (i.e. emulating favourable, natural conditions) (Nagaraj 1993). However, a recent study by Spitzner et al. (2019) demonstrated that survival of early larval stages up to zoea IV at low salinity was enhanced by exposure to higher temperatures, as observed in other marine invertebrates (Gonzalez-Ortegon and Giménez 2014). The same authors observed variations in responses from marked effects of salinity to no effects amongst broods. Spitzner et al. (2019) attributed such variation in salinity-sensitivity to maternal effects, as well as genetic variability.

A similar response was observed in Experiment A, as the strong effect of low salinity on larval survival at lower temperatures weakened at higher temperatures (i.e. high temperatures seemed to ameliorate the effect of low salinity on survival). Temperature increases the rate of biological processes and is well known to increase metabolic rates, growth and rates of development of larvae up to a critical temperature (e.g. (Jackson, Torres et al. 2014, Castejon, Rotllant et al. 2015, Castejón, Rotllant et al. 2018). Both feeding rate and activity will also increase with an elevation in temperature.

Moreover, the interaction between temperature and salinity suggests that temperature also influences the ability to tolerate low salinities. The effect of temperature on the megalopae is easier to explain, as increasing temperatures are likely to increase Na^+/K^+ ATPase activities involved in osmoregulation. Increased environmental temperature can also shift the iso-osmotic point protecting against the damaging effects of reduced salinity on body fluid osmolality and cell volume regulation, especially in those stages unable to osmoregulate. Such changes in the iso-osmotic point correspond with seasonal shifts of the brown shrimp, *Crangon crangon*, into estuarine environments enabling survival in brackish water because of increased summer temperatures (Weber and Spaargaren 1970). A similar response could occur in *C. maenas* larvae, although the underlying mechanisms are unknown.

The effect of temperature, salinity and food depended on developmental duration and developmental stage. At early stages, developmental duration decreased at high temperatures, but increased at low salinity or after food limitation. At 15°C, limited food had no effect when the larvae were reared in seawater, but was strongly affected when larvae was reared at 15°C in dilute seawater. Therefore, an increase in duration in larvae reared at 15°C in dilute seawater suggests that developmental processes are delayed, probably due to disruption by the lowered salinity, or a trade- off between development and the necessity to osmoregulate. Low salinity exposure can be energetically costly, not only in terms of the mechanisms responsible for osmoregulation, but also in terms of any resulting damage and subsequent repair mechanisms (Whiteley 2018). Previous studies have suggested that duration of development could be affected by offspring size or biomass, with higher biomass, and hence energy reserves leading to shorter durations of development (Giménez and Anger 2003, Gonzalez-Ortegon and Giménez 2014). However, there was no evidence of such a relationship in the present study or in the study conducted by Spitzner et al. (2019). The effect of food limitation reflects the increasing costs of coping with reduced salinities and also corresponds to the decrease in survival rates observed in low salinity larvae at 15°C. Moreover, developmental duration was longer under limited food conditions at 18°C and 21°C suggesting that limited energy availability was influencing the time taken to develop. This is not surprising given the expected increase in energy consumption with increase in temperature. Breteler (1975) studied the effects of salinity, temperature and limited access to prey on the developmental duration of early

juveniles of *C. maenas*. It was found that effects were more pronounced at the 7th stage of development (Breteler 1975). Moreover, experiments on the effects of food limitation in the larvae of both the European lobster (*Homarus gammarus*) and the velvet swimming crab (*Necora puber*) showed a reduction in duration of development from zoea III onwards (Jackson, Torres et al. 2014). Larval survival was high, but the larvae took longer to develop and reached smaller sizes when compared to larvae with access to food. Giménez & Anger (2005) report a general trend of prolonged developmental duration in treatments with limited access to prey in *C. maenas* (Giménez and Anger 2005). *Artemia* sp. quality has been reported to affect survival in late stage brachyuran larvae (Sulkin and McKeen 1999). Collectively, these experiments suggest that food limitation and low prey quality could give rise to fitness costs and lower recruitment of juvenile populations (Giménez, Anger et al. 2004, Jackson, Torres et al. 2014).

Duration of development to the zoea II varied significantly among berried females, due to variations in the magnitude of the effect of low salinity or food limitation on development. Such variation may be due to differences in energy reserves brought about by maternal effects, with differences in the temperatures and salinities experienced by the females being an important issue (e.g. Spitzner et al. 2019). Changes in environmental conditions are known to influence larval performance in decapod crustaceans either by affecting the females during reproduction or whilst the embryos are incubated in the egg mass (Giménez and Anger 2003, Gonzalez-Ortegon and Giménez 2014). A decrease in performance is thought to be associated with a sub-optimal maternal environment (Spitzner, Giménez et al. 2019).

3.5.2 Experiment B: The Effect of pCO_2 and Salinity on Survival and Developmental Duration

At the pCO_2 and salinity levels used here, only pCO_2 had an effect on the survival of *C. maenas* larvae. Elevated pCO_2 has been shown to decrease larval survival in some crustacean species, but not in all (Walther, Anger et al. 2010, Gravinese, Kronstadt et al. 2018, Whiteley 2018). The negative effect of elevated pCO_2 levels on larval survival rates can be explained by a lack of ability to physiologically compensate for the

changing environmental conditions (Kelly and Hofmann 2013, Wittmann and Portner 2013). Adult *C. maenas* have well developed gills that are specialised for gas exchange and ion regulation, but become more specialised during ontogeny. In early stages of development, larvae do not have the capacity to regulate either ion or acid-base regulation. They are likely to be more vulnerable to external increases in pCO_2 because they do not have the mechanisms to buffer against the increasing pCO_2 levels. Increasing pCO_2 is key to survival as it can disrupt key physiological functions such as body fluid pH regulation and nerve function (Wittmann and Portner 2013, Moya, Howes et al. 2016). However, exposure to an external pCO_2 of ~1000 µatm (0.1 kPa) still suggests that a diffusion gradient existed across the body surface reducing the likelihood of internal acidification, and hence survival.

An examination of the combined effects of pCO_2 (pH 7.6, 7.3, 7.0) with salinity (35, 25 and 15) on the hatch-rate of the brine shrimp *Artemia franciscana*, reported that elevated pCO_2 led to a reduction of hatch success, irrespective of salinity (Salma, Uddowla et al. 2012). A similar response was observed in the present study with *C. maenas* larvae as salinity had no effect. Salma et al. (2012) argue such an observation supports the hypothesis that elevated pCO_2 elicits negative impacts on hatch success and that salinity has no synergistic relationship with pCO_2 in this species (Salma, Uddowla et al. 2012). The mechanisms linking ion and acid-base regulation are poorly developed in larvae (Cieluch, Anger et al. 2004) and therefore the lack of effect of salinity is not surprising, although salinity has been shown to influence both survival and duration of development in *C. maenas* larvae at low temperatures (see Discussion for Experiment A). Moreover, the number of studies on the combined effects of elevated pCO_2 and reduced salinity on crustacean larvae are limited, and it is possible that interspecific variability exists as observed with effects of elevated pCO_2 on its own (e.g. Wittmann and Pörtner 2013).

At the megalopa stage, there was significant variation in development duration in larvae hatched from different females, but neither salinity nor elevated pCO_2 significantly affected the duration of development. Differences among broods have previously been reported for the intertidal porcelain crab, *Petrolisthes cinctipes*, in terms of metabolic responses on exposure to elevated pCO_2 levels (Carter, Ceballos-Osuna et al. 2013). The authors attribute such variability in parental effects, as well as genetic differences amongst broods.

3.5.3 Conclusion

C. maenas is a highly adaptable species ensuring its successful globalised distribution. So, while *C. maenas* larvae from some individuals or populations may be detrimentally impacted by global changes in temperatures (global warming), salinity (increased rainfall/freshwater run off), elevated pCO_2 (OA) (IPCC 2014) and food distributions (match-mismatch hypothesis) (Cushing 1990), other cohorts may flourish. It is unknown if this would cause an evolutionary divergence to even more adaptable *C. maenas* in the future if the more resilient populations survive and thrive. This could have knock-on effects for species that are predated upon by *C. maenas* and therefore further disrupting ecosystem stability alongside these environmental stressors (Leignel, Stillman et al. 2014, Spitzner, Giménez et al. 2019). More studies are required to further explore the interacting effects of multistressors, plus the availability of food on larval survival and duration of development for a range of crustacean species. Moreover, the present intraspecific variation observed in both experiments deserves more attention to more fully understand the variability in response of animals in the field (Parker, O'Connor et al. 2017). These studies therefore highlight the importance of studying multiple females and broods, as incorrect conclusions could be drawn from studies on larvae restricted to the same individual.

3.6 Appendices (Chapter Three)

3.6.1 Experiment A: Effect of Temperature, Salinity and Food Regime on Larval Development

3.6.1.1 Survival

Table 3.1: *Carcinus maenas*. Three-way ANOVA to evaluate the effects of temperature, access to food and salinity on the survival to the second zoea stage (Zoea II). Significant effects are given in **bold**.

Zoea II	Estimate	Std.	z	Pr(>|z|)
(Intercept)	0.03	0.18	0.18	0.8551
Food = 24	**-0.54**	**0.26**	**-2.07**	**0.0382**
Salinity = 33	**0.74**	**0.27**	**2.75**	**0.0061**
Temperature = 18	0.27	0.26	1.04	0.3004
Temperature =21	-0.23	0.26	-0.90	0.3660
Food =24 & Salinity =33	-0.56	0.38	-1.49	0.1358
ffood24:ftemp18	0.38	0.37	1.02	0.3095
ffood24:ftemp21	**1.26**	**0.37**	**3.38**	**0.0007**
fsal33:ftemp18	**-1.41**	**0.37**	**-3.76**	**0.0002**
fsal33:ftemp21	-0.67	0.37	-1.79	0.0729
ffood24:fsal33:ftemp18	**1.61**	**0.53**	**3.05**	**0.0023**
ffood24:fsal33:ftemp21	-0.35	0.53	-0.67	0.5037

Table 3.2: *Carcinus maenas*. Three-way ANOVA to evaluate the effects of temperature, access to food and salinity on the survival to the third zoea stage (Zoea III). Significant effects are given in **bold**.

III	Estimate	Std.	z	value	Pr(>|z|)
(Intercept)	**-1.44**	**0.23**	**-6.21**	**<0.0001**	*******
ffood24	0.25	0.32	0.79	0.4307	
fsal33	**2.02**	**0.30**	**6.74**	**<0.0001**	*******
ftemp18	**0.67**	**0.30**	**2.21**	**0.0274**	*****
ftemp21	**0.20**	0.32	0.64	0.5253	

ffood24:fsal33	**-1.53**	**0.42**	**-3.66**	**0.0003**	***
ffood24:ftemp18	-0.33	0.42	-0.78	0.4379	
ffood24:ftemp21	**0.92**	**0.43**	**2.16**	**0.0307**	*
fsal33:ftemp18	**-2.18**	**0.41**	**-5.30**	**<0.0001**	***
fsal33:ftemp21	**-1.48**	**0.42**	**-3.53**	**0.0004**	***
ffood24:fsal33:ftemp18	**2.33**	**0.57**	**4.08**	**<0.0001**	***
ffood24:fsal33:ftemp21	0.28	0.58	0.49	0.6275	

Table 3.3: *Carcinus maenas*. Three-way ANOVA to evaluate the effects of temperature, access to food and salinity on the survival to the fourth zoea stage (Zoea IV). Significant effects are given in **bold**.

IV	Estimate	Std.	z	value	Pr(>|z|)
(Intercept)	**-2.40**	**0.33**	**-7.26**	**<0.0001**	***
ffood24	0.53	0.43	1.24	0.2165	
fsal33	**2.84**	**0.38**	**7.48**	**<0.0001**	***
ftemp18	**1.01**	**0.40**	**2.52**	**0.0117**	*
ftemp21	0.29	0.44	0.66	0.5117	
ffood24:fsal33	**-1.74**	**0.50**	**-3.44**	**0.0006**	***
ffood24:ftemp18	-0.75	0.54	-1.38	0.1665	
ffood24:ftemp21	1.00	0.55	1.81	0.0698	.
fsal33:ftemp18	**-2.46**	**0.49**	**-5.04**	**<0.0001**	***
fsal33:ftemp21	**-1.58**	**0.52**	**-3.04**	**0.0024**	**
ffood24:fsal33:ftemp18	**2.67**	**0.67**	**4.01**	**0.0001**	***
ffood24:fsal33:ftemp21	-0.28	0.68	-0.41	0.6849	

Table 3.4: *Carcinus maenas*. Three-way ANOVA to evaluate the effects of temperature, access to food and salinity on the survival to the megalopa stage. Significant effects are given in **bold**.

M	Estimate	Std.	z	value
(Intercept)	**-4.78**	**1.00**	**-4.76**	**<0.0001**
ffood24	-14.64	910.00	-0.02	0.9872
fsal33	**4.16**	**1.02**	**4.07**	**<0.0001**
ftemp18	**2.76**	**1.04**	**2.64**	**0.0083**
ftemp21	1.12	1.16	0.96	0.3370
ffood24:fsal33	13.52	910.00	0.02	0.9881
ffood24:ftemp18	14.02	910.00	0.02	0.9877

ffood24:ftemp21	16.01	910.00	0.02	0.9860
fsal33:ftemp18	**-3.47**	**1.09**	**-3.20**	**0.0014**
fsal33:ftemp21	**-2.52**	**1.21**	**-2.08**	**0.0375**
ffood24:fsal33:ftemp18	-13.06	910.00	-0.01	0.9885
ffood24:fsal33:ftemp21	-15.65	910.00	-0.02	0.9863

Table 3.5: Post hoc test for the second stage of *C. maenas* using Bonferroni correction, focusing on the effect of access to prey discriminated by a combination of salinities and temperatures. Significant effects are given in **bold**.

ZII Post-hoc	Food	Estimate	Std. Error	z value	Pr(>|z|)
At 15C and 33ppm	6h	0.77	0.20	3.92	**0.0001**
	24 h	-1.11	0.27	-4.10	**<0.0001**
At: 18C and 33ppm	6h	-0.37	0.19	-2.00	**0.0458**
	24 h	0.88	0.26	3.33	**0.0009**
At: 21C and 33ppm	6h	-0.13	0.18	-0.73	0.4660
	24 h	-0.20	0.26	-0.78	0.4360
At 15C and 25ppm	6h	0.03	0.18	0.18	0.8551
	24 h	-0.54	0.26	-2.07	**0.0382**
At 18C and 25ppm	6h	0.30	0.18	1.64	0.1020
	24 h	-0.17	0.26	-0.65	0.5160
At: 21C and 25ppm	6h	-0.20	0.18	-1.09	0.2741
	24 h	0.71	0.26	2.70	**0.0069**

Table 3.6: Post hoc test for the third stage of *C. maenas* using Bonferroni correction, focusing on the effect of access to prey discriminated by a combination of salinities and temperatures. Significant effects are given in **bold**.

ZIII Post-hoc		Estimate	Std. Error	z value	Pr(>|z|)
Comparison 1: 15C and 33ppm	6h	0.58	0.19	3.06	**0.0022**
	24 h	-1.28	0.27	-4.70	**<0.0001**
Comparison 2: 18C and 33ppm	6h	-0.93	0.20	-4.58	**<0.0001**
	24 h	0.73	0.27	2.66	**0.0078**
Comparison 3: 21C and 33ppm	6h	-0.69	0.19	-3.58	**0.0003**
	24 h	-0.08	0.28	-0.28	0.7828
Comparison 4: 15C and 25ppm	6h	-1.44	0.23	-6.21	**<0.0001**
	24 h	0.25	0.32	0.79	0.4310
Comparison 5: 18C and 25ppm	6h	-0.77	0.20	-3.92	**0.0001**

	24 h	-0.08	0.28	-0.28	0.7800
Comparison 6: 21C and 25ppm	6h	-1.24	0.22	-5.66	**<0.0001**
	24 h	1.17	0.28	4.11	**<0.0001**

Table 3.7: Post hoc test for the fourth stage of *C. maenas* using Bonferroni correction, focusing on the effect of access to prey discriminated by a combination of salinities and temperatures. Significant effects are given in **bold**.

ZIV Post-hoc		Estimate	Std. Error	z value	Pr(>|z|)
Comparison 1: 15C and 33ppm	6h	0.44	0.19	2.35	**0.0186**
	24 h	-1.21	0.27	-4.46	**<0.0001**
Comparison 2: 18C and 33ppm	6h	-1.01	0.21	-4.90	**<0.0001**
	24 h	0.71	0.28	2.56	**0.0104**
Comparison 3: 21C and 33ppm	6h	-0.85	0.20	-4.25	**<0.0001**
	24 h	-0.49	0.30	-1.62	0.1040
Comparison 4: 15C and 25ppm	6h	-2.40	0.33	-7.26	**<0.0001**
	24 h	0.53	0.43	1.24	0.2170
Comparison 5: 18C and 25ppm	6h	-1.39	0.23	-6.07	**<0.0001**
	24 h	-0.22	0.33	-0.67	0.5050
Comparison 6: 21C and 25ppm	6h	-2.11	0.29	-7.18	**<0.0001**
	24 h	1.53	0.35	4.36	**<0.0001**

Table 3.8: Post hoc test for the Megalopa stage of *C. maenas* using Bonferroni correction, focusing on the effect of access to prey discriminated by a combination of salinities and temperatures. Significant effects are given in **bold**.

M Post-hoc		Estimate	Std. Error	z value	Pr(>|z|)
Comparison 1: 15C	(Intercept)	-5.48	1.00	-5.47	**<0.0001**
	fsal33	4.38	1.01	4.32	**<0.0001**
Comparison 2: 18C	(Intercept)	-2.29	0.22	-10.25	**<0.0001**
	fsal33	0.88	0.28	3.19	0.0015
Comparison 3: 21C	(Intercept)	-2.78	0.28	-10.10	**<0.0001**
	fsal33	0.44	0.36	1.22	0.2220

3.6.1.2 Duration of Development

Table 3.9: *Carcinus maenas*. Duration of development to the second zoea stage (Zoea II). Abbreviations: SS = Type I Sum of Squares, df = Degrees of Freedom, MS = Mean Squares. Significant effects are given in **bold**.

Source	SS	df	MS	F	p
Food	**611.78**	**1**	**612**	**185.30**	**<0.0001**
Salinity	**115.12**	**1**	**115**	**34.87**	**<0.0001**
Temperature	**2132.07**	**2**	**1066**	**322.89**	**<0.0001**
FxS	**13.62**	**1**	**14**	**4.13**	**0.0430**
FxT	**34.47**	**2**	**17**	**5.22**	**0.0060**
SxT	9.75	2	5	1.48	0.2290
FxSxT	7.50	2	4	1.14	0.3220
Error	2370.54	718	3		
Total	66788.00	730			

a R Squared = .552 (Adjusted R Squared = .545)

Table 3.10: *Carcinus maenas*. Duration of development to the third zoea stage (Zoea III). Abbreviations: SS = Type I Sum of Squares, df = Degrees of Freedom, MS = Mean Squares. Significant effects are given in bold.

Source	SS	df	MS	F	p
Food	**2176.94**	**1**	**2177**	**263.24**	**<0.0001**
Salinity	**204.85**	**1**	**205**	**24.77**	**<0.0001**
Temperature	**5356.00**	**2**	**2678**	**323.83**	**<0.0001**
FxS	**55.21**	**1**	**55**	**6.68**	**0.0100**
FxT	4.59	2	2	0.28	0.7580
SxT	**66.07**	**2**	**33**	**4.00**	**0.0190**
FxSxT	**121.47**	**2**	**61**	**7.34**	**0.0010**
Error	3986.06	482	8		
Total	160541.00	494			

a R Squared = .667 (Adjusted R Squared = .659)

Table 3.11: *Carcinus maenas*. Duration of development to the fourth zoea stage (Zoea IV). Abbreviations: SS = Type I Sum of Squares, df = Degrees of Freedom, MS = Mean Squares. Significant effects are given in **bold**.

Source	SS	df	MS	F	p
Food	**3042.47**	**1**	**3042.47**	**176.66**	**<0.0001**
Salinity	**136.87**	**1**	**136.87**	**7.95**	**0.0050**
Temperature	**10041.93**	**2**	**5020.97**	**291.54**	**<0.0001**
FxS	47.48	1	47.48	2.76	0.0980
FxT	**106.23**	**2**	**53.11**	**3.08**	**0.0470**
SxT	**111.35**	**2**	**55.67**	**3.23**	**0.0410**
FxSxT	**264.77**	**2**	**132.38**	**7.69**	**0.0010**
Error	6372.28	370	17.22		
Total	273439.00	382			

a R Squared = .683 (Adjusted R Squared = .674)

Table 3.12: *Carcinus maenas*. Duration of development to the megalopa stage. Abbreviations: SS = Type I Sum of Squares, df = Degrees of Freedom, MS = Mean Squares. Significant effects are given in **bold**.

Source	SS	df	MS	F	p
Food	**2016.74**	**1**	**2016.74**	**97.05**	**<0.0001**
Salinity	**79.85**	**1**	**79.85**	**3.84**	**0.0520**
Temperature	**7417.17**	**2**	**3708.59**	**178.47**	**<0.0001**
FxS	0.47	1	0.47	0.02	0.8810
FxT	39.83	2	19.92	0.96	0.3860
SxT	25.84	2	12.92	0.62	0.5380
FxSxT	0.08	1	0.08	0.00	0.9500
Error	3200.17	154	20.78		
Total	231617.00	165			

a R Squared = .750 (Adjusted R Squared = .733)

3.6.2 Experiment B: Effects of Elevated CO_2 and Salinity

3.6.2.1 Survival

Table 3.13: *Carcinus maenas*. Summary of the generalised linear model used to test effects of salinity and pCO_2 on survival from hatching to megalopa. Significant effects are given in **bold**.

Stage		**AIC by stage**		
Model	**II**	**III**	**IV**	**Megalopa**
Factorial	75.8	88.2	66.1	55.5
Additive	75.9	86.8	64.1	53.7
Parameter estimates				
Zoea II	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
Intercept	-0.67	0.23	-2.87	0.0041
Salinity =33	0.11	0.26	0.40	0.6914
pH = 8.1	0.45	0.27	1.71	0.0879
Zoea III				
Intercept	-1.42	0.27	-5.23	<0.0001
Salinity =33	0.17	0.29	0.58	0.5614
pH = 8.1	0.75	0.29	2.56	**0.0106**
Zoea IV				
Intercept	-1.74	0.30	-5.75	<0.0001
Salinity =33	0.00	0.32	0.00	>0.9999
pH = 8.1	0.72	0.33	2.20	**0.0278**
Megalopa				
Intercept	-2.28	0.38	-6.07	<0.0001
Salinity =33	-0.24	0.40	-0.60	0.5500
pH = 8.1	0.73	0.41	1.76	0.0790

3.6.2.2 Developmental Duration

Table 3.14: Zoea II *Carcinus maenas*. Two-way ANOVA to evaluate the effects of pH and salinity on the developmental duration to the second zoea stage. Abbreviations: SS = Type I Sum of Squares, df = Degrees of Freedom, MS = Mean Squares. Significant effects are given in **bold**. Female not significant.

	SS	Degr. of	MS	F	p
Intercept	**5325.26**	**1**	**5325.26**	**11810.89**	**<0.0001**
Salinity	0.18	1	0.18	0.39	0.5342
pH	0.08	1	0.08	0.18	0.6682
Salinity*pH	0.00	1	0.00	0.00	1.0000
Error	41.48	92	0.45		

Table 3.15: Zoea III *Carcinus maenas*. Two-way ANOVA to evaluate the effects of pH and salinity on the developmental duration to the third zoea stage. Abbreviations: SS = Type I Sum of Squares, df = Degrees of Freedom, MS = Mean Squares. Significant effects are given in **bold**.

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	**Fixed**	**14503.04**	**1**	**14503.04**	**0.98**	**29.61**	**489.75**	**0.0304**
Salinity	Fixed	0.04	1	0.04	0.65	0.76	0.05	0.8703
pH	Fixed	1.05	1	1.05	1.09	2.10	0.50	0.5993
Female	Random	27.33	1	27.33	0.00			
Salinity*pH	Fixed	2.34	1	2.34	0.77	1.53	1.53	0.4744
Female*Salinity	Random	0.86	1	0.86	0.97	1.60	0.54	0.6008
Female*pH	Random	0.39	1	0.39	0.76	1.58	0.25	0.7246
Female*Salinity*pH	Random	1.61	1	1.61	41.00	1.81	0.89	0.3516
Error		74.33	41	1.81				

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	**Fixed**	**14503.04**	**1**	**14503.04**	**0.99**	**29.74**	**487.62**	**0.0298**
Salinity	Fixed	0.04	1	0.04	44.07	1.76	0.02	0.8810
pH	Fixed	1.05	1	1.05	3.29	3.59	0.29	0.6224
Female	Random	27.33	1	27.33	44.00	1.75	15.58	0.0003
Salinity*pH	Fixed	2.34	1	2.34	44.00	1.75	1.34	0.2540
Error		77.19	44	1.75				

Table 3.16: Zoea III *Carcinus maenas*. Two-way ANOVA to evaluate the effects of pH and salinity on the developmental duration to the third zoea stage. Abbreviations: SS = Type I Sum of Squares, df = Degrees of Freedom, MS = Mean Squares. Significant effects are given in **bold**. No data for female 3, salinity 25: removed from analyses.

	SS	Degr. of	MS	F	p
Intercept	**7367.26**	**1**	**7367.26**	**4502.58**	**<0.0001**
Salinity	0.52	1	0.52	0.32	0.5773
pH	0.58	1	0.58	0.36	0.5565
Salinity*pH	0.00	1	0.00	0.00	0.9754
Error	37.63	23	1.64		

	SS	Degr. of	MS	F	p
Intercept	**7164.05**	**1**	**7164.05**	**3513.70**	**<0.0001**
Salinity	1.10	1	1.10	0.54	0.4726
pH	0.05	1	0.05	0.03	0.8724
Salinity*pH	3.10	1	3.10	1.52	0.2332
Error	36.70	18	2.04		

Table 3.17: Zoea IV *Carcinus maenas*. Two-way ANOVA to evaluate the effects of pH and salinity on the developmental duration to the fourth zoea stage. Abbreviations: SS = Type I Sum of Squares, df = Degrees of Freedom, MS = Mean Squares. Significant effects are given in **bold**.

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	14503.04	1	14503.04	0.99	29.74	487.62	0.0298
Salinity	Fixed	0.04	1	0.04	44.07	1.76	0.02	0.8810
pH	Fixed	1.05	1	1.05	3.29	3.59	0.29	0.6224
Female	Random	27.33	1	27.33	44.00	1.75	15.58	0.0003
Salinity*pH	Fixed	2.34	1	2.34	44.00	1.75	1.34	0.2540
Error		77.19	44	1.75				

Zoea IV females 1&2

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	30085.23	1	30085.23	0.87	20.54	1464.38	0.0261
Salinity	Fixed	7.94	1	7.94	0.00			
pH	Fixed	5.58	1	5.58	0.94	14.04	0.40	0.6476
Female	Random	19.88	1	19.88	0.79	12.74	1.56	0.4686
Salinity*pH	Fixed	8.14	1	8.14	0.12	0.63	12.82	0.6996
Female*Salinity	Random	0.75	1	0.75	0.59	1.37	0.55	0.6526
Female*pH	Random	13.57	1	13.57	0.22	0.89	15.32	0.5436
Female*Salinity*pH	Random	1.72	1	1.72	32.00	11.51	0.15	0.7015
Error		368.19	32	11.51				

Females not significant

	SS	Degr. of	MS	F	p
Intercept	40218.87	1	40218.87	4106.31	<0.0001
Salinity	10.33	1	10.33	1.05	0.3095
pH	0.57	1	0.57	0.06	0.8103
Salinity*pH	0.31	1	0.31	0.03	0.8601
Error	479.93	49	9.79		

	SS	Degr. of	MS	F	p
Intercept	13404.80	1	13404.80	904.68	<0.0001
Salinity	2.07	1	2.07	0.14	0.7127
pH	16.05	1	16.05	1.08	0.3111
Salinity*pH	8.06	1	8.06	0.54	0.4698
Error	281.53	19	14.82		

	SS	Degr. of	MS	F	p
Intercept	13200.82	1	13200.82	1980.12	<0.0001
Salinity	7.60	1	7.60	1.14	0.3051
pH	2.39	1	2.39	0.36	0.5596
Salinity*pH	0.76	1	0.76	0.12	0.7404
Error	86.67	13	6.67		

Table 3.18: Megalopa *Carcinus maenas*. Two-way ANOVA to evaluate the effects of pH and salinity on the developmental duration to the megalopa stage. Abbreviations: SS = Type I Sum of Squares, df = Degrees of Freedom, MS = Mean Squares. Significant effects are given in **bold**. Not sufficient df for interaction terms: those with df gave non-significant effects.

	Effect	SS	Degr. of	MS	Den.Syn.	Den.Syn.	F	p
Intercept	Fixed	39920.04	1	39920.04	0.66	6.83	5844.69	0.0353
Salinity	Fixed	35.8	1	35.8	20.10	13.46	2.66	0.1185
pH	Fixed	4.26	1	4.26	20.49	13.34	0.32	0.5782
Female	Random	7.52	1	7.52	20.00	13.49	0.56	0.4640
Salinity*pH	Fixed	17.57	1	17.57	20.00	13.49	1.30	0.2673
Error		269.81	20	13.49				

Female not significant

	SS	Degr. of	MS	F	p
Intercept	23285.4	1	23285.4	1258.52	<0.0001
Salinity	14.7	1	14.7	0.80	0.3918
pH	0.15	1	0.15	0.01	0.9302
Salinity*pH	9.23	1	9.23	0.50	0.4947
Error	203.52	11	18.5		

	SS	Degr. of	MS	F	p
Intercept	12623.05	1	12623.05	1269.36	<0.0001
Salinity	4.81	1	4.81	0.48	0.5126
pH	12.81	1	12.81	1.29	0.2996
Salinity*pH	8.33	1	8.33	0.84	0.3953
Error	59.67	6	9.94		

	SS	Degr. of	MS	F	p
Intercept	46800.86	1	46800.86	3883.80	<0.0001
Salinity	43.97	1	43.97	3.65	0.0676
pH	3.83	1	3.83	0.32	0.5779
Salinity*pH	15.08	1	15.08	1.25	0.2739
Error	301.26	25	12.05		

Analysed 3 females at salinity 33 only

	SS	Degr. of	MS	F	p
Intercept	12623.05	1	12623.05	1269.36	<0.0001
Salinity	4.81	1	4.81	0.48	0.5126
pH	12.81	1	12.81	1.29	0.2996
Salinity*pH	8.33	1	8.33	0.84	0.3953
Error	59.67	6	9.94		

	SS	Degr. of	MS	F	p
Intercept	22430.77	1	22430.77	4120.89	<0.0001
pH	17.36	1	17.36	3.19	0.1017
Error	59.88	11	5.44		

4 Chapter Four: Responses of *Carcinus maenas* Megalopae and Juveniles to pCO_2 and Salinity

4.1 Introduction

Climate driven changes in environmental variables are exposing organisms to conditions at or beyond the limits of tolerance. In marine environments, two important variables, temperature and the partial pressure of CO_2 are increasing globally. pCO_2 influences several additional variables such as pH and the concentration of carbonate ions in ocean waters. As environmental factors may lead to interacting effects (Darling and Cote 2008, Przeslawski, Byrne et al. 2015) (cf. Chapter 3) on survival and performance of early life stages, understanding such responses is one of the key challenges in marine ecology (Rudd 2014).

Knowledge surrounding the potential stage-dependent effects of elevated pCO_2 and reduced salinity on marine crustaceans is limited. It is known that the effects of salinity on performance of crustaceans is stage-dependent and correlates with the capacity to osmoregulate, along with the habitat occupied at each life stage (e.g. Charmantier, Giménez et al. 2002, Miller, Zarate et al. 2014). Expected changes in ocean pH due to elevated pCO_2 may also lead to stage specific effects on individuals. Juveniles and adults of intertidal benthic invertebrates may be better adapted to variations in such conditions as they may experience natural fluctuations in both pH and salinity in their natural habitat, especially in the summer associated with reduced oxygen levels in tide pools (Whiteley 2018). Larval stages, however, are more sensitive to such conditions (Byrne and Przeslawski 2013). Settling larvae and early juvenile stages are also expected to be sensitive to environmental variation: these stages suffer proportionally high mortality rates in the field and such mortalities may be increased under extreme conditions (Gosselin and Qian 1997, Walther, Anger et al. 2010).

In this chapter, the effects of elevated pCO_2 and salinity over the megalopa and early juvenile stages of the shore crab *Carcinus maenas* were investigated. Briefly, after successfully completing the fourth and final zoea instar stage (IV), shore crab larvae

undergo ecdysis to the megalopa stage that partially resembles the morphology of the adult counterparts with chelipeds and walking legs, but with a long pleon and pleopods used for swimming. Megalopa of shore crabs exhibit diel-vertical migrations (in order to feed) and circatidal rhythms, whereby larvae ascend to surface waters in nocturnal flood-tides (Moksnes 2002). Such behaviour enables larval transport and recolonization of estuaries or coastal intertidal zones, which usually occurs in Spring-Summer. Megalopae usually settle on complex substrata, such as filamentous algae or mussel beds, it is here that they then metamorphose into a juvenile crab. Juveniles grow quickly in the intertidal zone during summer and then migrate to the shallow subtidal in autumn-winter (Moksnes 2002). Juveniles will moult around 18 times on average (depending on a variety of factors such as temperature and food availability) until they become adult crabs (Moksnes 2002).

Longer term exposures to elevated pCO_2 levels are beginning to show that responses are more realistic if the individuals are exposed to seasonal changes in temperature (Godbold and Solan 2013). In order to study the effects of temperature in more detail, this chapter also investigated the effects of elevated pCO_2 and reduced salinity on later stages of development in two situations: individuals exposed to seasonal variations in temperature, and those held at a single controlled temperature.

4.2 Aims and Objectives

The main aim of this study was to evaluate the combined effect of elevated pCO_2 and salinity on the survival and development of the seldom studied late larval stages of *C. maenas* (Megalopa through to early Juveniles).

Chapter 4 had the following specific objectives:

- **Objective 1:** To determine the importance of natural variability in the capacity of individuals to develop under elevated pCO_2 and reduced salinity conditions, by testing if larvae settling at different times of the settlement season differ in their responses to pCO_2 and salinity (survival and duration of development).

- **Objective 2: Experiment C: Responses to seasonal temperature fluctuations:** To determine the effects of near-future (year 2100) predicted pCO_2 levels and low salinity on the duration of development and survival of *C. maenas* megalopa and early juveniles under near natural temperature cycles.

- **Objective 3: Experiment D: Responses to controlled temperature:** To determine the effects of near-future (year 2100) predicted pCO_2 levels and low salinity on the duration of development and survival of megalopa and early juveniles under controlled temperature conditions.

4.3 Materials and Methods

4.3.1 Collection of Megalopa of *Carcinus maenas*

Carcinus maenas megalopa were in the Menai Strait off St George's pier (Isle of Anglesey, North Wales, UK) in the periods of June to August 2014 and July to August 2015. Megalopae were predominantly located by collecting flotsam seaweed, namely *Fucus vesiculosus* and *Ulva lactuca* (favoured algae of the megalopae in this area, personal observation), both trapped in the collection region (Figure 4.1), or that floating by at flood tide. Animals were sorted where possible in the field, with correct species identified (e.g. *Necora puber* megalopae were also present) and unused seaweed returned into the water. This was deemed the most efficient method of collecting megalopa larvae, as opposed to performing zooplankton trawls in the area off the pier, which was also tried but resulted in much fewer larvae being found. Additional larval numbers were facilitated by small rib boat collection of floating algae. In addition to this, two boat-derived zooplankton tows were also performed, and samples sorted through in order to find more megalopa, but again this was not as efficient as the seaweed collection method performed off the pier.

Figure 4.1: Showing the collection area of the megalopae in the Menai Strait off St George's pier (Isle of Anglesey, North Wales, UK).

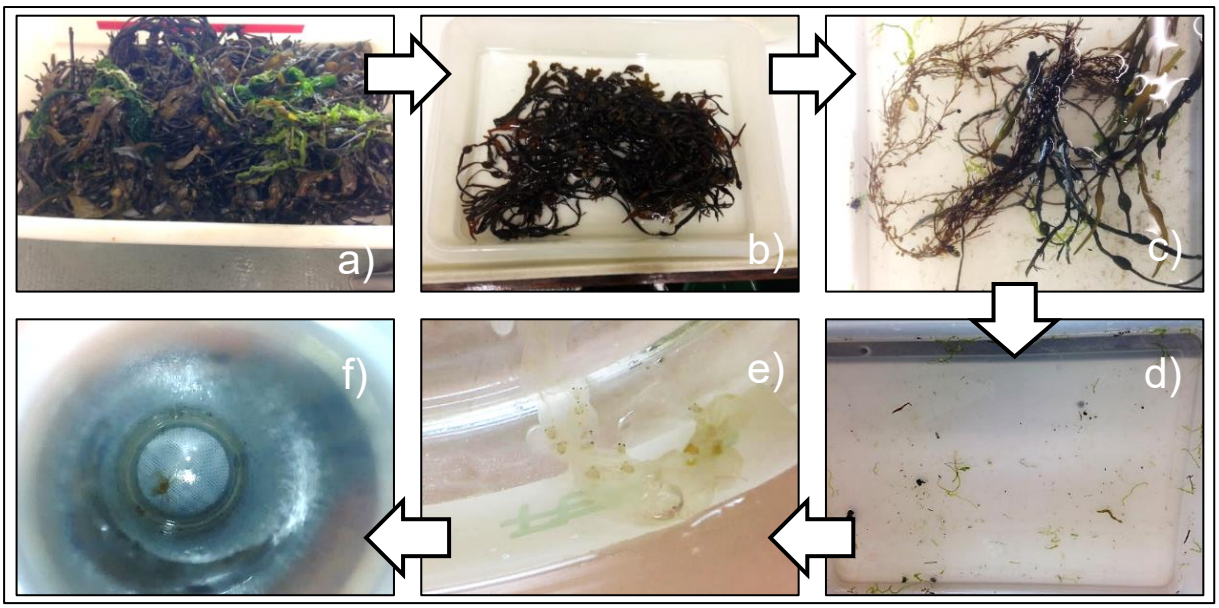


Figure 4.2: Collection method of *C. maenas* megalopa in the laboratory. After a collection of floating seaweed (a), sorting the seaweed into batches (b), then washing it in a tray of shallow filtered seawater (c), megalopa were then spotted (d) and pipetted using a glass pipette into a container containing filtered seawater (e), from here the megalopa were sorted into their experimental tubes, or pots for experiment 2 (f), where they remained for the duration of the experiment.

4.3.2 Larval Rearing/Experimental Conditions

4.3.2.1 Experiments with Seasonal Changes in Temperature

The following experiments were carried out in the main aquarium system responsible for providing elevated pCO_2 and reduced salinity conditions described in Chapter 2. Organisms were individually reared in modified 50ml falcon tubes with the lid removed and the opposite end cut, to form an open tube in which water could freely flow. A fine mesh on either end secured with non-toxic bands. Animals were transported in their experimental tank box from the lab submerged inside their tubes, after initial preparation in ambient seawater, to the aquarium system. The boxes containing the megalopae were then placed into tray tanks within the aquarium system and a tube of flowing water at the correct treatment condition was fed into the box. This ensured that the water conditions gradually changed to the treatment condition, as the boxes filled up, replacing the seawater inside on a continual basis. Boxes were cleaned every day to prevent build-up of sediment. Tubes were cleaned every other day to prevent accumulation of debris and algae. The salinity values chosen mimic conditions that could be experienced by larvae that are expelled in and along coastal area regions. The pCO_2 for current and near future scenarios.

Animals were fed ad libitum with freshly hatched *Artemia* sp. nauplii on a daily basis until they moulted to juveniles, they were then fed every other day with a piece of squid that was cut into a tiny portion, (less than) half the body size of the crab, prepared using a scalpel.

For the experiment, a selection of healthy megalopa (i.e. all limbs intact), were selected over a 6-week period. These larvae were maintained in the experimental system described in Chapter 2 with a 12:12 hour light:dark photoperiod.

Larvae were exposed to one of four experimental conditions, with different controlled conditions of salinity (33 and 25), and pCO_2 combinations (400 and 1,000 µatm) combinations (8.1 and 7.7). Response variables were mean duration of development through to Juvenile 5 stage, percentage survival through to Juvenile 5 stage and moult length from Juvenile 1-Juvenile 5. Larvae were reared individually in adapted

Eppendorf tubes with a mesh covering each end to enable a flow of water through the tube (Figure 4.3). The experiment used megalopa that were collected during June to August 2014, consisting of 184 megalopae in total (46 megalopa per treatment x 2 x 2 = 184 megalopa). This number was dependent upon how many megalopa could be found, ideally 10 per treatment per week, in accordance with objective 1.

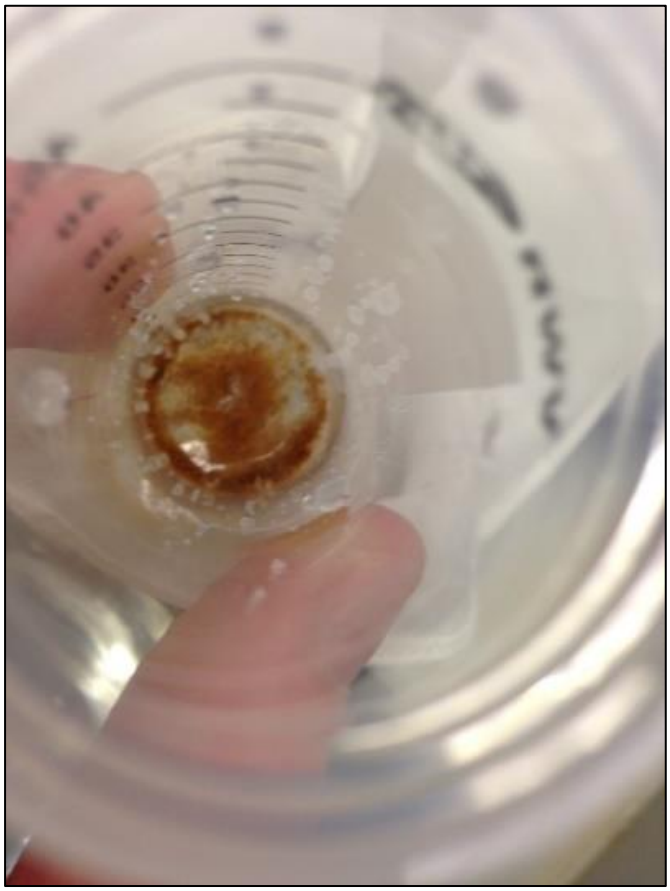


Figure 4.3: *Artemia* sp. nauplii in the adapted Eppendorf experimental tubes, with both ends open and a mesh on either side, from Experiment 1.

Larvae were checked at daily intervals, for moults (Figure 4.4 & Figure 4.5) and mortalities. Moults were stored in the freezer. Experiments continued until all the megalopa had either reached the Juvenile 5 stage or died. The duration that larvae were exposed to the different experimental conditions varied with treatment.

Animals were removed from the experiment upon reaching the juvenile 5 stage, any mortalities before this point were disposed of.

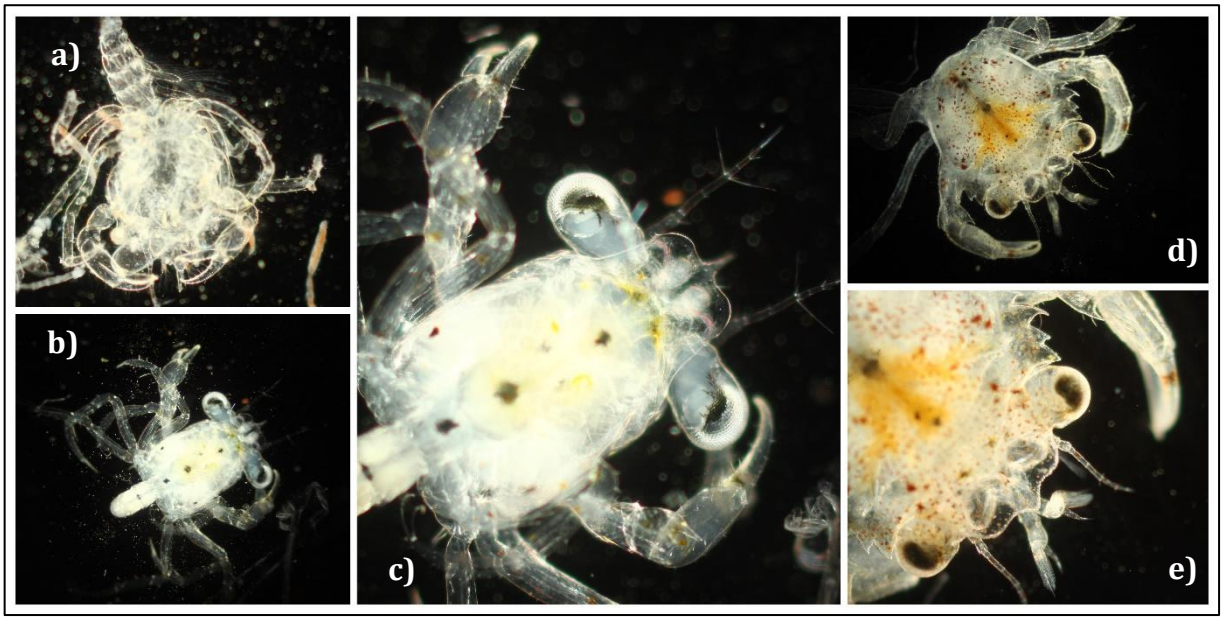


Figure 4.4: *Carcinus maenas*. a) exuvia of a megalopa; b) megalopa showing entire body; c) megalopa close up showing clear morphological details; d) early juvenile, stage collected for the purpose of photographing; e) early juvenile, note well developed marginal teeth and spines on the carapace indicating that this species is older than Juvenile 1 (photographed using dark-field microscopy with a Canon Eos 60D).

Figure 4.5: *Carcinus maenas* juvenile 1 – note lack of marginal teeth and spines on the carapace which is a morphological feature in juvenile 1. Experiments at Constant Temperature.

4.3.2.2 Experiments with Controlled Temperature

For this experiment, a selection of healthy megalopa (i.e. all limbs intact), was taken over a 6-week period. These larvae were maintained in fully programmable automated incubators and a 12:12 hour light:dark photoperiod at a temperature of 15ºC. The experiment used megalopa that were collected during July to August 2015, consisting of 120 megalopae in total (30 megalopa per treatment x 2 x 2 = 120 megalopa). This number was dependent upon; A) how many megalopa could be found, ideally 10 per

treatment per week, in accordance with objective 1. B) The experimenter effort in performing water daily changes of each individual pot.

These megalopae were maintained and individually reared in 100ml screw-capped containers. Water initially used was of ambient temperature (from the OA system). Larvae were then placed into the incubator at 15°C, this ensured a gradual change in temperatures over 4 hours, allowing larvae to adapt to experimental temperatures slowly. For subsequent daily water changes, water collected from the OA system was pre-heated to 15°C using carboys of water at the correct treatment condition within the incubator. The temperature was chosen because it is environmentally realistic and larval development has been shown previously to be successful at this temperature. The salinity values chosen mimic conditions that could be experienced by larvae that are expelled in and along coastal area regions, and the pCO_2 levels used were those for current and near future scenarios as explained in Chapter 2.

Other aspects of the experiments were carried out in the main aquarium under seasonal changes in temperature. Megalopa and juveniles were reared individually and exposed to one of four experimental conditions, with different controlled conditions of salinity (33 and 25), and pCO_2 combinations (400 and 1,000 µatm) up to the juvenile 5 stage. Animals were fed ad libitum with freshly hatched *Artemia* sp. nauplii on a daily basis until they moulted to juveniles, they were then fed every other day with a piece of squid. Individuals were checked at daily intervals, for moults and mortalities; moults were stored in the freezer until measurement could take place.

4.3.3 Data Analysis

For survival, the effect of salinity and pCO_2 were analysed though a generalised linear model based on the binomial distribution and logit link function using methods of model selection (see Chapter 3 for details) and following Zuur et al. 2009. See also Akaike (1998) regarding AIC (Akaike 1998, Zuur, Leno et al. 2009). Models were run in R (Team 2013). Effects on duration of development were tested using analysis of variance (ANOVA).

4.4 Results

4.4.1 Experiment C: Responses to Seasonal Temperature Fluctuations

Only at later juvenile stages studied (stages 4 and 5), survival responded to pCO_2 and/or salinity, but this was dependent upon collection week (Figure 4.6, Table 4.1 and Table 4.2). There was no effect of any variable at the earliest juvenile stages (stages 1-3). When data from different collection weeks was combined, there was significantly higher survival at stages 4 and 5 in low salinity seawater (Salinity = 25) (Figure 4.7, Table 4.5 and Table 4.6). Overall, effects were limited to later stages of development, depending upon animals collected at different points in the collection period. For survival to the fourth juvenile stage, the effect of pCO_2 was contingent on the week of collection (Table 4.1) and did not show consistent responses: for example, on week 2 and 3, survival was higher under normal pCO_2 conditions than on the low pCO_2 treatment, but such patterns were reversed on week 4 (Figure 4.6). Survival to the stage 5 responded to the combination of pCO_2 and salinity as well as salinity and week (Table 4.2) but again did not show any consistent pattern (Figure 4.6).

Duration of development varied significantly among individuals collected over different weeks at all stages studied (Tables 4.7 (stage 2), 4.8 (stage 3), 4.9 (stage 4), except for stage 5 (Table 4.10) (however, surviving to stage 5 was harder as temperatures had fallen in the system at this point, therefore low survivorship could have skewed the results). With the consideration of collection week, pCO_2 significantly affected stages 2 (Table 4.7), 3 (Table 4.8) and 4 (Table 4.9), with a significant interaction apparent at stage 4 (Figure 4.8, Table 4.9). When data were pooled, no effect at stages 2 (Table 4.11) or 4 (Table 4.13) was apparent. For stage 3 (Table 4.12), salinity and pCO_2 were significant when both the collection week was considered in addition to pooled data. In addition, for stage 5 (Table 4.15) salinity was significant when both the collection week was considered in addition to pooled data.

4.4.1.1 Survival

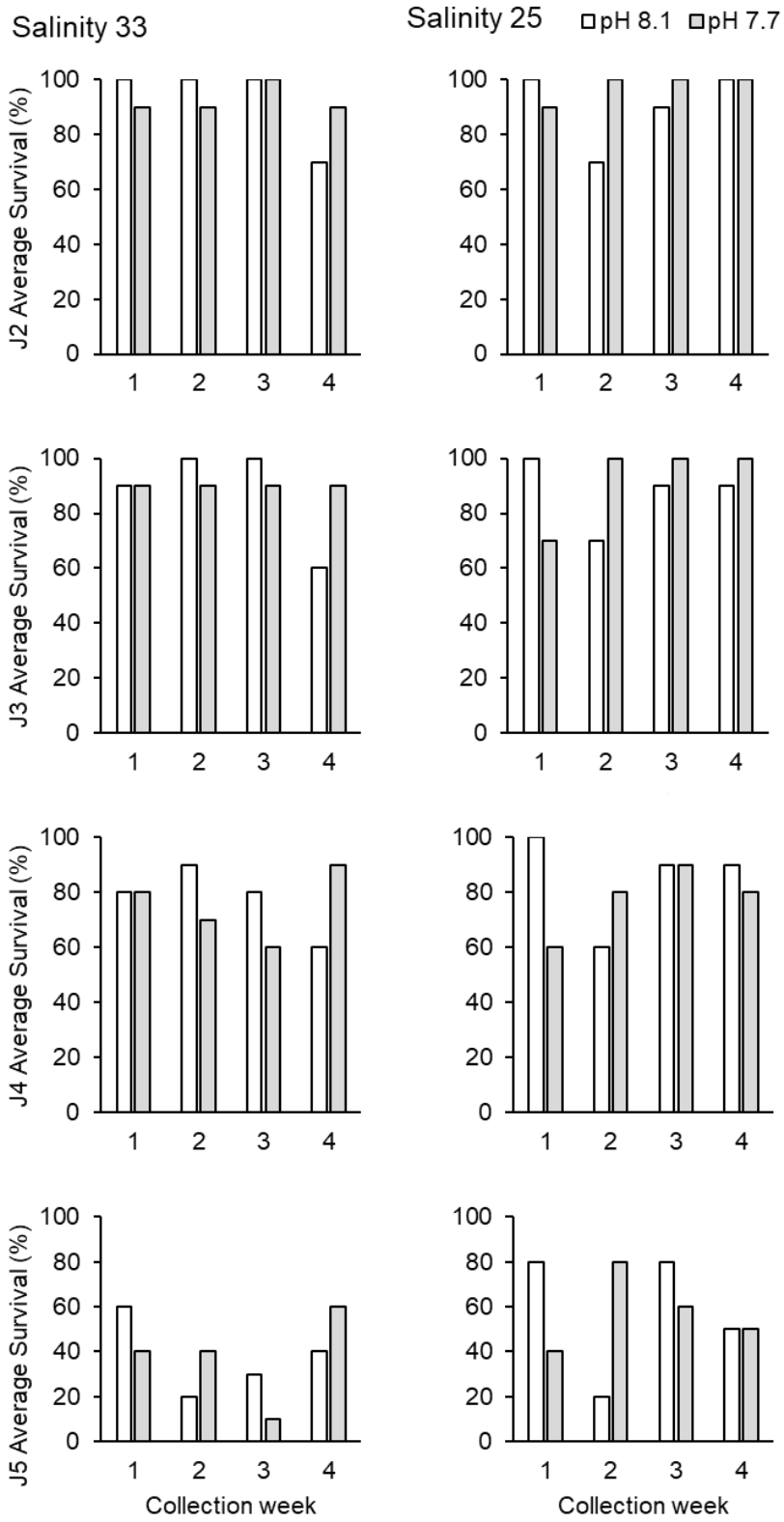


Figure 4.6: *Carcinus maenas*. Effect of pCO_2, salinity and collection week on average percentage survival up to juvenile 5 stage in a flow-through system.

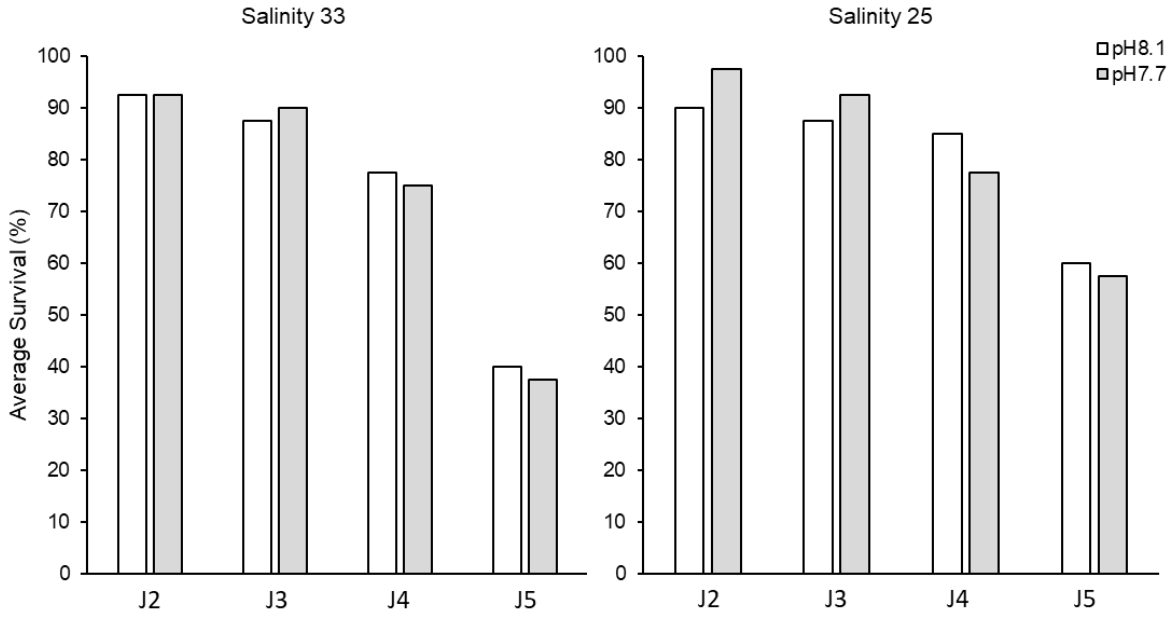


Figure 4.7: *Carcinus maenas*. Effect of pCO_2, salinity on average percentage survival up to juvenile 5 stage in a flow-through system.

4.4.1.2 Duration of Development

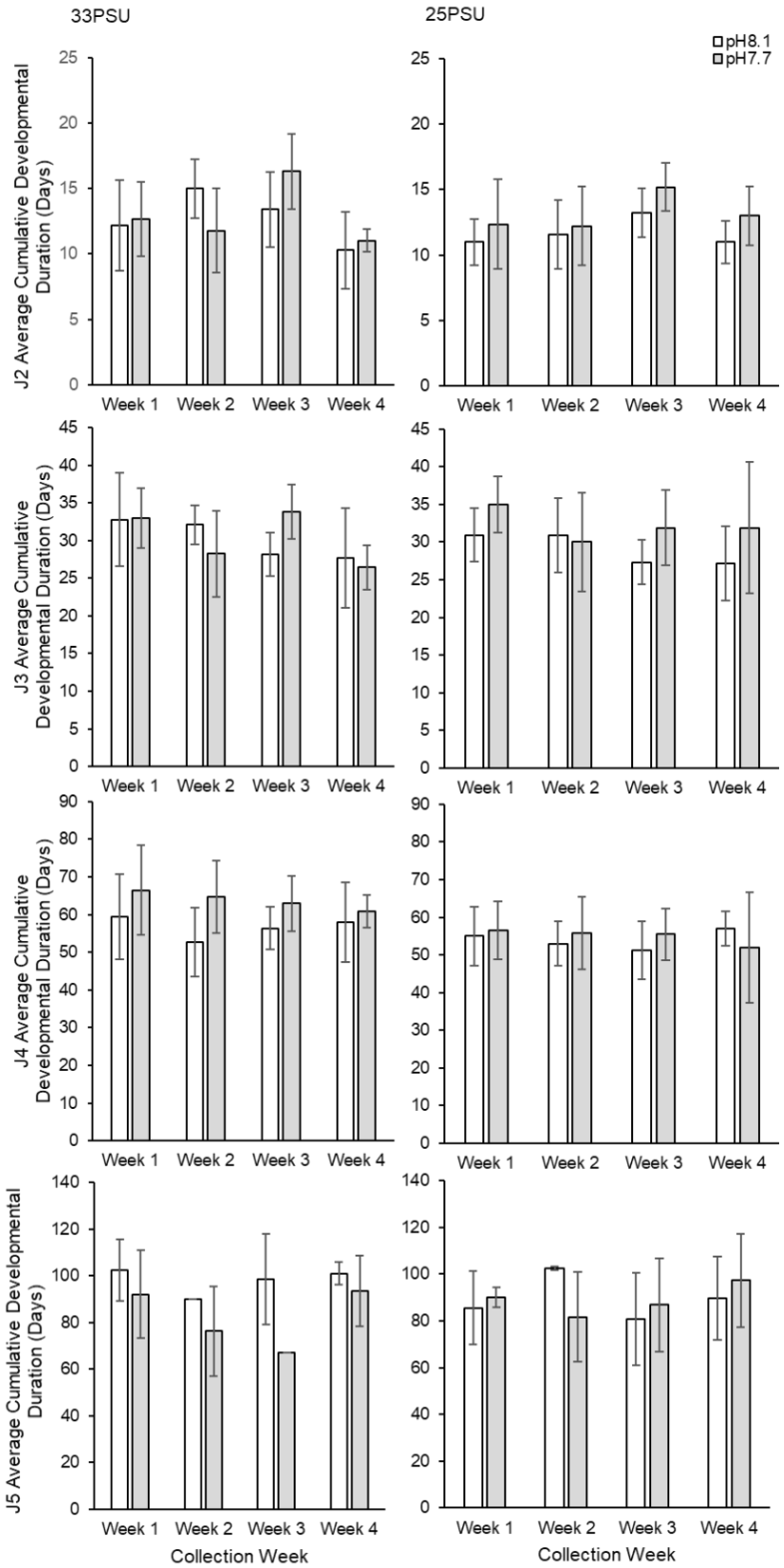


Figure 4.8: *Carcinus maenas*. Effect of pCO_2, salinity and collection week on the duration of juvenile development up to juvenile 5 stage in a flow-through system.

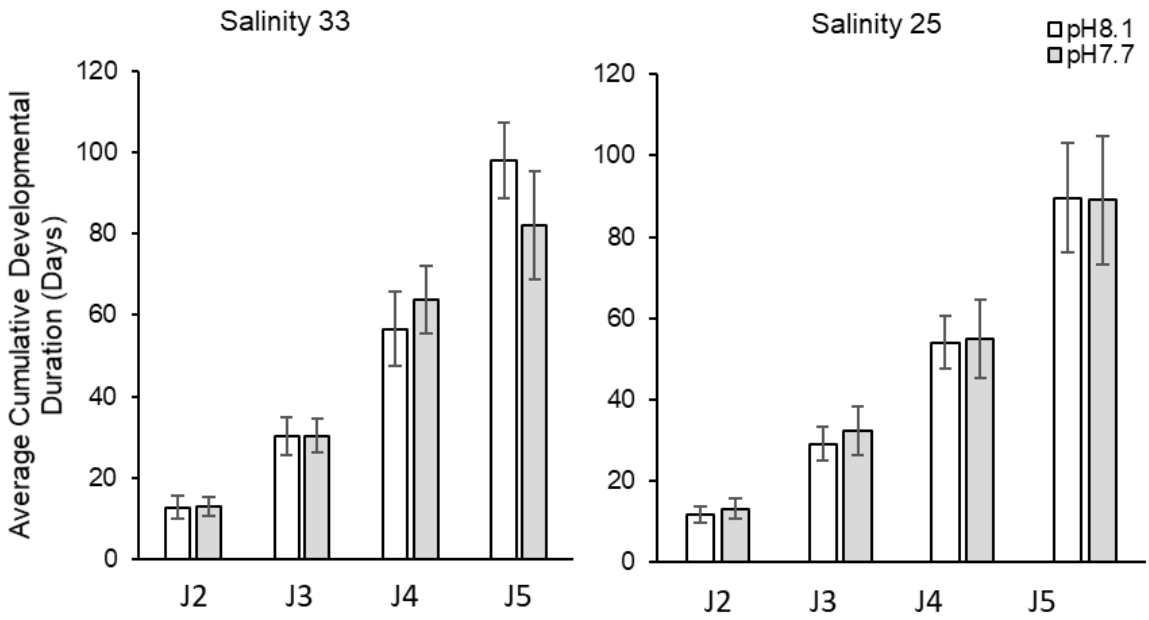


Figure 4.9: *Carcinus maenas*. Effect of pCO_2 and salinity on the duration of juvenile development up to juvenile 5 stage in a flow through system.

4.4.2 Experiment D: Responses to Controlled Temperature

Overall there is very little observed impact on survival at the earliest stages (J2, J3), with the exception of a significant impact of high pCO_2 on survival at stage 3 (Figure 4.10). Only at stages J3 and J5 were significant effects found when analysing collection week, at stage J3 (Table 4.15) there was a significant effect of pCO_2 1,000 µatm (i.e. pH 7.7) on survival and at stage J5 (Table 4.16). There was a significant effect of pCO_2 dependent upon collection week, with low pH typically causing a reduction in survival (Figure 4.10). There was no effect of any variable at the earliest juvenile stage 2, or at stage 4 when data from different collection weeks was combined, there was no significance found at any stage for survival (Figure 4.11; Tables 4.17 (J2), 4.18 (J3), 4.19 (J4), 4.20 (J5)).

Duration of development varied significantly among individuals collected over different weeks when combined with pH at stage 2. Duration of development also varied significantly among individuals collected over different weeks when combined with salinity at stage 2 (Figure 4.12, Table 4.21). For stage 3 (Table 4.22), duration of development varied significantly depending on collection week and salinity (Figure 4.12). For stage 4 (Table 4.23), developmental duration was significantly affected by collection week together with salinity and also when combined with pH. At stage 5 only (Table 4.24), salinity was significant with lower salinity generally showing a pattern of reducing developmental duration, however there were a lower number of crabs still alive at this stage, therefore low survivorship could skew the results. When collection week was removed from the analyses, at stage 2 nothing was significant (Figure 4.13, Table 4.25), with significant effects only being apparent for stage 3 (Table 4.26) (with lower salinity reducing the developmental duration) and at stage 4 (Table 4.27), which had a significant interaction of salinity and pH (at high salinity no obvious effect on developmental duration, but at low salinity, low pH increases developmental duration). Again, at stage 5 nothing was significant but the low number of crabs in these analyses could skew results (Table 4.28).

4.4.2.1 Survival

Survival (with week):

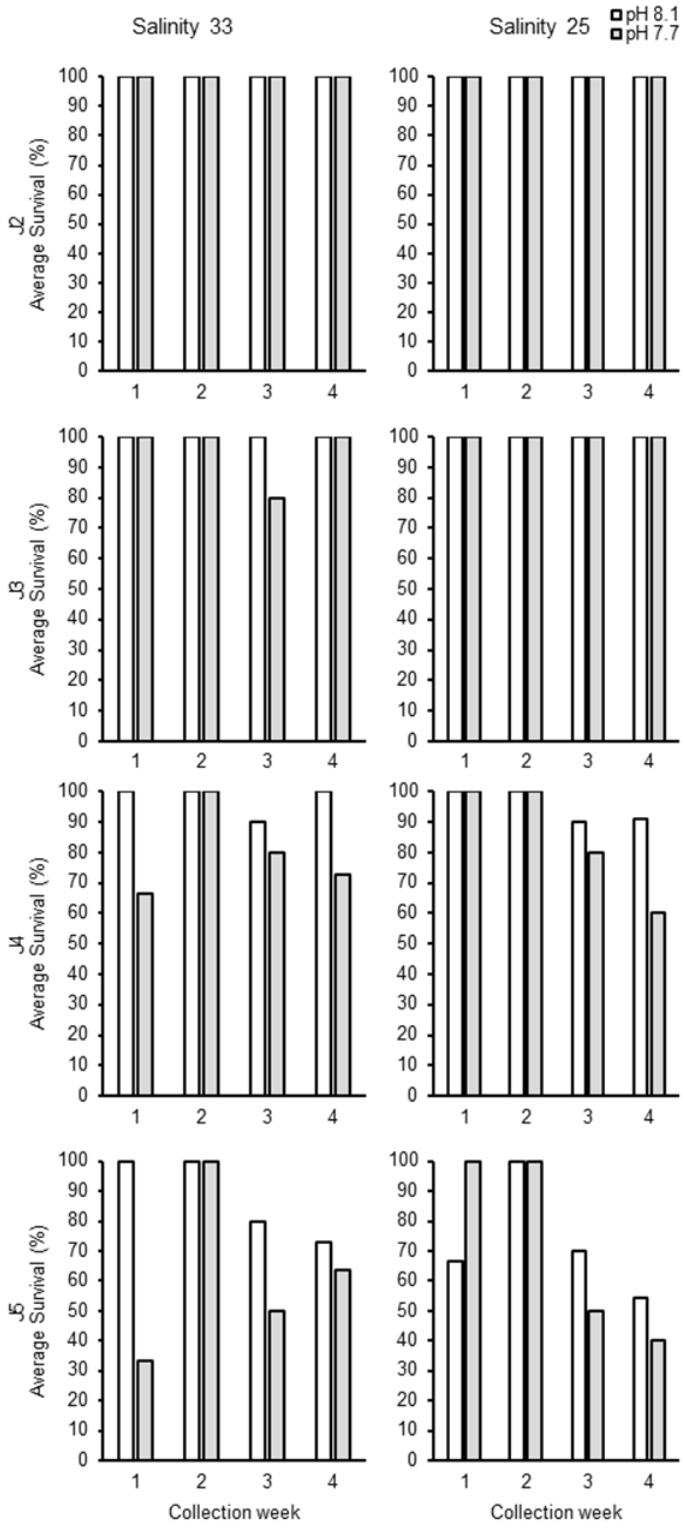


Figure 4.10: *Carcinus maenas*. Effect of pCO_2, salinity and collection week on average percentage survival up to juvenile 5 stage in a static system.

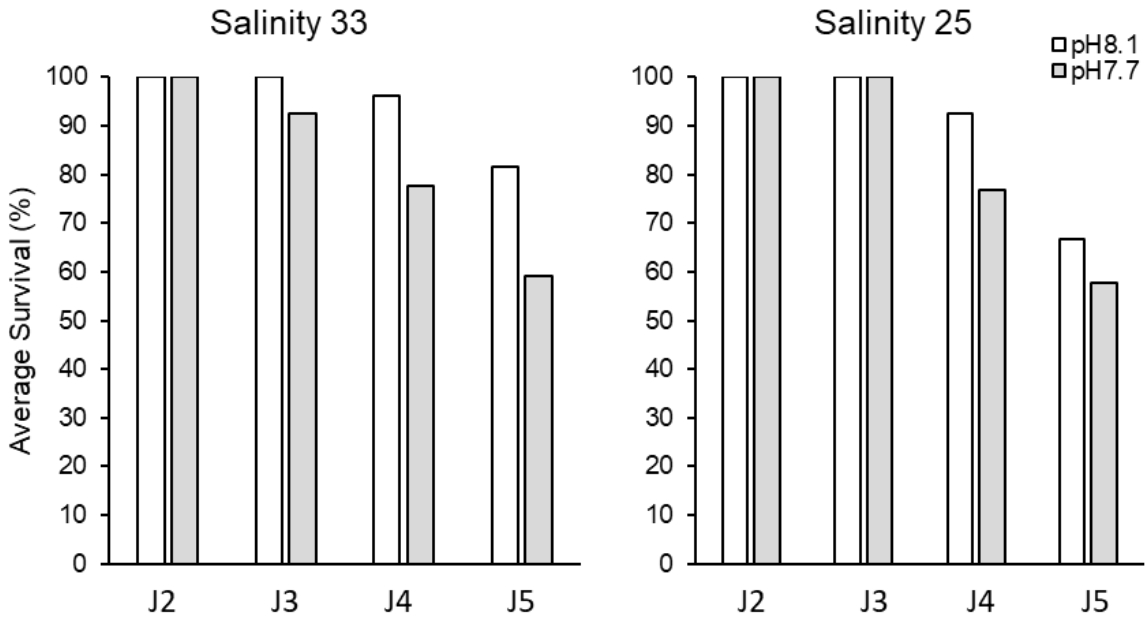


Figure 4.11: *Carcinus maenas*. Effect of pCO_2, salinity on average percentage survival up to juvenile 5 stage in a static system.

4.4.2.2 Duration of Development

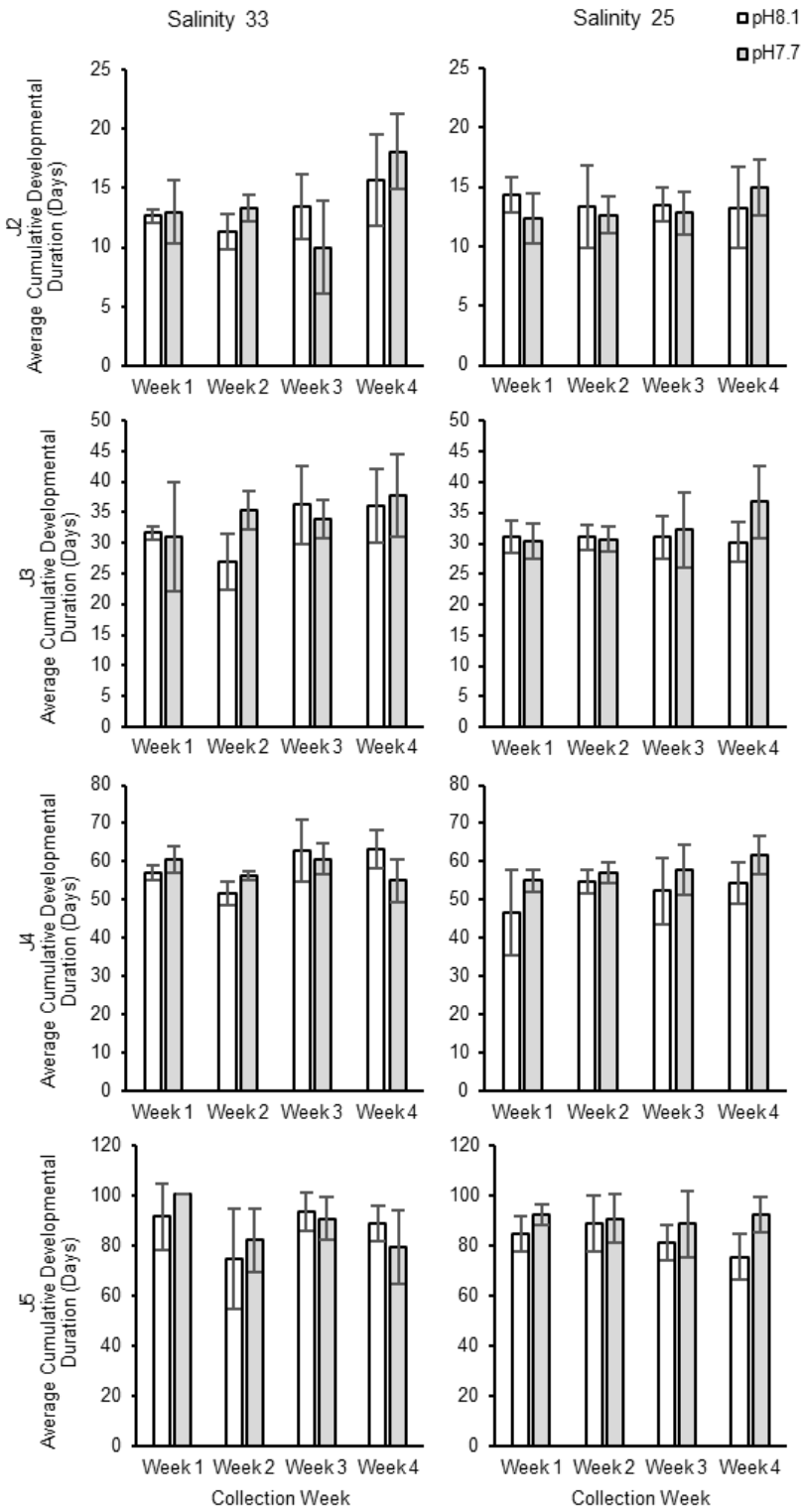


Figure 4.12: *Carcinus maenas*. Effect of pCO_2, salinity and collection week on the duration of juvenile development up to juvenile 5 stage in a static system.

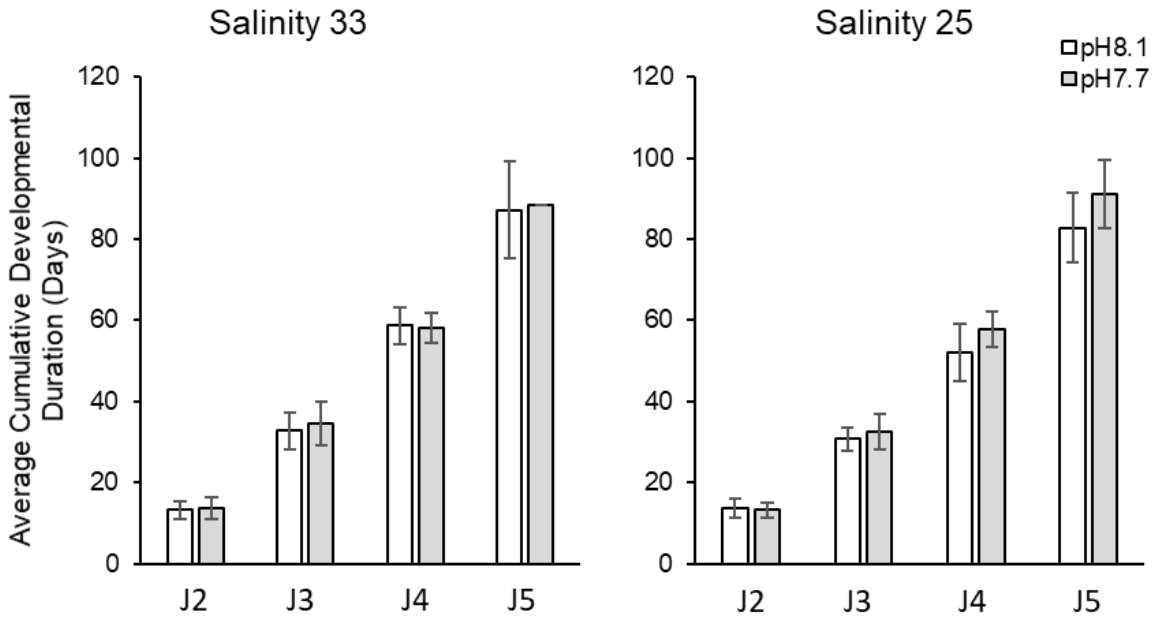


Figure 4.13: *Carcinus maenas*. Effect of pCO_2 and salinity on the duration of juvenile development up to juvenile 5 stage in a static system.

4.5 Discussion

These experiments provide information complimentary to experiments focusing on development from zoea to megalopa (Chapter 3). By initiating the experiment at the megalopa stage, a knowledge gap about responses of early juvenile stages to elevated pCO_2 and salinity is filled. Results indicate that developmental duration was significantly affected by elevated pCO_2 and/or salinity at varying levels, whereas, for survival, such influence was observed in the later juvenile stages. These results suggest the possibility of a potentially physiologically sensitive bottleneck within the life-cycle of *C. maenas* to environmental stressors, such as elevated pCO_2 and salinity, at the megalopa stage. In turn, successful recruitment of this species to adult populations may be impacted, but more research is required as this may not be the same for all populations of *C. maenas* globally.

In the first experiment, the focus surrounded the responses of *C. maenas* under near-natural seasonal variations in temperature. While such an experiment does not allow

us to fully identify the causes of the patterns of variability, it helps us to quantify responses under near natural conditions where temperatures will vary seasonally. Here, the main result was the context dependent nature of the effect of elevated pCO_2 on survival and duration of development, which might result from the combination of the temperatures experienced during the experiment (temperature varied over the summer). In the second experiment, temperature was constant in order to rule out the potential effects of ambient/seasonal temperatures on the responses of the juveniles. In both experiments, responses of juveniles depended on week of collection: this may point to either differences in larval or maternal experience (e.g. Pechenik 2006, Giménez 2010) or genetic differences among larvae settling at different times of the season. Seasonal variation in responses to elevated pCO_2 were also found in larvae of silverside *Menidia menidia* by Murray et al. (2014), who interpreted the increased capacity to tolerate reduced pH as adaptive and resulting from maternal conditioning (Murray, Malvezzi et al. 2014). For the case of shore crab juveniles, it is less likely that maternal conditioning plays a role as individuals must have developed for about 4-5 weeks in the sea before being collected for this experiment. It is more likely, however, that the observed variations are driven by changes in larval experience, for instance in the thermal or food environment. For example, Giménez (2010) and Rey et al. (2016) found important seasonal variations in the nutritional traits of settling megalopae of shore crabs (Giménez 2010, Rey, Neto et al. 2016). Giménez (2010) showed that such differences resulted in differences in body size of juveniles over the following three months post settlement reflecting differences in energy reserves (Giménez 2010). Giménez et al. (2004) showed that in brachyuran crabs, traits at metamorphosis can be driven by salinity conditions experienced during early larval stages (Giménez 2004, Rey, Neto et al. 2015). There is, however, little information on how larval experience drives natural variation in stressor tolerance in juvenile stages of crustaceans. Irrespective of the mechanism, these experiments show that there is important natural variation in the capacity of early stages to cope with conditions produced by elevated pCO_2, in addition to salinity. Moreover, they show that evaluations of the effects of climate driven variables on physiological performance need to incorporate seasonal (and possibly spatial) variability in responses; otherwise conclusions (e.g. based on individuals collected over a short time frame) will not represent the actual population average responses.

Overall, the magnitude of the effects of pCO_2 and salinity on survival and duration of development were much smaller in the juveniles than in the earlier zoeal stages. This result supports the hypothesis that larval stages tend to be more sensitive to environmental variations than juveniles of the same species (Byrne and Przeslawski 2013) and points toward the critical role of larvae as population bottlenecks in species with complex life cycles. Importantly, shore crab larvae have little capacity to osmoregulate and by extension have less well developed ionoregulatory systems. By contrast, megalopae and juveniles are osmoregulators. Indeed, for the juveniles, survival was greater at salinity 25 than at salinity 33, especially for juvenile 4 at ambient pCO_2 and juveniles 4 and 5 at elevated pCO_2. In this context, it is known that *Carcinus maenas* is a euryhaline species and in Europe is naturally found in sites with salinities of 9 to 35 (Winkler, Siebers et al. 1988). A previous study demonstrated that juveniles of *Carcinus maenas* are osmoregulators below a salinity of 31 but osmoconformers above this salinity (Cieluch, Anger et al. 2004). By inference, juveniles at a salinity of 25 are osmoregulators. In these experiments, survival was higher at this salinity, which may be related to the fact that they would be better at compensating for the effects of elevated pCO_2. However, it is apparent that this effect is dependent upon the particular week in which animals were collected, and in addition to this, these animals were collected in a region of great variability in salinity (the intertidal zone). In addition, the megalopa and early juveniles used in this study were reared after hatching from berried females and had probably spent several weeks in more variable salinity conditions. It is possible that they had greater tolerance levels than animals originating from eggs that had developed in a stable laboratory condition of salinity 32, prior to hatching.

Juvenile *Carcinus maenas* preferentially develop in brackish/variable coastal conditions, potentially explaining the salinity results here. The salinity impact mentioned is more pronounced in the elevated pCO_2 treatment. There is a clear interactive effect, with high pCO_2 amplifying the salinity effect. *Carcinus maenas* is relatively euryhaline, being able to tolerate a wide range of salinities. However, this capacity develops at different stages in different species. For *C. maenas*, this is particularly relevant due to the migration of its megalopa into coastal waters for development, prior to migrating to the shore. Compared with *C. maenas*, truly marine and stenohaline species show stronger effects of salinity on larval growth. The impact becomes more pronounced in the later developmental stages. In addition, pre-

exposure to low salinity, or other environmental variables, at the embryonic stage, can pre-adapt individuals to certain conditions (Charmantier and Charmantier-Daures 2001). As the animals used in these experiments were caught in the Menai Strait, it is possible that they could be pre-adapted as conditions within The Menai Strait are highly variable.

4.5.1 Conclusion:

C. maenas is a highly adaptable species, a trait which has ensured its successful globalised distribution. Response of juveniles to elevated pCO_2 seems to be influenced by seasonal settlement of megalopa. As recruitment of populations will be staggered over time, indicated by the occurrence of megalopa over a series of collection weeks, the responses may not be seen for all individuals of the species. While *C. maenas* larvae from some individuals or populations may be detrimentally impacted by global changes in temperatures (global warming), salinity (increased rainfall/land run off from melting ice), and elevated pCO_2 (IPCC 2014), other cohorts may flourish. It is unknown if this would cause an evolutionary divergence to even more adaptable *C. maenas* in the future, if the more resilient populations survive and thrive. This could have knock-on effects for species that are predated upon by *C. maenas*, or indeed those that rely on *C. maenas* as a source of nutrition, and therefore further disrupt ecosystem stability alongside these environmental stressors (Leignel, Stillman et al. 2014).

4.6 Appendices (Chapter Four)

4.6.1 Experiment C: Responses to Seasonal Temperature Fluctuations

4.6.1.1 Survival

Table 4.1: Significant effects of survival on stage 4 *C. maenas*, AIC model. Only week by pH (pCO_2). Chosen model (bold) is not significantly different from upper model. gIVg is significantly different from upper model. Effect of week by pH, in other words, the effect of pH depended upon collection week.

model term	model	df	AIC	P
2 way without fsal:fph	gIVc	18	231.88	
2 way without fsal:fweek and fsal:fph	**gIVd**	**13**	**232.32**	0.0636
fsal	gIVg	2	233.23	0.0180
2 way models	gIVb	19	233.87	
additive model fsal+fph+fweek	gIVe	8	234.02	
fsal+fph	gIVf	3	235.01	
null	gIVnull	1	239.27	
fph	gIVh	2	241.10	
3 way factorial	gIV	24	241.24	

Table 4.2: Significant effects of survival on stage 5 *C. maenas*, AIC model. Only pH:sal. gVb versus upper model – not significant so chose upper model gVc (bold).Effect of salinity depends on pH and effect of salinity depends on week**.**

model term	model	df	AIC	p
2 way -fph:fweek	**gVc**	**14**	**195.78**	
2 way models	gVb	19	199.99	0.3272
fsal+fweek	gVf	7	206.47	
2 way - fsal:fph:fweek-fph:fweek-fsal:fweek	gVd	9	206.57	
additive model fsal+fph+fweek	gVe	8	207.89	
fsal	gVh	6	208.46	
3 way factorial	gV	24	209.45	
null	gVnull	1	216.80	

Table 4.3: Effects of elevated pCO_2 and salinity on survival at stage 2 of *C. maenas* juveniles. Significant effects given in bold.

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	3.21	0.67	4.77	<0.001
fsal33	0.29	0.78	0.38	0.7060
fph8.1	-0.34	0.78	-0.44	0.6620

	Estimate	Std. Error	z value	Pr(>|z|)

(Intercept)	3.07	0.72	4.24	<0.001
fsal33	0.67	1.24	0.54	0.5900
fph8.1	-0.07	1.02	-0.07	0.9440
fsal33:fph8.1	-0.64	1.61	-0.40	0.6890

Table 4.4: Effects of elevated pCO_2 and salinity on survival at stage 3 of *C. maenas* juveniles. Significant effects given in bold.

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	1.64	0.36	4.53	<0.001
fsal33	-0.40	0.43	-0.91	0.3610
fph8.1	0.71	0.45	1.60	0.1090

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	1.53	0.39	3.93	<0.001
fsal33	-0.20	0.54	-0.37	0.7080
fph8.1	1.03	0.71	1.45	0.1480
fsal33:fph8.1	-0.54	0.92	-0.59	0.5540

Table 4.5: Effects of elevated pCO_2 and salinity on survival at stage 4 of *C. maenas* juveniles. Significant effects given in bold.

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	0.62	0.27	2.30	0.0217
fsal33	-0.88	0.31	-2.81	**0.0050**
fph8.1	0.15	0.31	0.47	0.6380

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	0.59	0.31	1.91	0.0562
fsal33	-0.83	0.44	-1.89	0.0582
fph8.1	0.21	0.46	0.46	0.6492
fsal33:fph8.1	-0.11	0.63	-0.18	0.8565

Table 4.6: Effects of elevated pCO_2 and salinity on survival at stage 5 of *C. maenas* juveniles. Significant effects given in bold.

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-0.63	0.28	-2.28	0.0226
fsal33	-0.65	0.34	-1.92	0.0544
fph8.1	0.28	0.33	0.85	0.3946

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-0.41	0.30	-1.33	0.1827
fsal33	-1.23	0.51	-2.40	**0.0163**
fph8.1	-0.18	0.44	-0.41	0.6807
fsal33:fph8.1	1.09	0.69	1.59	0.1124

4.6.1.2 Duration of Development

Table 4.7: Effect of pCO_2, salinity and collection week on the duration of juvenile development at Juvenile 2 stage of *C. maenas*.

Source	Type I Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	1383.05[a]	23	60.13	2.42	0.001
Intercept	191588.32	1	191588.32	7705.74	0
week	463.15	5	92.63	3.73	**0.003**
sal	2.34	1	2.34	0.09	0.759
ph	127.87	1	127.87	5.14	**0.025**
week * sal	208.49	5	41.70	1.68	0.142
week * ph	351.42	5	70.28	2.83	**0.018**
sal * ph	81.49	1	81.49	3.28	0.072
week * sal * ph	148.30	5	29.66	1.19	0.314
Error	4425.63	178	24.86		
Total	197397.00	202			
Corrected Total	5808.68	201			

a. R Squared = .238 (Adjusted R Squared = .140)

Table 4.8: Effect of pCO_2, salinity and collection week on the duration of juvenile development at Juvenile 3 stage of *C. maenas*.

Source	Type I Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	14530.97[a]	23	631.78	5.00	0
Intercept	495921.81	1	495921.81	3925.66	0
week	2146.69	5	429.34	3.40	**0.006**
sal	6283.09	1	6283.09	49.74	**<0.001**
ph	4064.32	1	4064.32	32.17	**<0.001**
week * sal	324.53	5	64.91	0.51	0.766
week * ph	583.70	5	116.74	0.92	0.467
sal * ph	387.78	1	387.78	3.07	0.082
week * sal * ph	740.86	5	148.17	1.17	0.325
Error	19328.22	153	126.33		
Total	529781.00	177			
Corrected Total	33859.19	176			

a. R Squared = .429 (Adjusted R Squared = .343)

Table 4.9: Effect of pCO_2, salinity and collection week on the duration of juvenile development at Juvenile 4 stage of *C. maenas*.

Source	Type I Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	14090.99[a]	23	612.65	1.65	0.054
Intercept	781112.51	1	781112.51	2103.21	0
week	4522.44	5	904.49	2.44	**0.042**
sal	1658.72	1	1658.72	4.47	**0.038**
ph	126.10	1	126.10	0.34	0.562
week * sal	1829.87	5	365.97	0.99	0.432
week * ph	3362.60	5	672.52	1.81	0.12
sal * ph	1639.01	1	1639.01	4.41	**0.039**
week * sal * ph	952.27	5	190.45	0.51	0.766
Error	28968.49	78	371.39		
Total	824172.00	102			
Corrected Total	43059.49	101			

a. R Squared = .327 (Adjusted R Squared = .129)

Table 4.10: Effect of pCO_2, salinity and collection week on the duration of juvenile development at Juvenile 5 stage of *C. maenas*.

Source	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2235.51[a]	18	124.20	0.96	0.522
Intercept	545815.57	1	545815.57	4217.20	0
week	1004.45	5	200.89	1.55	0.199
sal	424.27	1	424.27	3.28	0.079
ph	0.03	1	0.03	0.00	0.988
week * sal	424.40	3	141.47	1.09	0.365
week * ph	302.81	5	60.56	0.47	0.797
sal * ph	3.11	1	3.11	0.02	0.878
week * sal * ph	76.44	2	38.22	0.30	0.746
Error	4529.92	35	129.43		
Total	552581.00	54			
Corrected Total	6765.43	53			

a. R Squared = .330 (Adjusted R Squared = -.014)

Table 4.11: Effect of pCO_2 and salinity on the duration of juvenile development at Juvenile 2 stage of *C. maenas*.

Source	Type I Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	185.93[a]	3	61.98	2.11	0.1
Intercept	158041.47	1	158041.47	5391.64	0
sal	6.17	1	6.17	0.21	0.647
ph	66.42	1	66.42	2.27	0.134
sal * ph	113.35	1	113.35	3.87	**0.051**
Error	4748.60	162	29.31		
Total	162976.00	166			
Corrected Total	4934.53	165			

a. R Squared = .038 (Adjusted R Squared = .020)

Table 4.12: Effect of pCO_2 and salinity on the duration of juvenile development at Juvenile 3 stage of *C. maenas*.

Source	Type I Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	1624.89[a]	3	541.63	7.53	0
Intercept	492885.33	1	492885.33	6853.77	0
sal	970.68	1	970.68	13.50	**0**
ph	483.85	1	483.85	6.73	**0.01**
sal * ph	170.36	1	170.36	2.37	0.126
Error	10283.78	143	71.92		
Total	504794.00	147			
Corrected Total	11908.67	146			

a. R Squared = .136 (Adjusted R Squared = .118)

Table 4.13: Effect of pCO_2 and salinity on the duration of juvenile development at Juvenile 4 stage of *C. maenas*.

Source	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1925.15[a]	3	641.72	2.49	0.065
Intercept	793639.96	1	793639.96	3078.83	0
sal	695.02	1	695.02	2.70	0.104
ph	350.19	1	350.19	1.36	0.247
sal * ph	879.93	1	879.93	3.41	0.068
Error	23972.90	93	257.77		
Total	819538.00	97			
Corrected Total	25898.04	96			

a. R Squared = .074 (Adjusted R Squared = .044)

Table 4.14: Effect of pCO_2 and salinity on the duration of juvenile development at Juvenile 5 stage of *C. maenas*.

Source	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	815.29[a]	3	271.76	2.28	0.09
Intercept	545815.57	1	545815.57	4586.58	0
sal	806.29	1	806.29	6.78	**0.012**
ph	2.07	1	2.07	0.02	0.896
sal * ph	6.93	1	6.93	0.06	0.81
Error	5950.14	50	119.00		
Total	552581.00	54			
Corrected Total	6765.43	53			

4.6.2 Experiment D: Responses to Controlled Temperature

4.6.2.1 Survival

Survival (with week):

Table 4.15: Significant effects of survival on stage 3 *C. maenas*, AIC model. Chosen model (bold).

model term	model	df	AIC	p
fsal+fph+fweek	**gIIIe**	**7**	**86.36**	0.9471
fsal*fph*fweek-fsal:fph:fweek-fsal:fweek-fsal:fph	gIIId	11	92.18	
fsal*fph*fweek-fsal:fph:fweek-fsal:fweek	gIIIc	12	93.76	
fsal*fph*fweek-fsal:fph:fweek	gIIIb	16	98.74	
3 way factorial	gIII	20	106.39	

Table 4.16: Significant effects of survival on stage 5 *C. maenas*, AIC model. Chosen model (bold).

model term	model	df	AIC	p
fph+fweek	**gVf**	**6**	**121.05**	0.9695
fsal+fph+fweek	gVe	7	121.64	0.9760
fsal*fph*fweek-fsal:fph:fweek-fph:fweek-fsal:fweek	gVd	8	123.37	
Fweek	gVh	5	128.00	
fsal*fph*fweek-fsal:fph:fweek-fph:fweek	gVc	12	130.83	
fsal*fph*fweek-fsal:fph:fweek	gVb	16	137.49	
3 way factorial	gV	20	141.32	
Vnull null	gVnull	1	152.55	

Table 4.17: Effects of elevated pCO_2 and salinity on survival at stage 2 of *C. maenas* juveniles. Significant effects given in bold.

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	21.57	5428	0.004	0.997
fsal33	-18.93	5428	-0.003	0.997
fph8	<0.001	7613	0	1
fsal33:fph8	18.93	9297	0.002	0.998

Table 4.18: Effects of elevated pCO_2 and salinity on survival at stage 3 of *C. maenas* juveniles. Significant effects given in bold.

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	1.57	0.49	3.19	0.0014
fsal33	-0.18	0.67	-0.27	0.7858
fph8	1.07	0.88	1.21	0.2247
fsal33:fph8	0.91	1.42	0.64	0.5218

Table 4.19: Effects of elevated pCO_2 and salinity on survival at stage 4 of *C. maenas* juveniles. Significant effects given in bold.

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	0.64	0.39	1.64	0.1
fsal33	-0.24	0.54	-0.44	0.662
fph8	0.21	0.56	0.37	0.713
fsal33:fph8	1.00	0.83	1.20	0.229

Table 4.20: Effects of elevated pCO_2 and salinity on survival at stage 5 of *C. maenas* juveniles. Significant effects given in bold.

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-1.34	0.46	-2.93	0.0034
fsal33	0.15	0.63	0.25	0.8066
fph8	0.80	0.59	1.34	0.1801
fsal33:fph8	0.39	0.82	0.48	0.6325

4.6.2.2 Duration of Development

Table 4.21: Effect of pCO_2, salinity and collection week on the duration of juvenile development at Juvenile 2 stage of *C. maenas*.

Source	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	479.40[a]	19	25.232	3.289	0
Intercept	22190.126	1	22190.126	2892.574	0
Week	236.105	4	59.026	7.694	0
pH	1.316	1	1.316	0.172	0.68
Sal	8.277	1	8.277	1.079	0.301
Week * pH	99.278	4	24.82	3.235	**0.015**
Week * Sal	100.034	4	25.008	3.26	**0.015**
pH * Sal	1.251	1	1.251	0.163	0.687
Week * pH * Sal	33.144	4	8.286	1.08	0.371
Error	759.47	99	7.671		
Total	23429	119			
Corrected Total	1238.874	118			

a. R Squared = .387 (Adjusted R Squared = .269)

Table 4.22: Effect of pCO_2, salinity and collection week on the duration of juvenile development at Juvenile 3 stage of *C. maenas*.

Source	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	694.12[a]	19	36.53	1.97	0.019
Intercept		1		6036.10	0
Week	179.64	4	44.91	2.42	0.055
Sal	213.45	1	213.45	11.49	**0.001**
pH	62.50	1	62.50	3.36	0.07
Week * Sal	15.51	4	3.88	0.21	0.933
Week * pH	71.59	4	17.90	0.96	0.432
Sal * pH	19.95	1	19.95	1.07	0.303
Week * Sal * pH	131.48	4	32.87	1.77	0.143
Error	1523.37	82	18.58		
Total		102			
Corrected Total	2217.49	101			

a. R Squared = .313 (Adjusted R Squared = .154)

Table 4.23: Effect of pCO_2, salinity and collection week on the duration of juvenile development at Juvenile 4 stage of *C. maenas*.

Source	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1785.50[a]	19	93.974	3.485	0
Intercept		1		9599.185	0
Week	333.251	4	83.313	3.09	**0.022**
Sal	520.053	1	520.053	19.286	0

pH	32.002	1	32.002	1.187	0.28
Week * Sal	274.958	4	68.74	2.549	**0.048**
Week * pH	76.762	4	19.191	0.712	0.587
Sal * pH	161.513	1	161.513	5.99	**0.017**
Week * Sal * pH	386.959	4	96.74	3.588	**0.011**
Error	1590.932	59	26.965		
Total		79			
Corrected Total	3376.43	78			

a. R Squared = .529 (Adjusted R Squared = .377)

Table 4.24: Effect of pCO_2, salinity and collection week on the duration of juvenile development at Juvenile 5 stage of *C. maenas*.

Source	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1981.22[a]	17	116.543	0.926	0.559
Intercept		1		2620.578	0
Week	447.594	4	111.899	0.889	0.488
Sal	599.007	1	599.007	4.758	**0.041**
pH	6.785	1	6.785	0.054	0.819
Week * Sal	507.375	4	126.844	1.008	0.426
Week * pH	331.901	4	82.975	0.659	0.627
Sal * pH	49.168	1	49.168	0.391	0.539
Week * Sal * pH	39.394	2	19.697	0.156	0.856
Error	2643.75	21	125.893		
Total		39			
Corrected Total	4624.974	38			

a. R Squared = .428 (Adjusted R Squared = -.034)

Table 4.25: Effect of pCO_2 and salinity on the duration of juvenile development at Juvenile 2 stage of *C. maenas*.

Source	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	11.17[a]	3	3.72	0.35	0.79
Intercept	22190.13	1	22190.13	2078.56	0
salinity	9.34	1	9.34	0.88	0.351
pH	0.90	1	0.90	0.09	0.772
salinity * pH	0.92	1	0.92	0.09	0.77
Error	1227.71	115	10.68		
Total	23429.00	119			
Corrected Total	1238.87	118			

Table 4.26: Effect of pCO_2 and salinity on the duration of juvenile development at Juvenile 3 stage of *C. maenas*.

Source	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	310.21[a]	3	103.41	5.31	0.002
Intercept	112136.51	1	112136.51	5761.82	0
salinity	237.04	1	237.04	12.18	**0.001**
pH	53.78	1	53.78	2.76	0.1

salinity * pH	19.40	1	19.40	1.00	0.321
Error	1907.28	98	19.46		
Total	114354.00	102			
Corrected Total	2217.49	101			

Table 4.27: Effect of pCO_2 and salinity on the duration of juvenile development at Juvenile 4 stage of *C. maenas*.

Source	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	699.92[a]	3	233.31	6.54	0.001
Intercept	258841.57	1	258841.57	7253.13	0
salinity	506.854	1	506.85	14.20	**0**
pH	38.259	1	38.26	1.07	0.304
salinity * pH	154.804	1	154.80	4.34	**0.041**
Error	2676.514	75	35.69		
Total	262218	79			
Corrected Total	3376.43	78			

Table 4.28: Effect of pCO_2 and salinity on the duration of juvenile development at Juvenile 5 stage of *C. maenas*.

Source	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	513.75[a]	3	171.25	1.46	0.243
Intercept	329912.03	1	329912.03	2808.63	0
salinity	408.17	1	408.17	3.48	0.071
pH	43.97	1	43.97	0.37	0.545
salinity * pH	61.61	1	61.61	0.53	0.474
Error	4111.22	35	117.46		
Total	334537.00	39			

5 Chapter Five: Varying Responses of the Saltmarsh Shrimp *Palaemon varians* and the Subtidal Coastal Shrimp *Palaemon serratus* to pCO_2 and Salinity

5.1 Introduction

Ocean Acidification (OA) and concurrent changes that occur with it (e.g. pH, pCO_2) has been suggested to be an 'open-ocean syndrome' (Duarte, Hendriks et al. 2013) whereby the role of OA for species' performance is more important in the open ocean as compared to coastal species (Feely, Alin et al. 2010). Estuaries often experience fluctuations in pCO_2 (and indeed salinity) which may exceed those predicted for the open-ocean surface waters by the end of the century (Ceballos-Osuna, Carter et al. 2013, Duarte, Hendriks et al. 2013). Consequently, one may hypothesise that species living in such fluctuating environments, such as estuaries or saltmarshes, may already possess the adaptations needed to cope in a scenario of OA.

We still have limited information about the potential effects of habitat of origin on the performance of organisms under scenarios of OA. Research on the effects of OA on coastal species has found that responses are consistent with the above hypothesis, as long as pCO_2 levels remain constant. In the blue mussel, *Mytilus edulis*, environments with changing pH exhibit more negative effects (e.g. increased metabolic rates, antioxidant enzyme activities) than if pH remained at constant near-future pCO_2 scenarios (Mangan, Urbina et al. 2017). Similarly, larvae of the intertidal crab *Petrolisthes cinctipes*, exposed to elevated pCO_2 and optimal salinity, do not show significant change in respiration rate; those changes occur when larvae are exposed to elevated pCO_2 followed by a sequential exposure to stressful salinity levels (Miller, Zarate et al. 2014). In other words, coastal species appear to minimise effects of OA in scenarios of reduced but constant pCO_2 or in scenarios where additional environmental variables do not fluctuate. However, such a response may be related to the intertidal habitat where exposures to natural fluctuations in pCO_2 are a common occurrence (Carter, Ceballos-Osuna et al. 2013).

Knowledge on the effects of habitat on the performance at the larval stage is even more limited. When considering the potential role of habitat as a possible explanation for interspecific variation in the responses to OA in larval stages, it is important to consider both the role of the natal habitat and the role of ontogenetic migrations. The life cycle of many coastal-estuarine crustaceans follows an export strategy (Anger 2001), where larvae are released in brackish water but are then transported offshore. Larvae will then develop in continental shelf waters where environmental conditions are more stable. In such species, it may well be that the performance under scenarios of OA varies ontogenetically, in association with the conditions existing in the natural habitat where they develop. Alternatively, the performance may still reflect conditions existing in the parental habitat, especially because this is the habitat where larvae will be released. Existing information on responses to salinity point to both mechanisms being likely to play a role. On the one hand, a number of studies show that early life history of decapod crustaceans is characterised by ontogenetic patterns in the capacity to osmoregulate and in the patterns of salinity tolerance (Charmantier and Charmantier-Daures 2001, Charmantier, Giménez et al. 2002, Torres, Charmantier-Daures et al. 2007, Anger, Torres et al. 2008) and in the capacity to grow under low salinity (Torres, Gimenez et al. 2011). Conversely, some studies show that the ontogenetic patterns in the capacity to osmoregulate matches patterns of ontogenetic migration. For instance, species that release larvae in streams, but exhibit most zoeal development in open waters show increased capacity to osmoregulate in the first larval stage. This is followed by a reduction in the subsequent stages and a subsequent increase at the megalopa stage, which recolonises the estuarine habitat. On the contrary, the capacity to osmoregulate varies considerably depending on the natal habitat: for instance, the capacity to osmoregulate is higher in the zoea I of the saltmarsh crab *Neohelice granulata* (Charmantier, Giménez et al. 2002), than in the shore crab *Carcinus maenas* (Cieluch, Anger et al. 2004); such differences are likely to reflect the fact that the latter usually releases larvae in open waters.

5.2 Aims and Objectives

The aim of this chapter was to compare the performance of larvae of two coastal shrimps, *Palaemon serratus* and *Palaemon varians* in scenarios of elevated pCO_2 and reduced salinity. Detailed descriptions of both the life-histories and physiology of

Palaemon varians (Leach, 1813-1814) and *Palaemon serratus* (Pennant, 1777) were outlined in Chapter 1. *P. varia*ns inhabits saltmarshes, characterised by low and variable salinities and reduced pH levels. *P. varians* is highly tolerant to hypoxia (Hagerman and Uglow 1984), a condition that is usually associated with reduced pH. By contrast, *P. serratus* occur in estuarine-coastal waters where salinity and pH levels may be more stable. Tolerance to low salinity is higher in *P. varians* than in *P. serratus* (Panikkar 1941). Adults of *P. serratus* are, however, able to tolerate hypercapnia through elevation of [Ca^{2}+] in haemolymph (Dissanayake, Clough et al. 2010). The larval development of *P. varians* is shorter and consists in a lower number of stages than that of *P. serratus*. Oliphant (2013) demonstrated that *P. varians* larvae are extremely resistant to starvation, because individuals are facultatively lecithotrophic during the first two stages (Oliphant 2013, Oliphant and Thatje 2014) and vulnerability to food limitation exists only in the remaining three to four stages. By contrast, lecithotrophy in *P. serratus* is restricted to the first larval stage, and individuals remain vulnerable to food limitation over more than 5 stages (Gonzalez-Ortegon and Giménez 2014).

Larvae of both *P. serratus* and *P. varians* are able to develop under access to prey limited to 4 hours per day (Gonzalez-Ortegon and Giménez 2014, Oliphant, Ichino et al. 2014). Because larvae of many planktonic species perform diurnal vertical migrations that restrict the feeding for a few hours every day, a realistic simulation of feeding regimes needs to consider scenarios of limited access to prey (Giménez and Anger 2005), which is used as the standard food condition in the current chapter. Therefore, I did not include a treatment of full access to prey because preliminary experiments showed that food for 24 hours led to important changes in seawater pH.

Chapter 5 had the following specific objectives:

- **Objective 1:** To determine the effects of near-future (the year 2100) predicted pCO_2 levels and low salinity on the developmental duration and developmental pathway of larvae of a subtidal coastal marine species, *P. serratus* and a salt marsh shrimp, *P. varians*.

- **Objective 2:** To determine whether the larvae of the facultative lecithotrophic shrimp of *P. varians* are more tolerant to elevated pCO_2 and reduced salinity than the planktotrophic larvae of *P. serratus*.

5.3 Materials and Methods

5.3.1 Collection of Ovigerous Females of *P. varians*

Ovigerous females of *P. varians* were collected using push nets from Newborough saltmarshes (Isle of Anglesey, North Wales, UK) in July 2014. Females were kept in individually aerated aquaria (volume = 2 litres) at 18˚C, throughout embryonic development, until larvae hatched. They were fed green algae (*Ulva* sp.). Water was changed daily to ensure removal of waste material.

Figure 5.1. Ovigerous *Palaemon varians*, with eggs distinctly visible, immediately after capture in a saltmarsh creek at Newborough.

Figure 5.2. Top: Overview of saltmarsh creek at Newborough, Isle of Anglesey UK. Bottom: push net used to catch shrimp.

5.3.2 Collection of Ovigerous Females of *P. serratus*

Ovigerous females of *P. serratus* were obtained from fishermen at Amlwch Port (Isle of Anglesey, North Wales, UK) over the winter-early Spring months of 2015 (January-March). The shrimps were transported dry, on ice, and immediately transferred to cold ambient seawater upon return to the laboratory. Over time, the temperature of the seawater was increased to 18°C (to facilitate embryonic development). Females of this species are particularly sensitive to rapid changes in temperature (personal observation), therefore embryonic acclimation occurred over several weeks. Females were maintained in individually aerated aquaria (volume = 2 litres), throughout embryonic development, until larval hatching. They were fed green algae (*Ulva* sp.) which was replaced as required. Water was changed daily to ensure removal of waste material.

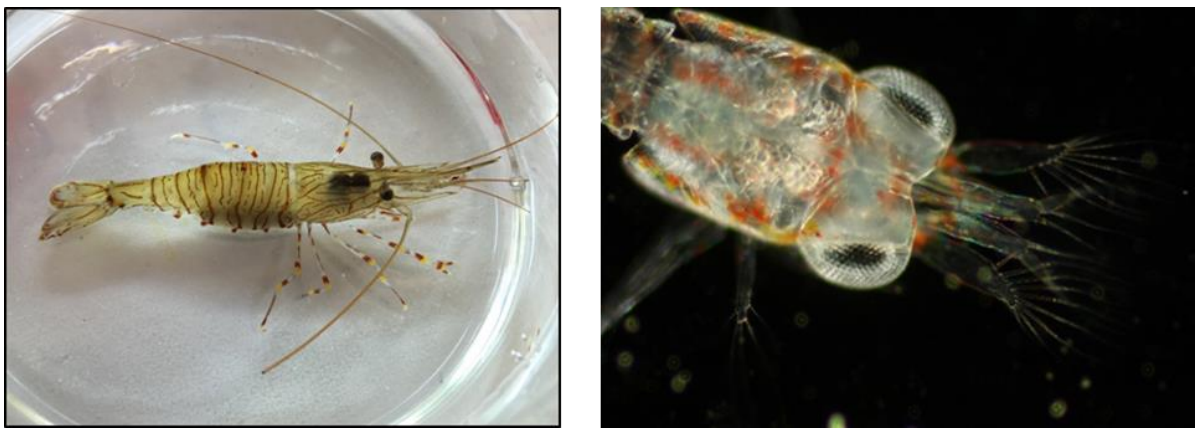


Figure 5.3. Left: Ovigerous *Palaemon serratus*, with eggs distinctly visible, in the laboratory after collection from Amlwch Port. Right: dorsal view of *P. serratus* zoea.

5.3.3 Handling and Rearing of Larvae

5.3.3.1 Experiment E: Effect of Elevated CO_2 and Salinity on Larval Development of *P. varians* Larvae

For the experiment, a selection of larvae from each female shrimps' individual brood was used upon hatching. Larvae were reared in a fully programmable automatic

incubator with a temperature of 18°C and a 12:12 hour light:dark photoperiod and exposed to the experimental conditions outlined below. A 4-hour feeding regime was used (to mimic natural environmental conditions of limited access to prey) and larvae were exposed to different controlled conditions of salinity and pCO_2 combinations. Response variables consisted of mean duration of development through to juvenile 1 stage, number of larval instars through to juvenile 1 stage, larval developmental pathways (the number of moults can be variable depending on when zoea reach an appropriate mass to reach the subsequent stage – typically less than 8 moults in total) through to juvenile 1 stage and percentage survival through to juvenile 1 stage.

Larvae were reared individually, following the methods detailed in Gonzalez-Ortegon and Giménez (2014), but using 100ml screw top rearing vessels. Larvae were assigned to a combination of salinities (salinity = 33 and 25) and pCO_2 levels (400 and 1,000 µatm). The experiment used larvae that hatched from 4 individual ovigerous females, consisting of 40 larvae per female (10 larvae per treatment combination × 2 × 2 = 40 larvae) and therefore resulting in a total of 160 larvae, with 40 larvae per female (40 × 4 females = 160 larvae).

Water initially used was pre-heated to 18°C using carboys of water at the correct treatment condition within the incubator (i.e. the same temperature as that in which the embryos developed). Water was treated in the same way for subsequent daily water changes. The temperature was chosen because it is environmentally realistic and larval development has been shown previously to be successful at this temperature (Oliphant 2013). The salinity values chosen so as to remain within the range of tolerance of both species. The pCO_2 level was chosen to mimic current and near future scenarios as outlined in Chapter 2.

Larvae were fed every day throughout the experiment, with 4-hour exposures to freshly hatched *Artemia* sp. nauplii (with an ad libitum concentration of around 10 nauplii ml-1, see Chapter 2), this maintained pH until the daily water change took place, as outlined in Chapter 2. Water changes were performed daily across all treatments, whereby *Artemia* sp. was fully removed after 4 hours of food exposure by replacing the water in each rearing vessel with the required treatment condition. The 4-hour feeding regime simulates what could be expected of temporal patterns of access to

prey on a daily basis due to patchiness in plankton distributions and of diel-vertical migration of larvae (e.g. Sulkin, Blanco et al. 1998, see also Chapter 2). No control feed group was used (i.e. 24 hours access to food), due to issues of maintaining pH in small static vessels as outlined in Chapter 2. Treatment water was obtained from the OA system outlined in Chapter 2.

Larvae were checked at daily intervals in order to record moults and mortalities. Newly hatched fresh *Artemia* sp. nauplii was added for a period of 4 hours and then subsequently removed when the culture water was replaced. Experiments continued until all the larvae had either reached the first juvenile stage or died. The stage of the juvenile was identified by looking at key morphological features (i.e. resembling an adult counterpart with very long antennae, pereiopods and pleopods that are both functional and well-developed) and observing benthic behaviour, as opposed to free-swimming (Gonzalez-Ortegon and Giménez 2014). The duration that larvae were exposed to the different experimental conditions varied with treatment, according to the moult cycles of individuals.

5.3.3.2 Experiment F: Effect of Elevated CO_2 and Salinity on Larval Development of *P. serratus* Larvae

For the experiment, a selection of larvae from each female shrimps' individual brood was used upon hatching. Larvae were reared in a fully programmable automatic incubator with a temperature of 18°C and a 12:12 hour light:dark photoperiod and exposed to the experimental conditions outlined below. A 4-hour feeding regime was used as in the case of *P. varians* and larvae were exposed to different controlled conditions of salinity and pCO_2 combinations. Response variables consisted of mean duration of development through to juvenile 1 stage, number of larval instars through to juvenile 1 stage, larval developmental pathways through to juvenile 1 stage (i.e. the number of moults that occurred before reaching the first juvenile stage; see Chapter 1 Section 1.9.2) and percentage survival through to juvenile 1 stage.

*There are four main developmental stages in the life cycle of *P. serratus*. Larvae, known as zoea, are adapted to swimming among the plankton. There are usually around 8-9 of these zoeal instars (Fincham 1983, Fincham and Figueras 1986), however this is largely governed by environmental variables such as salinity, temperature (Kelly, Tully et al. 2012) and availability of food (Reeve 1969). The final instar metamorphoses and moults into a stage similar to adults, a post larval instar (Reeve 1969, Kelly, Tully et al. 2012).

Larvae were reared individually as detailed in Gonzalez-Ortegon & Giménez (2014) and using the protocol employed for *P. varians* (e.g. 100ml screw top vessels; combination of salinities of 33 and 25; pCO_2 levels 400 and 1,000 µatm). The experiment used larvae that hatched from 3 individual ovigerous females, consisting of 40 larvae per female (10 larvae per treatment combination × 2 × 2 = 40 larvae) and therefore resulting in a total of 120 larvae, with 40 larvae per female (40 × 3 females = 120 larvae).

Larvae were placed into the incubator at 18°C, this ensured a gradual change in temperatures over 4 hours, allowing larvae to adapt to experimental temperatures more slowly. For subsequent daily water changes, water was pre-heated to 18°C using carboys of water at the correct treatment condition within the incubator (i.e. the same temperature as that in which the embryos developed). Water was treated in the same way for subsequent daily water changes. As in the case of *P. varians*, *P. serratus* develop successfully at 18ºC and salinity 25 (Gonzalez-Ortegon and Giménez 2014). Larvae were reared under limited access to prey of 4 hours per day as in the experiment of *P. varians* and larvae were checked daily for moults or dead individuals.

5.3.4 Data Analysis

The effect of environmental variables on survival was analysed through a generalised linear model, based on the binomial distribution and logit link function. Statistical analysis was based on model selection using the Akaike Information Criteria (AIC) (Zuur, Leno et al. 2009). Models were run in R (Team 2013). Model selection was as follows: when the AIC of two models differed by >3 the model with lower AIC was

selected; if the difference was <3 and the simpler model had the lowest AIC, that model was selected; if by contrast, the more complex model had the lower AIC the log-likelihood test to check for significance was used. The factors tested were pH (fixed), salinity (fixed) and female of origin (= “female”; random).

The effects of environmental variables on the duration of development were tested using mixed modelling using the package lm4 (function glmer for survival) and nlme (function lme or gls, for duration of development) considering female or origin as a random factor. Generalised linear model was used for survival (based on the binomial distribution) because each individual was reared in a separate container and thus each replicate unit can give only two values (1= alive, 0= dead). Duration of development was assumed to follow normal distribution. In the initial stage, a model with random terms was compared with one not containing random terms. For duration of development, models with variance heterogeneity were also considered and implemented using the varIdent constructor function. In the second stage, the best random structure was set and then the model comparison focused on models differing in the random structure. Examples of R code are given in the Table 5.1.

Table 5.1. Examples of R code showing the structure of the full model, containing random terms depending on the female of origin (ffem) but by salinity (fsal) and pCO_2 (fph).

Model type	Fixed part	Random part
Full mixed model	D~fph*fsal,	random= list(ffem = pdDiag(~fsal*fph))
Reduced mixed model	D~fph*fsal,	random=~1+fsal|ffem
Variance heterogeneity	D~fph*fsal,	weights=varIdent(form=~1|fsal)

5.4 Results

5.4.1 Experiment E: Effect of Salinity and pCO_2 on *P. varians*

5.4.1.1 Survival

Survival and development of larvae of *Palaemon varians* was little affected by pCO_2 or salinity in terms of survival and development: none of these factors were retained in the best models. Survival decreased progressively from hatching to the fourth stage

(Fig 5.4), but it was consistently high (> 70% Table 1.). Lower salinity seems to influence developmental pathway.

Table 5.2. Percentage survival of *P. varians*, showing very high survival across all treatment conditions

Treatment	Salinity 33		Salinity 25	
	pH 8.1	pH 7.7	pH 8.1	pH 7.7
% Survival	100	100	100	90
	87.5	100	100	75
	70	100	100	90
	100	90	90	100
Average %	89.375	97.5	97.5	88.75

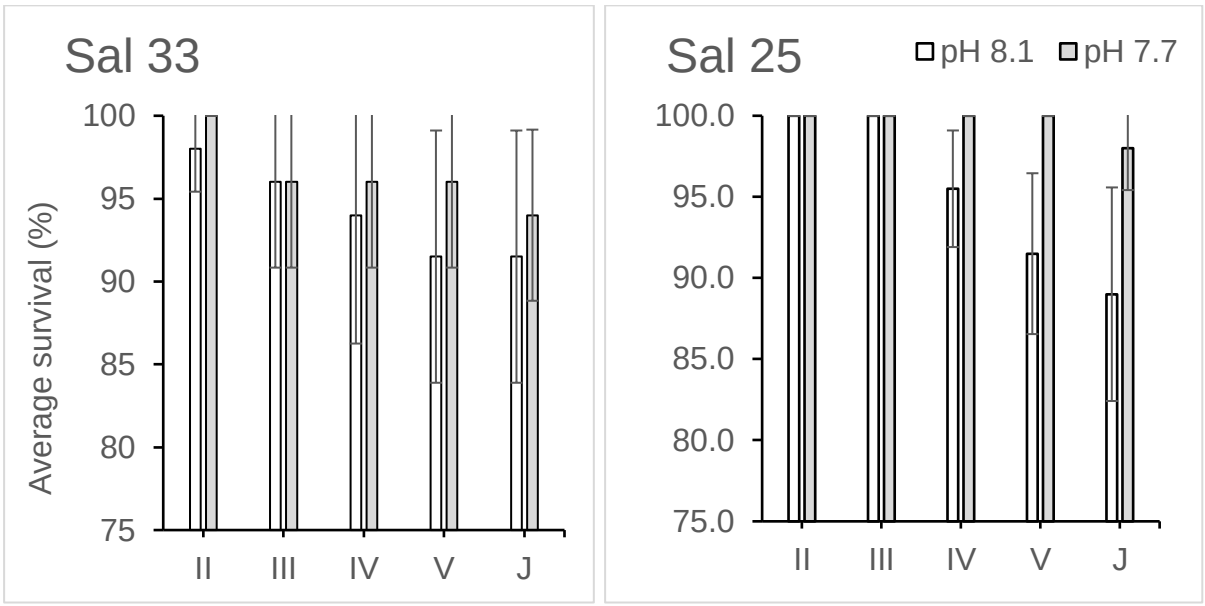


Figure 5.4: *P. varians*. Changes in average number of survivors from hatching to the juvenile stage in response to pCO_2 and salinity. Values are averages from larvae obtained from (n = 3 females); error bars are standard deviation.

5.4.1.2. Duration of Development

Duration of development was little affected by salinity or pCO_2 (e.g. zoea III and juvenile, Fig. 5.5) at all but one of the stages studied: in those cases, none of the factors were retained in the model (Table 5.3). The only factor retained in a model (for stage IV) was salinity but such effect disappeared at the juvenile stage.

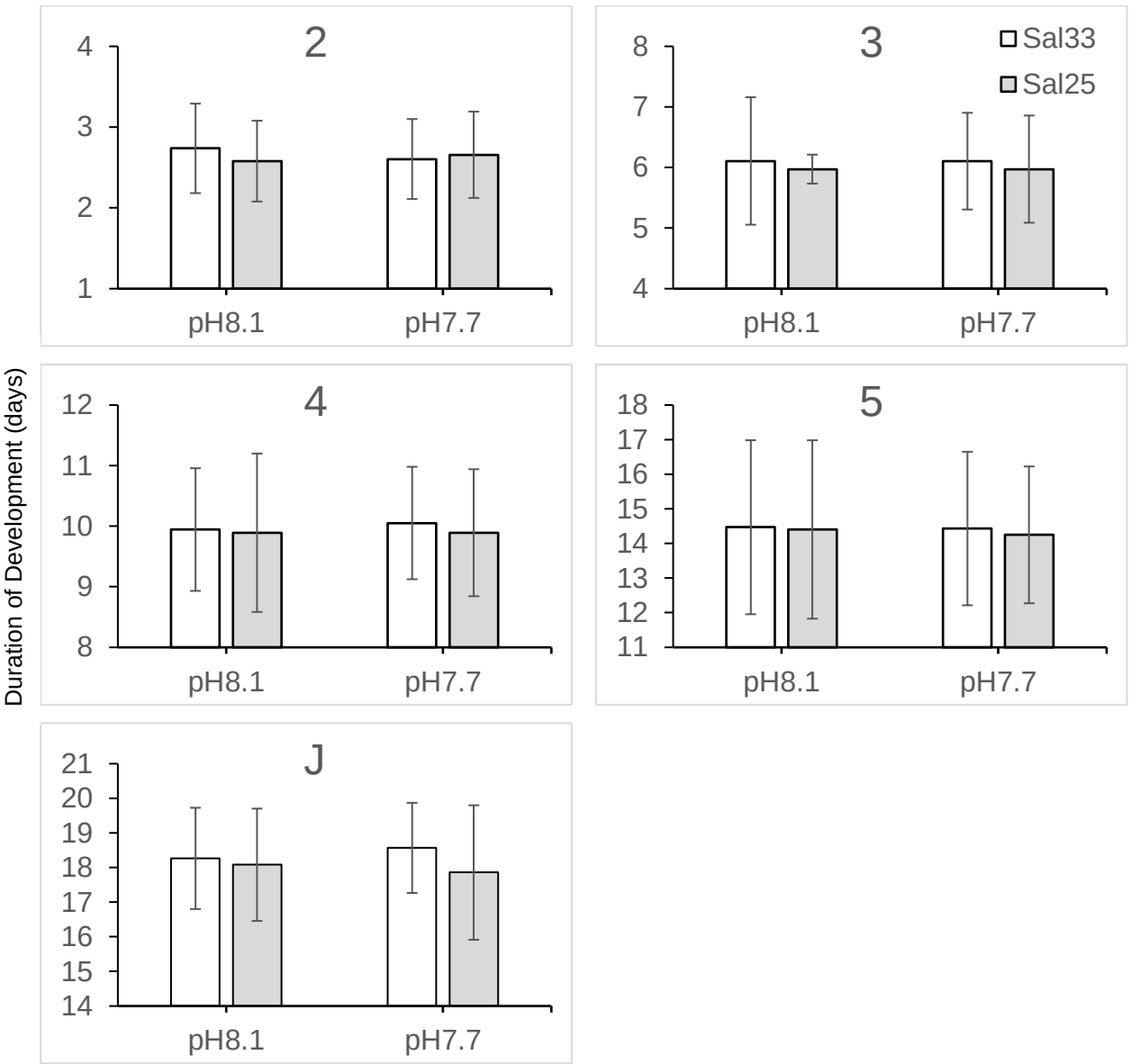


Figure 5.5: *P. varians*. Effect of pCO_2 and salinity on developmental duration from stage 2 to the first juvenile stage over time (n = 3 females). Non-significant effects.

P. varians is able to develop through alternative developmental pathways characterised by a different number of zoeal instars. The combination of low salinity and pCO_2 appeared to increase the porportion of larvae following longer pathways, but such proportion was still low (Fig. 5.6)

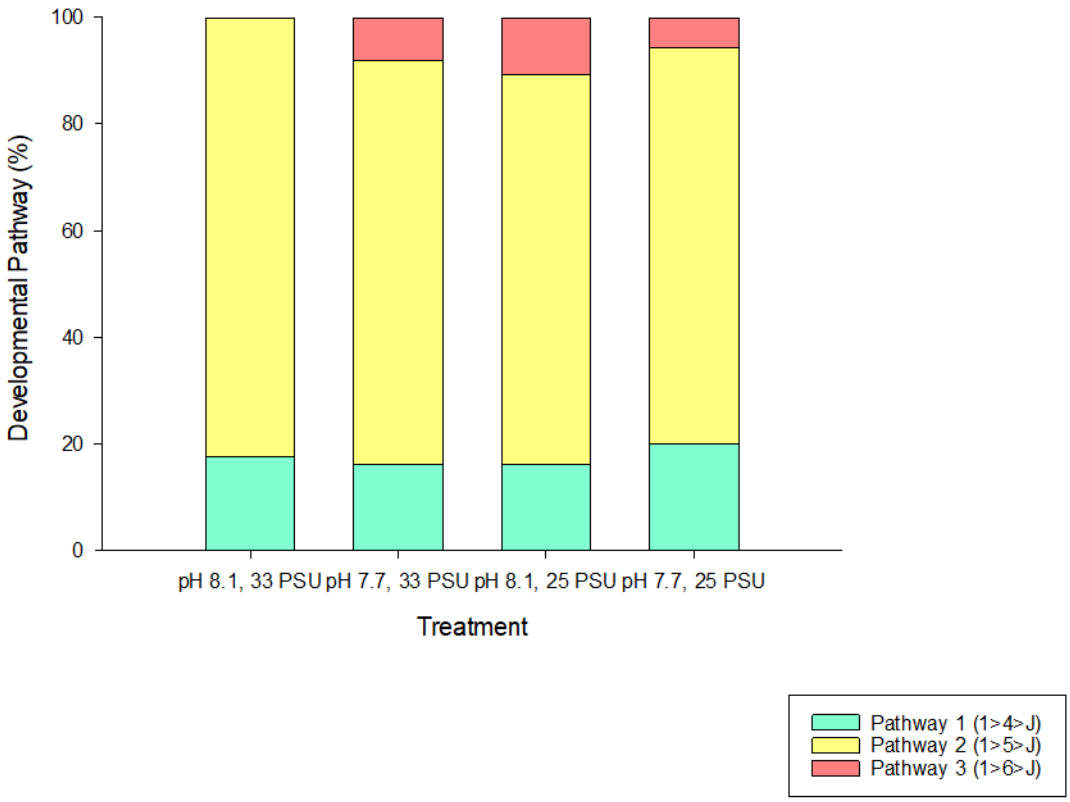


Figure 5.6. *P. varians*. Effect of pCO_2 and salinity on developmental pathway (% of individuals following a certain pathway of development) from hatching to the first juvenile stage over time (n = 3 females).

5.4.2 Experiment F: Effect of Salinity and pCO_2 on *P. serratus*

5.4.2.1 Survival

Effects of pCO_2 and salinity on survival of larvae of *P. serratus* were minimal (Fig. 5.7) and none of those factors were retained in the models (Table 5.5). Over the ca. 50 days of larval development, the number of surviving larvae decreased to 50%-60% in all treatment combinations.

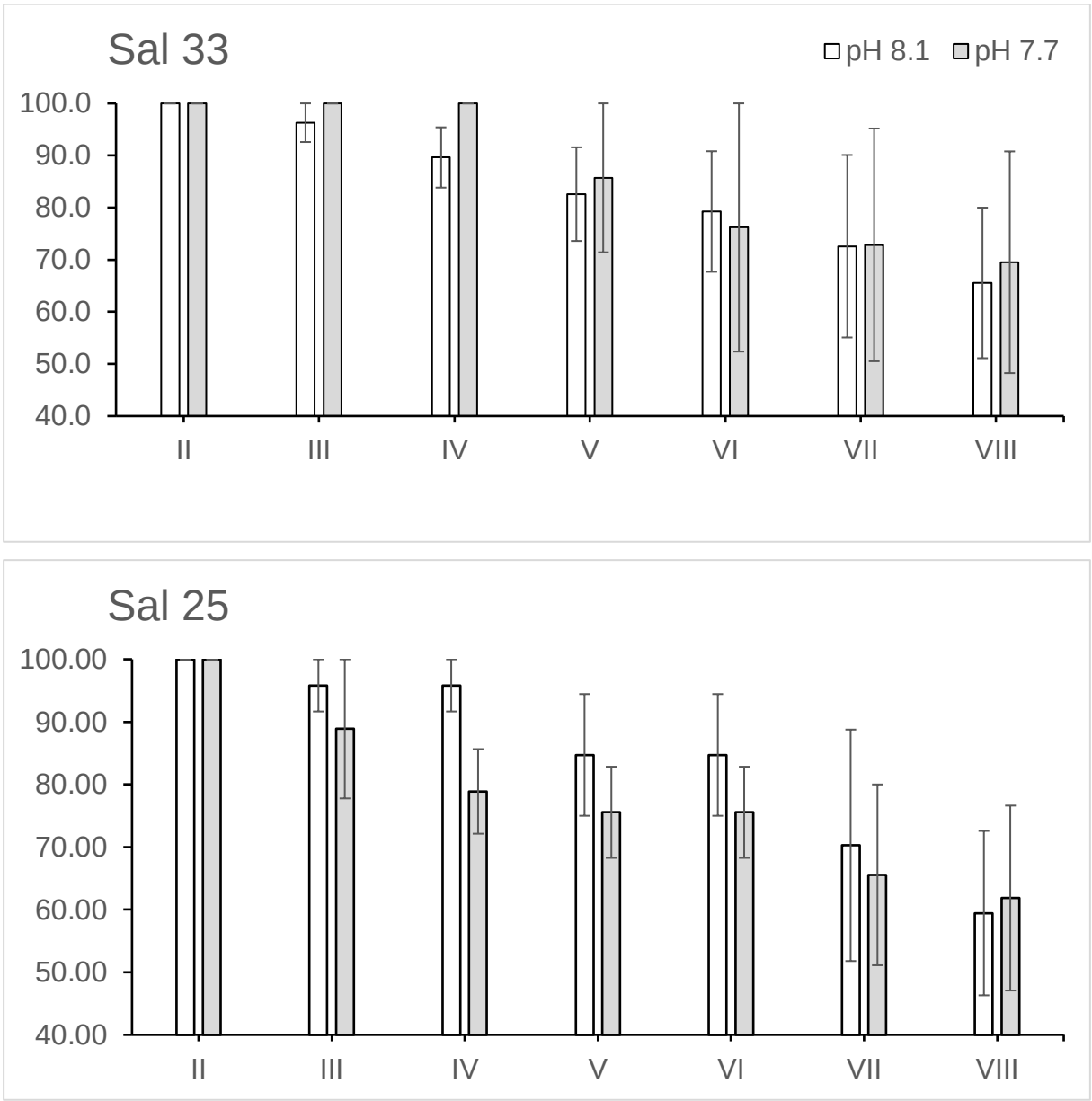


Figure 5.7: *P. serratus*. Effect of pCO_2 and salinity on survival from hatching to stage 8 (n = 3 females). Non-significant effects.

5.4.2.2 Duration of Development

For *Palaemon serratus,* duration of development varied little with pCO_2 and salinity (Fig. 5.8): the best model retained the interaction term of salinity and pH for development to zoea IV (Table 5.6); however, for other stages none of the terms representing treatment effects were retained. Instead, duration of development to the juvenile stage varied between 35-40 days across stages (Fig 5.8).

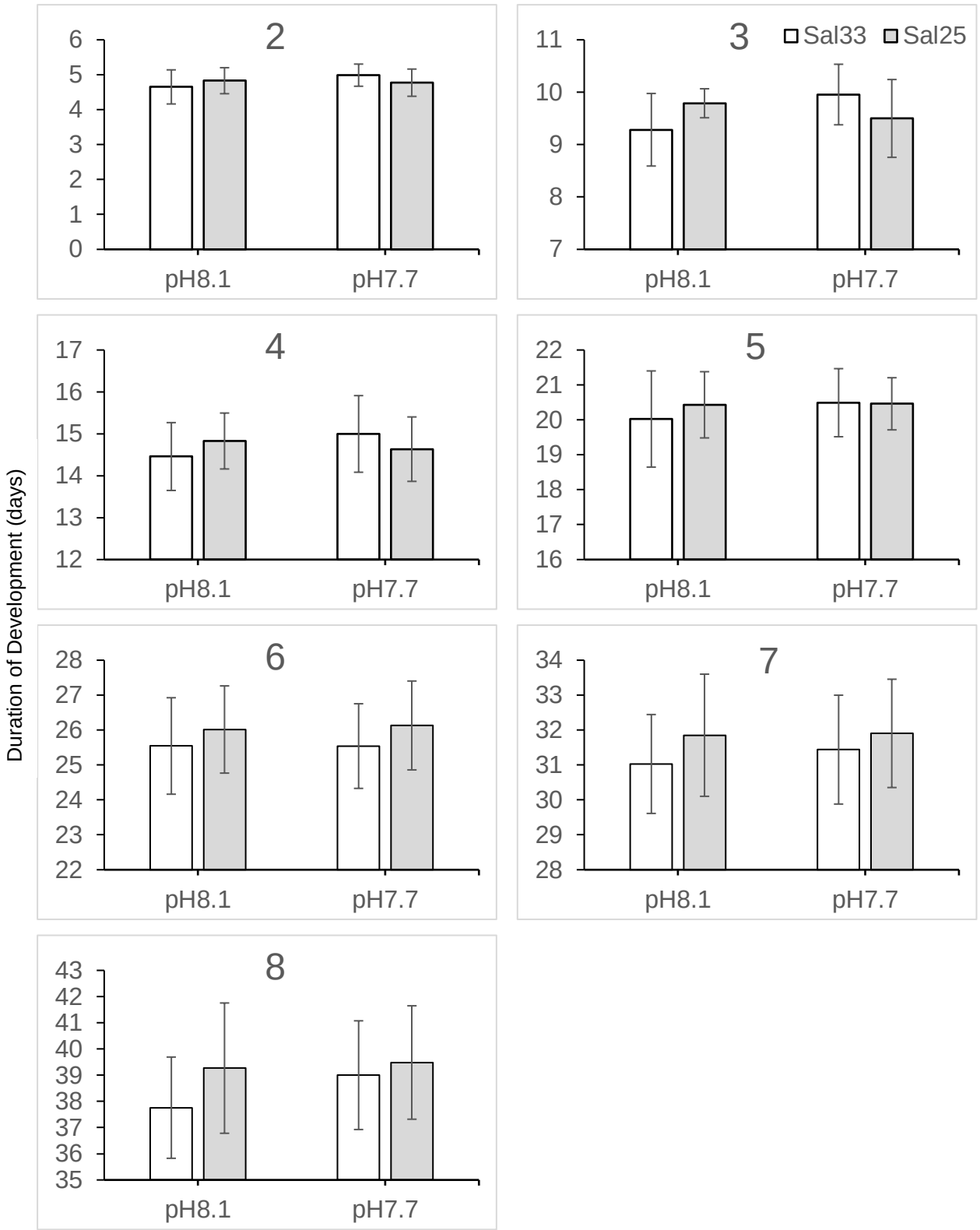


Figure 5.8: *P. serratus*. Effect of pCO_2 and salinity on developmental duration from stage 2 to 8 over time (n = 3 females). Significant effect for pH x Salinity interaction for developmental duration at early stages, stage 4, non-significant effects at later stages, stage 8.

5.5 Discussion

The experimental results show that larvae of both *P. varians* and *P. serratus* are highly tolerant to increased pCO_2 (reduced pH) when reared under both seawater and moderately low salinity (25), under a realistic temperature (18ºC) and under restricted access to prey. Neither salinity nor pH were retained in my best statistical model. In addition, the differences among treatment means were small: for instance, survival of *P. varians* at the end of development varied between 80-90% depending on treatment. In addition, the trend was consistent across larvae hatching from different females. We therefore conclude that larvae of both species are highly tolerant to end of century conditions of elevated pCO_2. In addition, this result reinforces the hypothesis that crustaceans are one of the groups of marine organisms that will be able to better cope with climate change (Kroeker, Kordas et al. 2010, Whiteley 2011). The relevance of such results for understanding interspecific variation in responses to ocean acidification is discussed below, along with the potential mechanisms driving tolerances to change.

The original hypothesis was that larvae of *P. varians* would be more tolerant than those of *P. serratus*. Indeed, larvae of *P. varians* showed high tolerance but also very high survival rates during the experimental periods. I expected a high level of tolerance because waters at saltmarshes are characterised by variations in salinity and pH. At Newborough, where egg carrying females were collected, values of pH recorded at the time of collection were lower than 7.7. Hence, embryos are already developing in conditions that may be considered well beyond those predicted for end of century pH levels in ocean waters. I hypothesised that such a capacity to tolerate low pH is transmitted to the larvae and maintained at least over the first zoeal stage. I based my hypothesis on the fact that in other estuarine decapod crustaceans, tolerance to temperature or salinity at early larval stages is enhanced if embryos experience similar temperatures or salinities (Giménez and Anger 2003). In the crab *Petrolistes cinctipes*, embryos are exposed to variable conditions of pH in intertidal zones; their larvae appear to be highly tolerant to low pH (Ceballos-Osuna, Carter et al. 2013). My data shows however, that tolerance is high during the entire period of larval development; otherwise, survival should have decreased in response to low pH in the advanced larval stages. Overall, the present results also confirm the high tolerance of *P. varians*

larvae to food limitation as has been found previously (Oliphant 2013, Oliphant and Thatje 2014). However, we expected reduced tolerance to low pH in *P. serratus*. For the studied population, egg carrying females occur in open waters where salinities are around salinity 35. Hence, embryos should develop under conditions of present day pCO_2 and high salinity; at least from the perspective of post-zygotic maternal effects, we should have found reduced survival in the advanced larval stages.

Both *P. varians* and *P. serratus* showed much higher tolerances to end of century elevated pCO_2 levels than larvae of other decapods. This contrasts slightly with observations on effects of OA on larval stages of marine coastal decapods and other crustaceans. For instance, Bechmann et al. (2011) studied larvae of the shrimp *Pandalus borealis* and found that survival was unaffected by near-future pH levels, but there was a significant delay in developmental duration after a 5-week exposure. Arnberg et al. (2013) also studied the effects of temperature and pH on larvae (up to stage 4) of *P. borealis* and also found that pH increased developmental duration. Effects of OA on larvae of the spider crab were recorded as increases in duration of development (Walther, Anger et al. 2010). Effects of OA on larvae of barnacle *Amphibalanus amphitrite* appear to depend on temperature, but most are reflected as changes in duration of development (McDonald, McClintock et al. 2009, Pansch, Nasrolahi et al. 2012). From the examples above it is not possible to make firm conclusions, due to the diversity of methods and experimental conditions used in the different studies. However, we can formulate the hypothesis that estuarine species should be better suited to tolerate the defined “end of century” levels of ocean acidification, than marine species, if such larvae develop in coastal estuarine waters. Whether actual climate change impacts will influence such species by, for example, further reducing pH in estuarine or saltmarsh waters, is still be ascertained.

It is difficult to establish the factors responsible for explaining the high tolerance of *P. serratus* and *P. varians* to end of century pCO_2 levels. Such tolerances may be provided by the already existing strong capacity of their larvae to tolerate low salinity. Tolerance of low salinity is based on iono-regulation, mainly at the extracellular level, by means of active uptake of ions taking place in specialised cells called ionocytes in the gill epithelia. Dissanayake et al. (2010) studied adult *P. serratus* and established that Palaemonids are efficient hypo-ionic/osmotic regulators in marine waters, with the

predominantly subtidal species (*P. serratus*) being similarly tolerant to the intertidal species *P. elegans* – although with potentially differing mechanisms of regulation (Dissanayake, Clough et al. 2010). The ability to iono-regulate is associated with the ability to regulate acid-base changes in the extracellular compartment (Whiteley 2018) and may help to avoid the effects of elevated external pCO_2. The tolerance of the subtidal species to elevated pCO_2 suggests that other mechanisms may also be involved in providing protection against external changes in carbonate seawater chemistry.

5.5.1 Conclusion

Larvae of both *P. varians* and *P. serratus* were highly tolerant to end of century changes in ocean chemistry. This reinforces the current thinking that crustaceans are one of the groups that are better pre-adapted to future ocean acidification.

At the qualitative level, comparisons of responses between the Palaemonids studied here and other crustaceans, suggest that estuarine – euryhaline species are better prepared to withstand the conditions of elevated pCO_2 defined as end of century. However, it is still not clear how actual changes in pCO_2 in estuarine-saltmarsh waters will impact such species, especially in the longer term.

5.6 Appendices (Chapter Five)

5.6.1 Experiment E: Effects of Salinity and pCO_2 on *P. varians*

5.6.1.1. Duration of Development

Table 5.4. *Palaemon varians*: model selection for duration of development to selected stages. Model selection was carried out using the Akaike information criteria (AICc). Selection for random terms were based on restricted maximum likelihood fitting (REML) while that of fixed terms was based on maximum likelihood (ML) fitting. Abbreviations: ♀: female of origin; s: salinity; pCO_2: temperature; H: variance heterogeneity depending on combinations of salinity and pCO_2 (pCO_2:S), salinity or pCO_2. Homogeneity: variance homogeneity); Null: overall mean). The best overall model contains both the best random and fixed term as highlighted in red.* significant effect of salinity (p=0.0042).

Model selection: Random (REML)	AIC			
Term	II	III	IV	J
♀: pCO_2:S	141	245	345	516
♀:S		241	350	514
♀: pCO_2	137	244	344	513
♀	136	240	347	510
H(pCO_2:S)	239	371	446	525
H(pCO_2)	235	369	442	523
H(S)	236	367	448	521
Homogeneity	234	367	447	521
Model selection: Fixed (ML)				
Full factorial	124	232	339	508
F+T	123	230	337	509
pCO_2	122	230	344	507
S	121	228	336*	507
Null	120	228	342	505

5.6.2 Experiment F: Effect of Salinity and pCO_2 on *P. serratus*

5.6.2.1 Survival

Table 5.5. *P. serratus*: model selection for survival through first nine stages using generalised linear model, with binomial family. Model selection was carried out using the Akaike information criteria (AIC). The mixed model contained female of origin as random factor. Abbreviations: ♀: female of origin; s: salinity; pCO_2: temperature; Null: overall mean. For all stages but Stage IV, the best model (in red) contained female as random factor.

Model selection:	AICc						
Term Stage	III	VI	V	VI	VII	VIII	XIX
Mixed full factorial	40	67	104	108	119	141	152
Fixed full factorial	45	65	108	117	140	150	160
F+T	39	65	102	106	117	139	150
pCO_2	39	**64**	100	105	116	138	148
S	39	69	101	105	115	137	148
Null	**39**	69	**99**	**103**	114	136	**146**

5.6.2.2 Duration of Development

Table 5.6. *Palaemon serratus*: model selection for duration of development to selected stages. Model selection was carried out using the Akaike information criteria (AICc). Selection for random terms were based on restricted maximum likelihood fitting (REML) while that of fixed terms was based on maximum likelihood (ML) fitting. Abbreviations: ♀: female of origin; s: salinity; pCO_2: temperature; H: variance heterogeneity depending on combinations of salinity and pCO_2 (pCO_2:S), salinity or pCO_2. Homogeneity: variance homogeneity); Null: overall mean). The best overall model contains both the best random and fixed term as highlighted in red.

Model selection: Random (REML)	AIC						
Term	III	VI	V	VI	VII	VIII	XIX
♀: pCO_2:S	221	273	294	319	306	329	291
♀:S	220	270	292	317	304	327	289
♀: pCO_2	218	269	290	317	304	326	291
♀	216	267	288	313	300	323	287
H(pCO_2:S)	231	319	314	357	343	334	292
H(pCO_2)	233	315	313	353	340	330	288
H(S)	232	316	314	353	340	330	289
Homogeneity	231	314	315	351	338	328	287
Model selection: Fixed (ML)							
Full factorial	209	264	286	313	302	327	292
F+T	223	268	286	312	301	326	293
pCO_2	221	266	285	313	302	327	293
S	223	267	285	310	300	327	294
Null	221	265	284	311	301	328	294

6 Chapter Six: Summary and Conclusions

Ocean acidification (OA) is occurring on a globalised scale and may cause disruptions to crustacean larval performance. As many marine crustacean larvae develop in a relatively stable pelagic environment, they are likely to be sensitive to perturbations in their surrounding environmental conditions. However, despite the critical role such species play in marine ecosystem functioning, little robust information exists on the impacts of OA on survival and development of brachyuran crustacean larvae, especially in conjunction with additional environmental stressors, such as salinity, temperature and food availability, which are predicted to co-vary with OA. A failure to completely understand the range of species' tolerance capacities in a comprehensive manner may result in flawed inferences into the effects of marine environmental changes being made (Kroeker, Micheli et al. 2013, Wittmann and Pörtner 2013, Whiteley 2018).

From previous research, it is clear that species and/or life history stages are not likely to respond uniformly to near-future predicted climatic induced changes in ocean chemistry, particularly in conjunction with multiple stressors (Przeslawski, Byrne et al. 2015). While many adult crustaceans are likely to be capable of coping with fluctuations in their environmental conditions, embryonic and larval stages may potentially be more susceptible. Additionally, if species lack a compensatory capacity to cope with the changing conditions, their performance may be detrimentally affected, which in turn may impact recruitment, exacerbating potential early stage impacts (Whiteley 2018).

The aim of this thesis was to elucidate the effect of elevated pCO_2 in conjunction with multiple stressors on a variety of decapod crustacean species, in terms of the little studied early life stages, with potentially differing abilities to tolerate changes in salinity levels, depending on their ecological niche. The species studied were: *Carcinus maenas*, *P. serratus* and *P. varians*, which are important species in terms of the roles they play in ecosystem functioning, over trophic food webs and as commercially important fisheries.

Exposure of early larval stages to combinations of salinity, temperature and food limitation in *C*. *maenas* revealed that high temperature ameliorated the effect of low salinity on survival and developmental duration. Limited access to food also affected developmental duration, but exposure to pCO_2 alone in a second experiment only affected survival, and low salinity alone had no effect.

Exposure of early juvenile stages of *C. maenas* collected from the field to elevated pCO_2 and reduced salinity, in an aquarium exposed to seasonal variations in temperature, revealed that developmental duration was significantly affected by both factors, but to varying levels. Survival, on the other hand was only influenced by pCO_2 and salinity in the later juvenile stages. In the situation where temperatures were constantly held at 15ºC. These results suggest the possibility of a potentially physiologically sensitive bottleneck within the life-cycle of *C. maenas*.

Exposure of early larval stages of the estuarine species, *P. serratus* and *P. varians*, to pCO_2 and salinity had no effect on either survival or developmental duration (Chapter 5). In *P. serratus*, developmental duration was negatively influenced by the interaction of elevated pCO_2 and low salinity, but the effect was weak and restricted to the early stages studied.

When the effects on all species examined are considered together, no consistent inter-species pattern is evident (Table 6.1). While shrimp larvae, when compared to crab larvae, appear to be generally more resilient to the perturbations examined, there were clear differences between the two members of the *Palaemon* genus examined, with the estuarine species being the most resilient to multiple stressors (Table 6.1). As such, it is important to determine species-specific effects, avoiding drawing conclusions from even closely related species.

It is clear, however, that multiple stressor interactions play a critical role in determining OA effects. Additionally, based on the observation that female brood and also collection week influenced results, there are clear phenotypic, spatial and maternal variability effects. This is an important consideration for future research, as conclusions based on individuals collected over a short time frame are unlikely to represent the actual average population response.

Furthermore, experimental design, based on Chapter 4, looking at *C. maenas* megalopa, is an important consideration, with seasonal changes in temperature having different effects than constant temperatures.

Table 6.1: Effects of multiple stressors (pCO_2, Sal [salinity], T [temperature], Fo [food], Fe [Female], W [week]) on Developmental Duration (DD) and Survival (S). Green = the effect of the variable is significant in isolation. Blue = the effect of the variable is only significant as part of an interactive effect. Variables that have significant effects in isolation, may also have interactive effects with other variables. Where there are interactive effects, interacting variables are indicated with asterisk (*) or sets of asterisks (**, ***, ****) where multiple interactions are present. Not all stages shown for *P. serratus* shrimps as they follow variable developmental pathways often exceeding 5 stages. - = a negative influence

		Stage II		Stage III		Stage IV		Stage V	
		DD	S	DD	S	DD	S	DD	S
Chapter 3: *C. maenas* larvae Exp. A	Sal	*	* ***	*	* *** ****	** ***	* ** ***		*
	T	**	* ** ***	*	** *** ****	* ** ***	** ***		*
	Fo	* **	* ***	*	* ** ****	* ***	* ***		
	Fe			*					
Chapter 3: *C. maenas* larvae Exp. B	pCO_2				-		-		
	Sal								
	Fe								
Chapter 4: *C. maenas* juveniles (Seasonal temperature with week)	pCO_2	*				*	*		*
	Sal					*			* **
	W	*					*		**
Chapter 4: *C. maenas* juveniles (Seasonal temperature no week)	pCO_2								
	Sal								
	pH	*				** ***			*

		Stage II		Stage III		Stage IV		Stage V	
		DD	S	DD	S	DD	S	DD	S
Chapter 4: *C. maenas* juveniles (Constant temperature with week)	Sal	**				* ** ***			
	W	* **				* ***			*
Chapter 4: *C. maenas* juveniles (Constant temperature no week)	pCO_2					*			
	Sal					*			
Chapter 5: *P. varians* larvae	pCO_2								
	Sal								
Chapter 5: *P. serratus* larvae	pCO_2	*-		*-		*-			
	Sal	*-		*-		*-			
	F	*							

6.1 Perspectives

The approaches used during this thesis, in order to investigate the effects of elevated pCO_2, salinity, food availability and temperature on a variety of early life stages of decapod crustaceans, could be used to examine the potential effects of these multiple stressors on other marine invertebrates that have complex life cycles; encompassing embryos, meroplanktonic larval stages, and how they could affect successful recruitment of other species or populations.

It might be hypothesised that other species that live in stable marine environments, may be more susceptible to climatic induced and other environmental stressors, as they may lack the adaptive mechanisms to cope with stressful conditions, that perhaps species living in more changeable environments may possess. For successful recruitment, it is important that the influx of juveniles into an adult population are maintained. For the species studied here, berried females are present twice a year in the study region, therefore there is potential for two chances of recruitment.

Often in OA (either pH or elevated pCO_2) studies, conclusions have been acquired from evidence derived in acute (i.e. short-term) exposure studies (such as hourly, daily or weekly). In addition to often measuring the responses of single taxa, at only one life-history stage (Whiteley 2011). Responses of species to exposure to elevated pCO_2 over prolonged time scales, in addition to encompassing several life stages, or even generations are not well known (Whiteley 2011, Gunderson, Armstrong et al. 2016).

It is important that studies with chronic, or longer exposure time-frames (such as over months, or perhaps less feasibly, years), ideally with successive generational studies (although in decapod crustaceans this is not likely to be feasible), is required to be able to effectually understand what the persistent, or longer-term, implications are of species in response to changes in the oceanic chemistry (Whiteley 2011, Suckling, Clark et al. 2014, Suckling, Clark et al. 2015).

A variety of marine invertebrates and life-history stages should be assessed to identify the most sensitive species and/or developmental stages, thereby infering where population bottlenecks may arise (Ries, Cohen et al. 2009, Whiteley 2011, Suckling, Clark et al. 2014, Suckling, Clark et al. 2015). This has particular importance for zooplanktonic crustaceans and those that form a part of the marine nekton and benthos. For example, as they provide a vital protein source for taxa at higher trophic-levels, in addition, healthy populations of larvae/juveniles ensure the maintenance of healthy adult populations of marine invertebrates (Whiteley 2011).

6.2 Thesis Specific Future Work

Here, I list a number of potential studies arising specifically from this thesis:

1) Develop a stable flow-through system for food limitation experiments in conjunction with elevated pCO_2. This was not possible for the larval experiments here due to temperature limitations and potential damage of mesh to rostral spines. This would reduce the researcher effort, in terms of performing water changes, and potentially enable longer term studies, with larger numbers of larvae.

2) To determine if multiple stressors affect embryonic development (Ellis, Bersey et al. 2009). For instance, expose subsequent larvae after hatching to determine if pre-exposure influences response to multiple stressors.

3) Determine if prolonged juvenile exposure to multiple stressors e.g. for shrimps some effects only become pronounced at or after the second juvenile stage (Gonzalez-Ortegon, Blasco et al. 2013). But this would require high survival of larvae from hatching, so the use of greater numbers at the experimental offset is important, or perhaps rearing in high density batches at the start of the experiment in order to enable high survival to later stages.

6.3 General Conclusions

Serious scientific concern surrounds the increasing pCO_2 levels in Earth's atmosphere and oceans (Caldeira and Wickett 2003, Caldeira and Wickett 2005). The effects of CO_2 acidified seawater are likely to significantly influence life in the marine environment (Caldeira and Wickett 2003, Fabry, Seibel et al. 2008, Gattuso and Hansson 2011). Some species, populations and life-history stages may be exhibit resilience and prosper. However, others may be adversely impacted, potentially causing implications for other trophic levels and regime shifts may arise (Wood, Spicer et al. 2008, Ries, Cohen et al. 2009). If early life-history stages are detrimentally impacted, a population bottleneck could occur, which may subsequently impair successful recruitment, biodiversity and therefore species composition and ecosystem functioning (Kurihara 2008, Dupont and Thorndyke 2009, Byrne and Przeslawski 2013, Gunderson, Armstrong et al. 2016).

As a result of the accumulation and potential interactions of a host of direct and indirect climate change derived effects (e.g. OA, global warming, changing currents, sea level rise, species' shifts pole-wards) (Caldeira and Wickett 2003), in conjunction with additional anthropogenic influences (e.g. over-fishing, habitat destruction, pollution and exponentially increasing human populations); for many ocean regions, resilience may be severely jeopardised over the coming centuries (Fabry, Seibel et al. 2008, Gattuso and Hansson 2011, IPCC 2014). The synergistic interplay of natural factors in addition to these anthropogenic influences may fuel a cumulative or snowballing effect

that could result in severe and potentially irreversible consequences (Gattuso and Hansson 2011, Harvey, Gwynn-Jones et al. 2013).

If the diversity of roles that marine crustaceans play in the oceans is impacted, important repercussions may occur ecologically, as crustaceans are fundamental in marine food chains for species at higher trophic-levels and because they are important primary/secondary consumers (Whiteley 2011). Socio-economic effects may also arise if fisheries/aquaculture or tourism industries are affected, this is important because optimally functioning marine ecosystems provide a fundamental importance for the maintenance of human livelihoods, health and wellbeing (Gattuso and Hansson 2011). More scientific research, education, stakeholder and government collaboration on both regional and globalised scales is crucial to be able to inform on the consequences of the globalised changes and determine effective mitigation pathways and successful marine management strategies (Gattuso and Hansson 2011, Whiteley 2011).

6.4 Thesis Specific Conclusions

This research highlighted that to be able to gain accurate predictions of responses to climatic induced changes, that are species specific, it is important to consider different life stages, over a variety of species and to consider intraspecific variation within a species and also to consider maternal influences and spatial and temporal variations.

The main conclusions are as follows:

1) *C. maenas* larvae are able to develop under limited access to prey over a wide range of salinities and temperatures.

2) For *C. maenas* zoea larvae exposed to elevated pCO_2 at different salinities there is a consistent stress effect, shown here as a reduction in survival and development.

3) For *C. maenas* megalopa and juveniles, exposed to elevated pCO_2 and different salinities over an extended exposure period, there is no consistent stress effect; instead the effect varies among cohort of larvae settling at different times of the season, perhaps as a result of variations in larval experience or due to genetic variability.

4) Overall, zoea larvae of *C. maenas* appear to be more sensitive to elevated pCO_2 than juveniles, which is consistent with the hypothesis that larvae represent a critical phase of the life cycle with regards to sensitivity to elevated pCO_2.

5) Larvae of *Palaemon serratus* and *Palaemon varians* were not affected by elevated CO_2. Perhaps larvae of *P. varians* are slightly less sensitive to elevated pCO_2 and salinity stress than the coastal shrimp species of the same genus, *P. serratus*, but the difference was minimal.

Whether the results shown here would give rise to populations of more adaptable species, of the species studied in this thesis, remains unknown. However, this could have confounding environmental impacts if, for example, hardier populations of *C. maenas* are more effectively able to decimate prey species that may be more susceptible to climate induced changes, such as several bivalve mollusc species.

7 References

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8 Thesis Appendices

8.1 Appendix 1

Abstract and poster presented at the **Aquatic Biodiversity and Ecosystems:** Evolution, Interactions and Global Change Conference at The University of Liverpool, 30th August-4th September 2015.

Effects of multiple stressors on the development and performance of the early life stages of decapod crustaceans.

Curry, A.E., Whiteley, N.M., Gimenez, L.

Many marine decapod crustacean larvae develop in relatively stable pelagic environments; therefore, they are likely to be sensitive to perturbations in environmental conditions. The effects of climate change induced ocean acidification (OA) on decapod crustacean larvae are not well understood, particularly when combined with other co-varying environmental stressors. However, species and/or life history stages are not expected to respond uniformly to predicted changes (~2100).

Early life stages of three decapod crustacean species common to Europe (*Palaemonetes varians, Palaemon serratus and Carcinus maenas*) were exposed to ambient (pH 8.1) and near-future (pH 7.7) OA conditions, in conjunction with variations in salinity, temperature and food availability. While larvae of *P. varians* develop in estuarine conditions, those of *P. serratus* and *C. maenas* develop in more stable coastal marine waters.

Responses varied across species, with no apparent affects on development and survival of the early life stages of *P. varians*, and varied affects on development and survival of both *P. serratus* and *C. maenas*. Effects were more apparent with multiple stressors.

Results suggest important intraspecific differences in the capacity to tolerate OA conditions, particularly when combined with other environmental stressors, perhaps reflecting phylogenetic or intraspecific differences in the larval adaptations to variable environments.

8.2 Appendix 2

Effects of multiple stressors on the development and performance of the early life stages of decapod crustaceans

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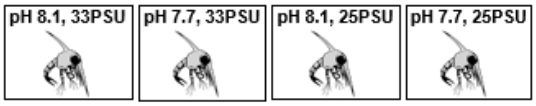
1. Introduction:

- The effects of ocean acidification (OA) on decapod crustacean larvae are not well understood, particularly in combination with other environmental stressors such as salinity.
- Salinity is an important environmental factor for coastal marine species.

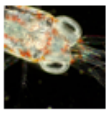
2. Objectives:

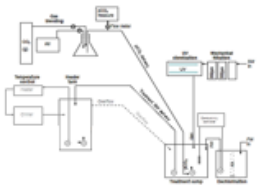
Evaluate the effects of OA and salinity on three species of decapod crustaceans, with differing life histories:
Carcinus maenas, *Palaemonetes varians*, *Palaemon serratus*.



3. Method:

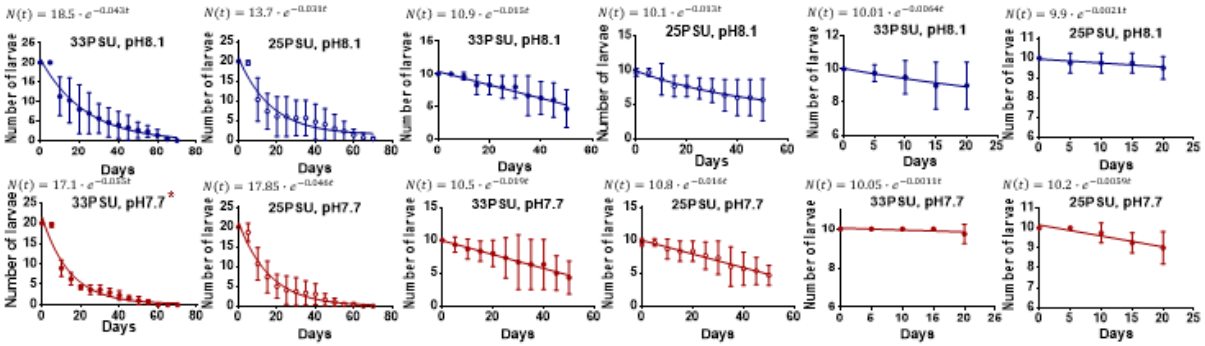
- Experiments: 3-5 egg carrying females were collected from sites around Anglesey, North Wales UK.
- Larvae exposed to the following conditions:
- Statistical analysis:
  Survival - ANCOVA, time as covariate, salinity and pH as factors.
  Duration of development – 3-Way ANOVA, pH and salinity as fixed factors, female random factor.





4. Results:

Survival:

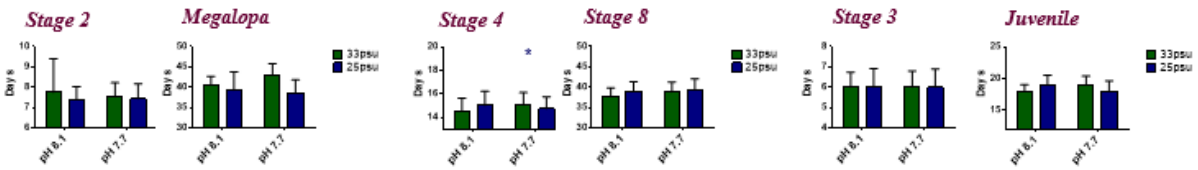


*Significant interaction OA x time (slope)

Non significant effects

Non significant effects

Duration of development:



Non significant effects

*Stage 4: Significant pH X salinity interaction

Non significant effects

5. Conclusions:

P. varians larvae unaffected by OA and salinity

P. serratus larval developmental duration significantly affected by OA and salinity, in early stages.
Experiment ongoing.

C. maenas larval survival significantly affected by OA (larvae from Winter hatches).
Experiment looking at Autumnal hatches ongoing.

Important interspecific and intraspecific differences in the capacity to tolerate OA conditions of the three species, with differing life histories.

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