UNIVERSITY OF SOUTHAMPTON

FACULTY OF ENVIRONMENTAL AND LIFE SCIENCES

Ocean and Earth Science

THE EFFECT OF TEMPERATURE THROUGHOUT INTRACAPSULAR DEVELOPMENT AND ADULT LIFE OF THE EUROPEAN STING WINKLE, *OCENEBRA ERINACEUS* (LINNAEUS, 1758) IN THE GLOBAL WARMING CONTEXT

by

Maria Loreto Mardones Velozo

Thesis for the degree of Doctor of Philosophy

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University of Southampton

Abstract

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Climate change is shifting temperature regimes across Earth, which will result in impacts on the physiology, ecology and distribution patterns of organisms, especially for ectotherms species were nearly all-physiological and behavioural traits are sensitive to temperature. Many predictions assume that each population of a particular species has the same environmental niche; however, these predictions do not consider that across species' geographic distributions, species exhibit intraspecific differences among populations. This could be more pronounced for species that undergo direct development, with restricted connectivity and low gene flow, which increase the potential for individuals to evolve local adaptations to native environmental conditions. This thesis aimed to understand the effect of temperature during the early and adult stages in two populations with different thermal histories of the European winkle *Ocenebra erinaceus* (Linnaeus, 1758).

The results showed that *O. erinaceus* exhibits intraspecific differences during early development in terms of its thermal tolerance and physiological response. Embryos from the warm water population showed a wide, eurythermal tolerance range, exhibiting metabolic adjustment to increased temperatures. On the contrary, embryos from the cold water population showed narrow thermal tolerance windows, showing limited metabolic compensation to high temperatures. The thermal tolerance variation among populations of *O. erinaceus* might be a result of physiological adaptations to local thermal conditions. Moreover, local adaptation during early stages can persists in later ontogenetic stages. Adults from the thermal sensitive population (i.e. cold water) exposed to future ocean conditions showed a partial acclimation and cessation of reproductive investment.

This study demonstrated that the impact of global warming will depend on the physiological adaptation of embryos as well as adults to local environmental conditions. *O. erinaceus* exhibits intraspecific differences at population-scale in its optimal temperatures and thermal limits. In warming scenarios, local extinctions will be expected in northern populations with the potential of the expansion of the distribution range of southern populations to fill the niche space left empty by northern populations.

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Research Thesis: Declaration of Authorship

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Title of thesis: The effects of temperature throughout intracapsular development and adult life of the European sting winkle, *Ocenebra erinaceus* (Linnaeus, 1758) in the global warming context

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Definitions and Abbreviations

β-act	β-actin gene
β-tub	β-tubulin gene
BLAST	Basic Local Alignment Search Tool
CO_2	Carbon dioxide
CT_{min}	Critical minimum temperature
CT_{max}	Critical maximum temperature
cDNA	Complementary DNA
CNRQ	Normalized and relative quantities of gene expression
C_q	Quantification cycle
CO_3^{-2}	Carbonate ion
CVH	Climatic variability hypothesis
DIC	Dissolved inorganic carbonate
EBI	European Bioinformatics Institute
EBUS	Eastern Boundary Upwelling Systems
ES	Percentage of embryos survival per egg mass
ERG	Endogenous reference gene
FAO	Food and Agriculture Organization of the United Nations
Gadph	Glyceraldehyde-3-phosphate dehydrogenase generalist
GCC	Global Climate Change
GOI	Gene of interest
HSP	Heat shock proteins
HSP70	Heat shock protein 70-Isoform
*hsp*70	Heat shock protein 70-Isoform gene
IPCC	Intergovernamental Panel on Climate Change
IT	Intracapsular time
MMR	Maximum metabolic rate
MIQE	Minimum standard for the provision of information for qPCR experiments
MHW	Marine heatwaves

Definitions and Abbreviations

MCS	Marine cold spells
N	The percentage of capsules with embryos with normal developmental
NOAA	National Oceanic and Atmospheric Administration
NOCS	National Oceanography Centre
NCBI	National Centre Biotechnology Information
NTC	No Template Control
OA	Ocean acidification
OCLTT	Oxygen and Capacity Limitation of Thermal Tolerance
OCR	Oxygen consumption rate
ppm	Parts per million
ppb	Parts per billion
PCR	Polymerase Chain Reaction
pCO_2	Partial pressure of CO_2
qPCR	Quantitative real-time PCR
RCP8.5	Representative Concentration Pathways, 'worst case scenario 8.5'
RMR	Routine metabolic rate
SMR	Standard metabolic rate
SST	Sea Surface Temperatures
TPC	Thermal performance curve
T_{opt}	Optimal temperatures
T_{pejus}	Pejus temperatures
TTM	Time to metamorphosis
TA	Total alkalinity
T°/CO_2	Experimental treatment (+ 3 °C; ~900 µatm)
Ωca	Saturation state of calcite minerals
Ωar	Saturation state of aragonite minerals

Chapter 1 Introduction

1.1 Coastal marine environments

Coastal environments are among some of the most ecologically and socio-economically important systems on the planet, providing over US$14 trillion of ecosystem goods (e.g. food and raw materials) and services, e.g. CO_2 sequestering (Costanza et al., 1997). The coastal area encompasses the narrow transition connecting terrestrial and marine environments, with a high variety of different ecosystems (e.g. coral reefs, mangroves), habitats (e.g. rocky shore, tide pools, subtidal zone, sediments) and oceanographic processes (e.g. upwelling events) producing large fluctuations in their physical and chemical parameters (Feely et al., 2008, Helmuth et al., 2002). Most commercial marine species are harvested from marine coastal systems and specifically from benthic environments, which are the economic livelihood for almost 40% of the human population living in this area (Agardy et al., 2005). Given their global importance for the provision of ecosystem goods and services, the coastal marine environment is a major concern regarding the potential impacts of climate change (Harley et al., 2006).

From a geographical perspective, the coastal zone can be arranged along a latitudinal gradient from the equator to the poles (Kelletat et al., 2014). Tropical coastal environments are normally found from the equator to 23.5° north and south latitude with sea surface temperatures (SST) > 20 °C, high precipitations, large freshwater runoff and high solar radiation (Nittrouer et al., 1995). Coral reefs, seagrasses and mangroves are the dominant ecosystems along the tropical coast (Kelletat et al., 2014). Temperate coastal environments fall between latitudes from 23.5° to the polar circle at about 66.5° north and south latitude. Temperate coasts experience warm to hot summers and cool winters, with high SST seasonality (i.e. highest at polewards boundaries and lowest at equatorial boundaries at both hemispheres; Kelletat et al., 2014). For example, along the European Atlantic coast, the seasonal fluctuation is lowest at highest temperate latitudes (~ 8 °C), highest at intermediate temperate latitudes (~ 15 °C) and intermediate at low temperate latitudes (~ 10 °C; Campos and Van de Veer 2008). However, in some locations, such as the Mediterranean, daily SST fluctuations in summer can even be greater with maximum values above 20 °C (Campos and Van de Veer 2008). Salt marshes, wetlands, upwelling and seagrass are the main ecosystems along the temperate coastline (Kelletat et al., 2014). On the contrary, polar coastal zones extend from 66.5° north and south latitudes to the poles, which can be subdivided into upper, medium and low subzones depending on extension and intensity of terrestrial ice, sea ice and permafrost (Kelletat et al., 2014). Polar coast zone is characterized by low annual SST, strong seasonality in radiation balance, wind and snowfall (Hansom et al., 2014). In Antarctica, for example, the SST range is over

less than 4 °C annually anywhere and commonly range over only 2 °C (Barnes et al., 2006). In certain areas, such as the Ross Sea, SST variation is even less than 1 °C (Peck et al., 2010).

Along the marine coastline, the intertidal zone is one of the most ubiquitous habitats which includes rocky shores and sandy beaches (Agardy et al., 2005). The intertidal zone extends between the low and high tides and is the home to several species from different phyla (e.g. Mollusca, Cnidaria, Arthropoda, Porifera). It can be divided into three subdivision based on the vertical exposure of the zone: high, middle and low intertidal (Little and Kitching 1996). The high intertidal zone is the highest still-tide level that received wave splash and it is dominated by small gastropods and crustaceans. The middle zone is regularly exposed and submerged by tides and is dominated by mussels, algae, gastropods and the low intertidal zone, also known as the shallow subtidal zone, is only exposed to air at the lowest low tides, and is dominated by anemones, brown seaweed, chitons, crabs, mussels, amongst others (Little and Kitching 1996).

Due to their characteristics (e.g. tide, wave action), the intertidal zone is a very stressful and fluctuating environment in their physical and chemical parameters (e.g. temperature, salinity, UV, oxygen availability and pH; Helmuth et al., 2006). The rocky intertidal habitat, for example, is exposed to rapidly fluctuating and often extreme environmental stresses such as high temperatures, desiccation, UV exposure, and oxygen availability (Helmuth and Hofmann, 2001; Przeslawski, 2004). Low tides can generate complex mosaic of stressful thermal environments for intertidal organisms (Helmuth et al., 2002); e.g. during period of highest solar heating, intertidal organisms can reached temperatures of 33.8 °C during aerial exposure (Helmuth and Hofmann, 2001). To cope with this fluctuating environments, intertidal species have evolved physiological adaptations such as: decreasing their metabolic rate and, or in some cases, resorting to anaerobic metabolism during aerial exposure (Bayne et al., 1976). However, the energetic costs of living in these challenging environments are very high. Some coastal organisms are already living at the extreme of their physiological capacity (Somero, 2002, 2010; Stillman, 2003); which makes them at risk in future ocean conditions.

1.1.1 Climate change in coastal marine environments

Global Climate Change (GCC) is one of the most important issues facing mankind; human anthropogenic greenhouse gas emissions (carbon dioxide CO_2, methane CH_4 and nitrous oxide N_2O) have risen to unprecedented levels in recent decades (Gattuso et al., 2015). This dramatic increase has produced changes in the weather, air and sea-surface temperature, sea level and ocean acidification, amongst others (IPCC 2013). Estimations made in open ocean systems have shown that prior to the industrial revolution, respective concentrations were 280 ppm (CO_2), 722 ppb (CH_4)

and 273 ppb (N_2O); however these have increased by 40%, 150%, and 20%, respectively, because of human activity (Meinshausen et al., 2011). Carbon dioxide (CO_2) is one of the most important greenhouse gases in terms of its effects on climate change (e.g. regulating Earth's surface temperature) and in regulating the chemistry of the global ocean. The increase of CO_2 emissions into the Earth's atmosphere has accelerated from 280 ppm in the pre-industrial period to 410.27 ppm at November 2019, according to the Mauna Loa Observatory CO_2 record (NOAA). It is true that in the geological past both the concentration of CO_2 and the global mean temperature have oscillated over time and, at times, have exceeded present concentrations (Lüthi et al., 2008). Periods with high concentrations of CO_2 has been observed in the past and have produced mass extinctions, e.g. the Palaeocene-Eocene thermal maximum (Widdicombe and Spicer, 2008). Nevertheless, throughout glacial-interglacial cycles, CO_2 concentration has varied between 180 and 290 ppm (Doney et al., 2009) and at present the concentration of CO_2 exceeds that observed during the last 800,000 years (Lüthi et al., 2008). By the end of the twenty-first century, CO_2 concentrations have been predicted to reach 936 ppm (Meinshausen et al., 2011), sea surface temperature increase by 2.73 (± 0.72) °C (Bopp et al., 2013; Meinshausen et al., 2011;) and sea surface pH to decrease by 0.33 (± 0.003) units (Bopp et al., 2013; Orr et al., 2005).

The effects of climate change on coastal systems will be more pronounced and even worse than expected for open oceans (Harley et al., 2006). For example, as a result of global temperature increases and freshwater inputs from ice-melt, the world's oceans are expanding, which has resulted in a mean sea level rise to 2 mm per year (IPPC 2013). Greenhouse warming will intensify coastal upwelling by strengthening winds along the ocean margin (Bakun, 1990); which will upwelled cold and nutrients-rich waters with high CO_2, low pH and low oxygen concentration from deep waters into the photic zone impacting the physiology of coastal organisms. Studies on the likely effects of climate change on Eastern Boundary Upwelling systems (EBUS) showed strong and consistent changes in the time, intensity and spatial heterogeneity of coastal upwelling (Bakun et al., 2015; García-Reyes et al., 2015; Wang et al., 2015). Wang et al., (2015) evidenced that the upwelling season will be longer and more intense at high latitudes than low latitudes. This will drive more nutrient-rich cold deep water to the surface at high latitudes. At the same time, however, the increase in solar heating due to global warming could enhance stratification (i.e. deepening the thermocline) preventing cold and nutrient-rich water from being upwelled (Wang et al., 2015). The increase in the intensity of upwelling in spring and summer will increase hypoxic events in the coastal region (Bakun et al., 2015). Atmospheric circulation will increase precipitation patterns by increasing the frequency of winter storms that affect salinity, turbidity and terrestrial-derived nutrients (Bromirski et al., 2003).

Extreme ocean temperature events, i.e. marine heatwaves (MHW) and marine cold spells (MCS) will increase in frequency, intensity and longevity in future climate change scenarios (Hobday et al., 2016; Shlegel et al., 2017). These anomalous events cause a variety of impacts on coastal organisms, including shifts on species distribution ranges, rapid changes in biodiversity patterns and local extinctions (Hobday et al., 2016). MHW, i.e. a prolonged period of anomalously high SST, are increasing due to climate change causing catastrophic effects for coastal organisms (Oliver et al., 2018). For example, the MHW over Europe in the summer of 2003 increased the SST by 2-3 °C in the northern area of the Mediterranean Sea, causing high benthic mortality and loss of seagrass meadows (Shlegel et al., 2017, Darmaraki et al., 2019). Another MHW along the Western Australian coast in 2011, caused shifts in the benthonic ecosystem structure and functioning (Hobday et al., 2016). Although MHWs are more expected in climate change scenarios (Oliver et al., 2018), MCS events have been projected as a result of the strengthening of coastal upwelling (Gershunov and Douvelle 2008). MCSs have the potential to impact coastal ecosystems and organisms especially on the intertidal and coastal biota locally (Shlegel et al., 2017). For example, during the cold-water event of January 2020 in Florida, United State, coastal STT below 16 °C were recorded, causing high coral mortalities and dramatic losses in coral cover (Lirman et al., 2011).

All these effects of climate change on coastal systems will jeopardize the persistence of coastal species with a direct impact in the biogeographic distributions and, therefore on the functioning of coastal marine ecosystems (Harley et al., 2006).

1.2 Thermal response of marine organisms

Temperature plays an important role regulating multiple levels of biological organization, from limiting physiological and biochemical processes to driving the distribution patterns of species across latitudes (Jones et al., 2009; Somero, 2005; Weiss et al., 2009). As aquatic invertebrates are unable to regulate body temperatures independently from the marine environment (i.e. ectothermic), nearly all-physiological and behavioural traits (e.g. growth, reproduction, predation) are sensitive to temperature (Hochachka and Somero, 2002). Thus, temperature is the main physical driver controlling the physiology of ectothermic species.

1.2.1 Physiological response to temperature

Most biochemical reactions increase with increased temperature (Hochachka and Somero, 2002), although physiological functions such as heart or ventilation rate are complex interactions compromising biochemical and physical processes (Schulte 2015). One of the simplest ways to describe the acute effects of temperature on biochemical processes is to generate a Thermal

Performance Curve, TPC (Fig. 1.1, Schulte et al., 2011). TPCs tend to be unimodal and left-skewed with three different regions (Dell et al., 2013):

(1) Ascending phase: the rate of a specific physiological trait increases as temperature increases within an organism's thermal range. The rising phase can be explained by the Arrhenius equation, in which the rate exponentially increases up to an optimal temperature, T_{opt} (Schulte, 2015).

(2) Thermal optimum: the intermediate level that encompasses the temperatures that optimize the physiological rate (T_{opt}).

(3) Falling phase: at higher temperatures, the physiological rate drops rapidly to reach the critical temperature that permits the performance (Angilletta et al., 2002; Schulte et al., 2011). Discontinuities in this relationship, also known Arrhenius breakpoints, indicate the destabilizing effect of temperature at the molecular level (e.g. protein denaturation).

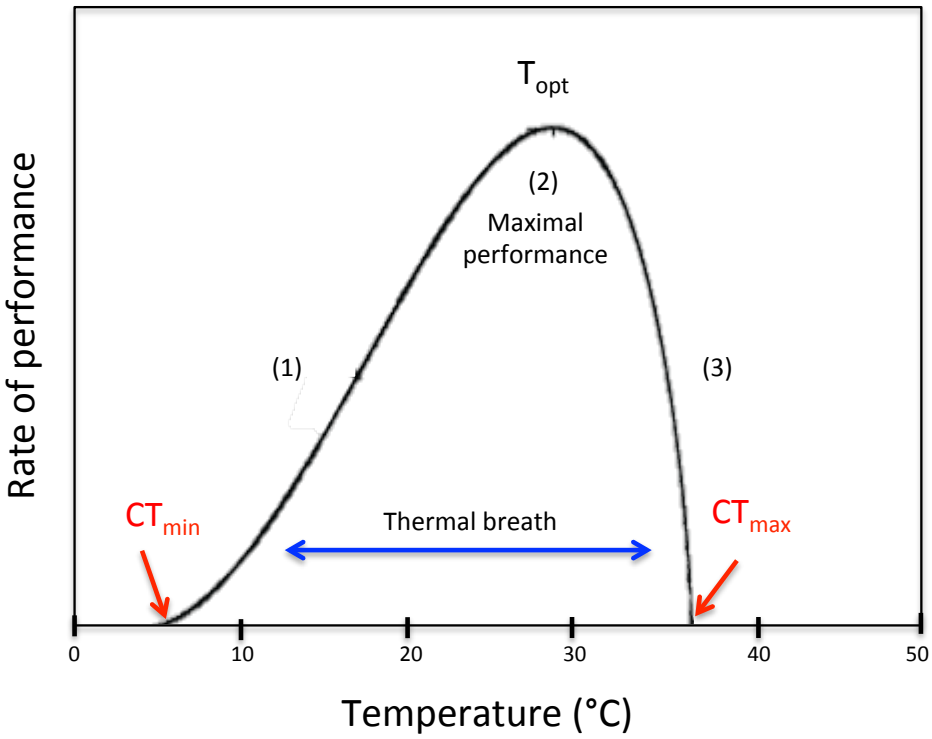

Figure 1.1 Typical thermal performance curve (TPC) with unimodal shape: (1) Ascending phase, (2) thermal optimum and (3) falling phase. The thermal breath or performance breath defines the ranges of temperatures that the performance is allowed, i.e. between critical temperatures (CT_{min} and CT_{max}). Modified from Schulte et al. (2011) and Dowd et al. (2015).

In theory, TPCs tend to take similar shapes across different levels of biological organization (Dell et al., 2013; Schulte, 2015). The exponential and decline phase of TPCs fit very well at the molecular level, for example, with the catalytic function of an enzyme (Schulte et al., 2011). However, over long time scale, thermal sensitivities are more complex. The organisms can acclimatize and adapt

in response to prior thermal exposure, resulting in changes in the shape of TPC. For example, the environment experienced by parents can alter the tolerance limits and TPC shape of the offspring and subsequent generations (Schaefer and Ryan, 2006). Thus, the shape of TPC is not just a result of a simple thermodynamic effect on a single protein or suite of proteins/pathways, the shape and characteristics vary according to different levels of biological organization (Schulte et al., 2011).

Effect of temperature at whole-organism level

One approach that unifies the effects of temperature at organism level is the hypothesis of Oxygen and Capacity Limitation of Thermal Tolerance (OCLTT, Fig. 1.2, Eliason et al., 2011, Fusi et al., 2016, Pörtner 2001, 2017). The OCLTT hypothesis proposes that the thermal performance curve in animals is shaped by the capacity for oxygen delivery, i.e. ventilatory oxygen uptake and cardiovascular oxygen transport, in relation to the oxygen demand at the mitochondrial level (Pörtner, 2001, 2002, 2010). This hypothesis also proposes the physiological limits for thermal tolerance ranges, which constrain the distribution of organisms. Ectothermic organisms have optimal temperatures (T_{opt}), where they can perform critical functions such as reproduction and growth, which are correlated with the maximal aerobic scope, i.e. the difference between standard metabolic rate (SMR) and the maximum metabolic rate (MMR). In ectotherms, SMR reflects the energetic cost associated with the maintenance of an organisms, which increases with temperature, and MMR represents the maximal rate at which oxygen can be transported to the mitochondria, which does not always increase with temperature. The difference between MMR/SMR represents the energy available to perform life functions (Fig. 1.2a; Pörtner, 2001). When the optimal temperatures are reached, oxygen supply and aerobic scope are maximal and oxygen levels in body fluids are high.

Outside these optimal temperatures, the oxygen delivery through heart and ventilation rate is unable to match the oxygen demands in tissues, producing a reduction in aerobic scope and a slight decline in the performance rate. These temperatures are known as 'pejus temperatures' and indicate the early limitations to thermal tolerance. Beyond the pejus temperatures, the aerobic scope reaches critical temperatures (CT_{min} and CT_{max}) due to the oxygen deficiency, experiencing hypoxic conditions and producing a decline in the performance rate (Pörtner, 2001). Beyond critical temperatures, the circulatory and ventilatory systems fail as they reach the limits of their capacity to deliver oxygen, aerobic metabolism disappears and mitochondrial function declines making a transition to anaerobic metabolism, which over the long-term can produce a systemic failure (Pörtner, 2002). The classic example conducted by Frederich and Pörtner (2000) measured the oxygen tension (i.e. oxygen partial pressure) in the spider crab, *Maja squinado,* and revealed that oxygen levels in the haemolymph and oxygen supply are maximal within optimal temperatures.

Beyond the optimal temperatures (i.e. during cooling or warming acute exposure) at pejus temperatures (T_{pejus}), the oxygen saturation decreased and the concentration of lactate and succinate (i.e. anaerobic end-products) increased in the haemolymph. At this stage, ventilation and heart rates became less constant; thus, the oxygen demand was no longer met by respiratory capacity. Beyond critical temperatures (i.e. CT_{min}-CT_{max}), ventilatory and circulatory activity collapsed and the whole organism succumbed (Frederich and Pörtner, 2000). The OCLTT has been proposed to be applicable across all animal taxa, with oxygen delivery as the primer driver to set up the thermal tolerance limits (Pörtner et al., 2017). However, insufficiency in oxygen delivery could just be one of several physiological processes (e.g. protein failure function, ion homeostasis) constraining thermal tolerance limits (Verberk et al., 2016b; Clark et al., 2013).

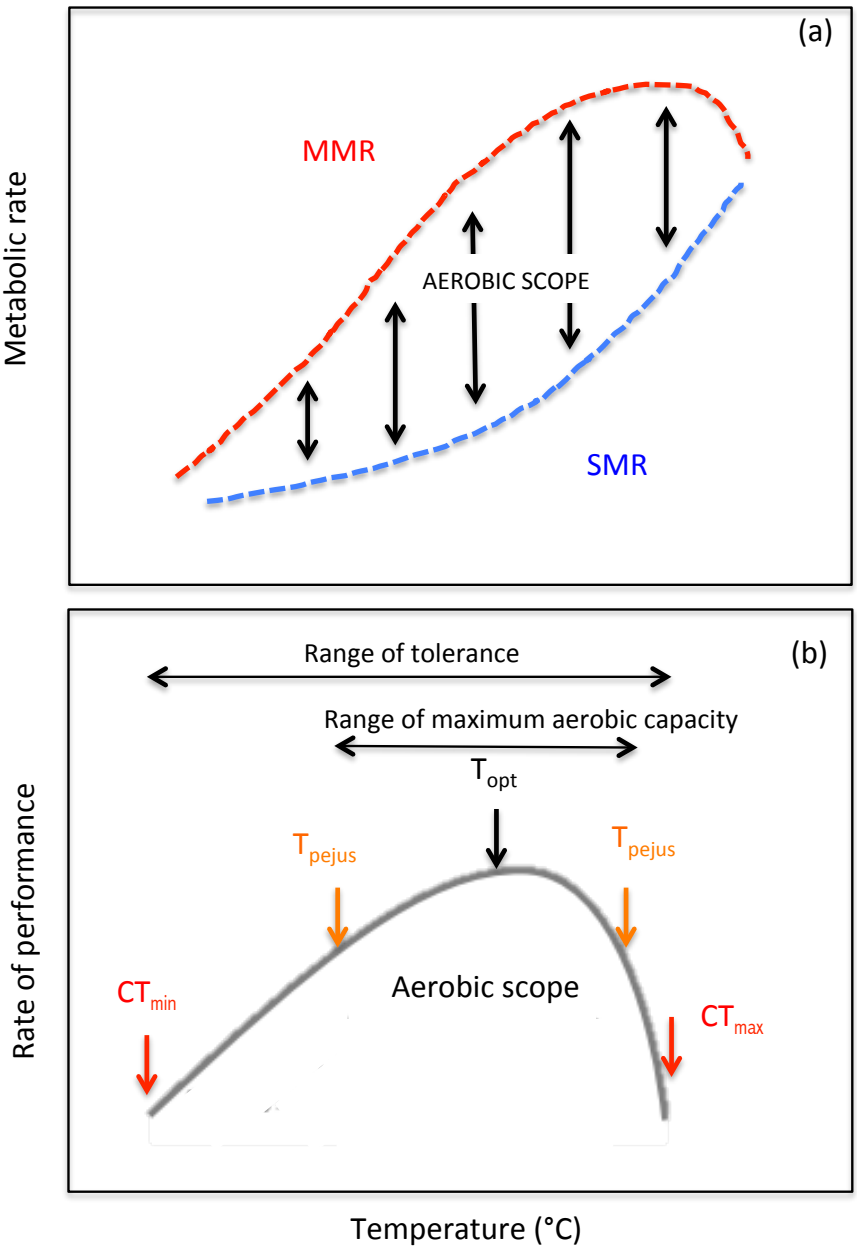

Figure 1.2 (a) Thermal dependency of standard metabolic rate (SMR) and maximum metabolic rate (MMR). The difference between MMR/SMR represents the aerobic scope. **(b)** Conceptual model of the oxygen and capacity limited thermal tolerance hypothesis. Aerobic scope is maximized at optimal temperatures (T_{opt}). Aerobic scope declines at T_{pejus} leading to a decrease in oxygen, which can no longer be sustained beyond critical temperatures (i.e. between $CT_{max-min}$). Modified from Pörtner (2002, 2010) and Verberck et al. (2016b).

Protective mechanism to thermal stress: heat shock proteins

Beyond CT_{max}, organisms have mechanisms at the cellular level to protect the macromolecular structure against heat stress. Cellular proteins are vulnerable to temperature increases because they operate in a very specific thermal range; thus, proteins are at risk of denaturation into a non-functional conformation (Whiteley and Mackenzie, 2016). The cellular stress response of most

organisms involves the synthesis and accumulation of a class of stress proteins called heat shock proteins (HSP), also known as chaperones (Kültz, 2005; Morris et al., 2013). These proteins are from a large family of ubiquitous proteins that are synthesized under environmental stresses, including thermal (Hofmann and Somero, 1996; Tedengren et al., 2000), chemical (Snyder et al., 2001), or acidic stress (Cummings et al., 2011; Lardies et al., 2014). The heat shock response is activated with the presence of denatured or abnormal proteins in the cell; HSPs then prevent the aggregation of unfolded proteins, re-fold thermally denatured proteins, or transport and degrade misfolded/aggregated proteins (Morris et al., 2013).

The stress-70 isoform (i.e. HSP70) is one of the most studied HSPs due to its role enhancing the thermal tolerance response of ectothermic organisms (Hofmann and Somero, 1995; Tomanek and Somero, 1999). The HSP70 family is ubiquitous and highly conserved in almost all organisms; for example, increased temperatures induce the synthesis of HSP70 in oyster, mussels, amphipods, and gastropods, amongst others (Hofmann and Somero, 1995; Bradley et al., 1998; Hauton et al., 2009; Lardies et al., 2014). HSP70 can persist for short periods from hours to days after the initial stress exposure. For example, high expression levels were observed in the abalone *Haliotis rufescens* after 2-3 days of exposition to thermal and chemical stress (Snyder et al., 2001) or 5 days exposure in mussel gills (Tedengren et al., 2000). Heat shock studies have demonstrated that the activation of the HSP70 in ectotherms is positively correlated with adaptation temperature (Somero, 2002); thus, the response can be affected by latitude thermal gradients, vertical zonation, and seasonality, amongst, others (Somero, 2002; Tomanek and Somero, 1999).

1.2.2 Ecophysiological response to natural thermal variation

Thermal gradients influence the distribution of marine species, species replacement (i.e. congeners) and intraspecific variability between populations of a single species (Castañeda et al., 2004; Hochachka and Somero 2002). In general, mean seawater temperatures decrease as latitude increases from tropical to polar zone, leading to a decrease in species tolerance thermal range, i.e. the distance between CT_{max} and CT_{min} (Addo-Bediako et al., 2000). Tropical and polar seas have small oscillations of the annual temperatures compared to temperate seas. For example, annual thermal variation in tropical seas is over 1 – 3 °C (Clarke & Gaston, 2006). Similar thermal variations are observed in Antarctica where annual temperatures range between 2 and 4 °C and in some locations the variation can be less than 1 °C (Barnes et al., 2006). However, in temperate regions, thermal annual oscillation is greater, e.g. the thermal variation along the European coast vary between 10 and 15 °C due to seasonal variation (Campos and Van der veer, 2008).

Whole-organisms response

According to the thermal adaptation theory, species inhabiting tropical or polar latitudes with stable thermal profiles have evolved a specialized thermal strategy (stenothermal), i.e. organisms are well adapted to local conditions but poorly adapted to alternative environments (Verberk et al., 2016a). For example, tropical stenothermal species showed high CT_{max} but narrow thermal tolerances (Somero, 2010). In fact, tropical species are living very close to their upper critical thermal limits (CT_{max}), they have evolved greater tolerance to high temperatures at expense of the acclimation capacity (Stillman and Somero, 1996; Stillman, 2003). A study conducted by Stillman and Somero (2000) in twenty species of the porcelain crab *Petrolisthes* spp. through a latitudinal and vertical gradient, demonstrated that porcelain crab species inhabiting the intertidal zone and from the tropical region showed higher CT_{max} and narrower thermal windows than temperate species; tropical species adapted their upper thermal limits to coincide with the microhabitat conditions. On the other hand, polar Antarctic teleost fishes normally live in temperatures between 2 °C and – 1.9 °C with a CT_{max} of 4 °C; temperatures outside of this normal range, above 2 °C, cause death (Hochachka and Somero, 2002). Following with this idea, a study conducted by Peck et al., (2010) on Antarctic organisms from the phyla Echinodermata, Mollusca, Brachiopoda and Crustacea showed poor physiological capacities to temperature only 3.5 °C above respect to the annual thermal average (1 – 2 °C). Moreover, two species, the sea urchin *Sterechinus neumayeri* and the amphipod *Paracedadocus gibber*, exposed to temperatures of 3 °C for 60 days showed no metabolic compensation. Polar and tropical species showed respectively lower and higher CT_{max}, narrower thermal breath (i.e. between CT_{min} – CT_{max}), and no metabolic compensation to temperatures outside of the normal thermal range, which make these species at greater potential risk of extinction under global warming. In temperate regions (i.e. latitude between 20° and 50°) species have evolved a generalist thermal strategy (eurythermal); i.e. temperate species can withstand thermal variation of 20 – 30 °C on a daily basis (Hochachka and Somero, 2002). Thermal tolerance ranges are broader, with high seasonal variations in CT values and high metabolic compensation (Sommer and Pörtner, 2002). For example, the temperate lugworm *Arenicola marina* exhibits high seasonal variability in their thermal tolerance windows and CT values along the western European coast. The lugworm showed broader thermal windows and higher CT values in summer than in winter (Wittmann et al., 2008). Therefore, temperate species showed a broader thermal tolerance range, variable CT_{max} and high metabolic compensation.

Previous studies have shown that across a species' distribution (i.e. within populations of single species), the amplitude of thermal tolerance or thermal breath is also associated with latitudinal patterns and seasonal thermal variation (Sunday et al., 2011, Kuo and Sanford 2009, Yu et al., 2018). The climate variability hypothesis (CVH) states that thermal adaptation is proportional to the

magnitude of the environmental variation that organisms of a single species experience (Yu et al., 2018). Hence, within the distribution of a temperate species, low-latitudes populations where seasonal variation is minimal exhibit narrower thermal tolerances compared to high-latitude populations, where seasonal variation is extreme (Gaitán-Espitia et al., 2013; Sunday et al., 2011). For example, in the temperate cold-water fish *Rhynchocypris oxycephalus*, widely distributed along the East Asia, a low-latitude population showed a narrower thermal tolerance range than the high-latitude population. Indeed, transcriptomic analyses evidenced that the high-latitude population expressed more temperature responding genes than low-latitude specimens (Yu et al., 2018). However, other studies have suggested that thermal tolerance variation among populations can be a result of genetic adaptations to local thermal conditions (Jansen et al., 2007; Gleason and Burton, 2013). For example, Reitzel et al., (2013) found in the temperate sea anemone *Nematostella vectensis* that low latitude populations have higher thermal tolerance as a consequence of the warm temperatures that they experienced. Zippay and Hofmann, (2010) reported that early stages also exhibit different thermal limits, for example, early stages (i.e. veliger) from northern latitude of the gastropod *Nucella ostrina* were less tolerant to heat stress than those from central latitude. Thus, early stages also displayed different thermal tolerance limits as a function of adaptation to the local thermal conditions at which they were collected.

Molecular response

At the cellular level, the synthesis of HSP also is influenced by latitudinal gradients (Hofmann and Somero, 1996; Somero, 2002; Tomanek and Somero, 1999). For example, Hoffman and Somero (1996) found that the HSP expression pattern of temperate congeneric mussels (*Mytilus trossulus* and *M. galloprovincialis*) depends on their latitudinal distribution. Northern mussels experiencing colder temperatures showed a lower threshold to induce the HSP70 synthesis and higher relative expression levels in gill tissues than southern mussels experiencing warmer temperatures. It was concluded that *Mytilus* spp. species experiencing colder temperatures are more thermally sensitive (Hofmann and Somero 1996). Similar findings were observed between congeners of *Tegula* spp., warm-adapted species induce HSP70 synthesis at higher temperatures than cold-adapted species (Tomanek and Somero, 1999). For example, *T. rugosa*, the most warm-adapted species activated the heat shock response at 30 °C, on the contrary, the *T. montereyi* induce *hsp*70 at 27 °C.

Seasonality and vertical gradients (i.e. intertidal height) also can influence the synthesis of HSP70 in marine organisms. For example, the intertidal mussel *Mytilus trossulus* exhibited high concentrations of *hsp*70 in the gill tissues in summer instead of in winter (Hofmann and Somero, 1995). Moreover, mussels inhabiting the intertidal zone showed a higher concentration *of hsp*70 than their conspecifics from the subtidal. It was concluded that changes in the HSP concentrations

depend on the environmental temperatures that organisms experienced and this may contribute to the development of thermal tolerance (Hofmann and Somero, 1995). Therefore, acclimatisation and adaptations of organisms to ambient temperatures can influence heat shock responses, leading to interspecific and intraspecific variation that can contribute to setting species' distribution patterns.

1.2.3 The interactive effects of temperature and other stressors

A stressor can be defined as "an environmental or genetic factor that causes a change in a biological system, which is potentially damaging and which has some consequence on the organisms" (Morris et al., 2013, modified from Hoffmann and Parsons 1991). Temperature is one of the most stressful physical parameters in marine environments shaping the distribution and abundance of marine organisms, with the potential to produce local extinction in marine organisms (Thomas et al., 2004). However temperature does not act in isolation, there are other additional stressors that can intensify or ameliorate the effects of increased temperature, e.g. oxygen, salinity or pCO_2. Therefore, it is imperative to have a good understanding of how temperature can act with other stressors, and how this will affect the tolerance and physiological limits of marine ectotherms.

Temperature and oxygen

As a result of the increase of global temperatures due to climate change, the world's oceans are deoxygenating leading to hypoxic/anoxic sea ocean conditions (Keeling et al., 2010; Matear and Hirst, 2003). Temperature plays an important role in controlling the extent of hypoxia, e.g. increasing the stratification and reducing the ventilation of marine waters (Vaquer-Sunyer and Duarte 2011). At the global scale, ocean models predict that over the next century, the dissolved oxygen concentration in the oceans will decrease by 1 to 7% on average (Keeling et al., 2010; Matear and Hirst, 2003). Indeed, in certain areas, e.g. the coastal zone, the frequency and intensity of hypoxic/anoxic events will be more intense under future ocean warming (Keeling et al., 2010). The physiological response of marine invertebrates to hypoxia includes adjustment of cardiorespiratory systems, changes in the pigment oxygen transportation (e.g. hemocyanin), metabolic depression and decrease in ATP generation, amongst others (Pörtner et al., 2005; Whiteley and Mackenzie, 2016; Guppy and Withers, 1999).

Previous studies have demonstrated that warming affects the vulnerability of ectotherms to hypoxia (Vaquer-Sunyer and Duarte, 2011; Tripp-Valdez et al., 2017). As temperature increases, the respiration rate and oxygen requirements increased, until organisms reach critical temperatures where oxygen requirements are not meet by oxygen supply (e.g. ventilation or cardiovascular transport; Pörtner, 2010). This mismatch between demand and oxygen supply leads to hypoxic

conditions and a transition to an anaerobic mitochondrial mode (Pörtner, 2010). However, when organisms are exposed to the combined effects of warming and hypoxia (~ 2 mg O_2 L^{-1}; Peruzza, 2017), critical temperatures (CT_{max}) are reached more quickly due to increased respiration rate and oxygen requirements; which causes an increase in the hypoxic thresholds and a narrowing of the thermal tolerance window (Vaquer-Sunyer and Duarte, 2011). For example, a meta-analysis conducted on benthic organisms have shown synergistic effects of hypoxia and warming; elevated temperatures increase the hypoxia threshold, reducing the thermal tolerance limits and leading to high mortalities, up to 74% (Vaquer-Sunyer and Duarte, 2011). Similar results were observed in juveniles of the temperate green abalone *Haliotis fulgens* exposed to a temperature ramp (+ 3 °C day^{-1}) and hypoxic conditions (50% air saturation; Tripp-Valdez et al., 2017). Juveniles were more vulnerable to warming under hypoxic conditions, exhibited metabolic depression and a downward shift of the upper critical temperatures, which reduced the juvenile's thermal tolerance response (Tripp-Valdez et al., 2017). Therefore, ocean warming is likely to amplify the intensity and severity of hypoxic events, posing a threat to the survival of marine biota.

Temperature and salinity

The world's oceans are undergoing unprecedented changes, such as rising temperatures, changing the chemistry (i.e. alkalinity, pH and salinity) and shifting weather patterns (e.g. high rainfall; Dickson et al., 2002; Doney et al., 2012). There is growing evidence showing that increases in precipitation and warming will result in tropical oceans facing large increases in temperature and saltier conditions, and at high latitudes facing freshening effects (i.e. decreased salinity; Helm et al., 2010, Holan et al., 2019). For example, areas across the Southern Ocean have experience unprecedent warming and changes in precipitation patterns in recent decades (Pendlebury and Barnes-Keoghan, 2007); indeed, rainfall has increased by 35% in this region since 1970 leading to freshening effects. Also, increases of run-off, glacier and ice shelf melting are contributing to the freshening of the Southern Ocean (Sylvano et al., 2018). In the North Atlantic Ocean, freshwater inputs have increased due to the increase in melting ice, leading to changes in seawater salinity and alkalinity (Dickson et al., 2002). These increases in precipitation and ice melt will lead to a decreased in salinity in nearshore marine environments that will have a negative impact on marine organisms. The decrease in salinity affects the physiological response of marine ectotherms, causing metabolic depression, increases in cellular energy expenditure to maintain osmoregulation and cell volume (Whiteley and Mackenzie 2016).

The concurring ocean warming and decreased salinity have shown synergistic and antagonist effects on marine ectotherms, especially at high latitudes (Détrée et al., 2020; Holan et al., 2019; Navarro et al., 2019). Even though warming has been considered one of the most important

physical parameters affecting the physiology of ectotherms under climate change (Sokolova and Lanning, 2008), decreased salinity also have pervasive effects on the physiology of marine ectotherms. For example, Navarro et al. (2019) conducted a study on the Antarctic shallow water fish *Harpagifer antarticus* to understand the effects of increasing seawater temperatures and decreasing salinity. The results showed that warming had an overriding effect on *H. antarticus* relative to decreased salinity, impacting all the physiological variables measured during the exposure, e.g. ingestion rate, oxygen consumption, mortality, absorption efficiency, the scope for growth. However, variables such as ingestion, feeding and absorption and oxygen respiration rate were affected by warming and decreasing salinity; which in long-term can have negative impacts on the physiology of this species (Navarro et al., 2019). On the contrary, no effects of decreased salinity or interactive effects of warming and decreased salinity were observed on the physiological energetics parameters (e.g. ingestion rate, absorption efficiency, oxygen uptake) of the edible temperate whelk *Trophon geversianus* from Southern Patagonia (Détrée et al., 2019). It was concluded that this species could be more robust to warming and future variation in salinity. Therefore, the response to the combined effects of temperature and decreased salinity is specie-specific and will depend on the physiological capacities of marine ectotherms.

Temperature and pCO_2

Oceans have absorbed approximately half of all anthropogenic CO_2 emissions to date, changing the chemistry of marine oceans and eliciting an increase in $[H^+]$ due to the dissociation of ion bicarbonate and carbonate, which has become known as ocean acidification (Gazeau et al., 2013). At the cellular level, hypercapnia (i.e. elevated CO_2 partial pressure) affects the acid-base regulation of marine ectotherms, which in normal pCO_2 conditions is compensated by the accumulation of bicarbonate anions in the intracellular compartment (Pörtner et al., 2004). However, when pCO_2 concentrations are elevated, organisms are not able to maintain the homeostasis between intra and extracellular pH, negatively impacting the physiology of marine organisms. For example, in the temperate squid *Sipunculus nudus*, high CO_2 elicited a decrease in extracellular pH leading to a metabolic depression (Reipschläger and Pörtner, 1996). At whole-organisms level, high pCO_2 concentrations affect growth, reproductive success (i.e. fertilization), calcification, ventilatory activity, and behaviour (e.g. prey detection) has been reported (Kelly et al., 2013; Kroeker et al., 2010; Manríquez et al., 2014).

Previous studies have demonstrated that concurring ocean acidification and global warming may have synergistic effects on marine ectotherms (Hofmann and Todgham, 2010; Reynaud et al., 2003). For example, Anthony et al., (2008) evidenced that prolonged exposure to high CO_2 and temperature acted synergistically causing coral bleaching (i.e. loss of pigmentation), and lowering

the thermal bleaching threshold. It was concluded that CO_2 effects might offset any potential for adaptation and acclimatization by coral reef to thermal stress. Synergistic effects were found in the temperate crab *Cancer pagurus*, the increased in *p*CO2 concentration caused enhanced sensitivity to thermal stress, narrowing the thermal tolerance windows (Metzger et al., 2007). On the other hand, the effects of temperature with high pCO_2 can be antagonistic. For example, the main factor impacting the shell strength (i.e. maximal load that each shell valve can endure) after 6 months exposure to increased temperature and reduced pH of the temperate bivalve *Mytilus edulis* was temperature (Mackenzie et al., 2014). In this study was concluded that warming has an indirect effect on the shell strength by mussels allocating energy in maintenance rather than in shell formation. On the contrary, the maintenance of shell strength under high pCO_2 treatments suggested that mussels were unaffected. Therefore, it can be concluded that the physiological response of marine ectotherms to the combined effects of warming and ocean acidification is highly variable and tend to be species-specific.

1.3 Phenotypic plasticity and local adaptation

Natural environments are a mosaic of biotic and abiotic factors acting on organisms via natural selection, generating a gradient of phenotypic responses. Organisms have two different mechanisms to survive novel environmental conditions: (1) phenotypic plasticity, by using existing physiological capacity (Thomas et al., 2018) or (2) adaptation, by changing the genetic composition of populations (Yu et al., 2018).

1.3.1 Phenotypic plasticity

Phenotypic plasticity, i.e. the ability of a genotype to produce distinct phenotypes when exposed to different environments throughout its ontogeny (Pigliucci, 2005) has been well studied in terrestrial environments, specially focused in insects and plants (Pigliucci, 2005; Whitman and Agrawal, 2009) and in marine organisms (Bourdeau et al., 2015). Plasticity is a property of the individual and it is a rapid-response mechanism that enables an organism to acclimate and survive environmental changes (Chevin et al., 2010). Environmental conditions (i.e. physical, chemicals or biological) can induce plastic changes in the morphology, physiology, behaviour and life history of organisms (Hollander, 2008). Indeed, the intraspecific differences observed within species' distribution (i.e. among populations) might reflect the plastic response of organisms under fluctuating environmental conditions (Osores et al., 2017; Lardies et al., 2014; Ramajo et al., 2016a). In the short-term, the phenotypic response can be reversible (i.e. phenotypic flexibility) and in long-term, irreversible (e.g. developmental plasticity) leading to adaptive plasticity or become a powerful adaptation (Nettle and Bateson, 2015, Bourdeau et al., 2015). However, phenotypic

plasticity can contribute or constrain adaptation. For example, when populations are faced with a new or altered environment, plasticity should promote establishment and persistence leading to adaptive divergence among populations (Ghalambor et al., 2007). However, also phenotypic plasticity can retard adaptation, because environmental conditions can lead organisms to produce maladapted phenotypes (Fox et al., 2019).

Maternal effects

Maternal effects have been extensively studied in terrestrial systems, mainly in plants and insects (Roach and Wulff, 1987), and in marine environments (Agrawal et al., 1999; Berkely et al., 2004; Bernando, 1996; Marshall et al., 2008a; Mousseau and Fox, 1998). Maternal effects can be defined as "a class of phenotypic effects that parents have on phenotypes of their offspring that are unrelated to the offspring's own genotype " (Bernando, 1996). Thus, a maternal effect is only considered when the phenotypic response of offspring is affected directly by the maternal phenotype. Thus far, research in maternal effects has focused on studying the impact during early development, because maternal influences are stronger at early stages of development. In subsequent stages, the offspring genotype itself makes a stronger contribution to environmental cues (Bernando, 1996; Mousseau and Fox, 1998; Roach and Wulff, 1987).

Maternal effects can be adaptive, that is, the mother's response to unpredictable environments adjusts the offspring phenotype to obtain high offspring fitness (Marshall and Uller, 2007). Indeed, research on animals and plants have demonstrated that maternal effects can result in transgenerational acclimation, involving morphological and physiological changes in next generations (Donelson et al., 2011; Munday, 2014; Shama et al., 2014. For example, daphnid mothers of the cladocera *Daphnia cucullata* induce transgenerational morphological defences in the progeny altering the phenotypic response to face potential hazards. Mothers of *D. cucullata* are capable of detecting chemical signals (kairomones) released by predators. Once mothers have perceived the presence of chemical signals from predators, they were able to induce morphological changes in their offspring, producing juveniles with larger helmets, offering them protection from predation (Agrawal et al., 1999). In the tropical spiny damselfish *Acanthochromis polyacanthus*, it has been demonstrated that thermal experience by parents can produce transgenerational acclimation in offspring (Donelson et al., 2011). After three generations of parents exposed to high temperatures (+1.5 and + 3 °C), the offspring's metabolic performance (i.e. aerobic scope) improved when offspring experienced the same thermal environment as the parents (Donelson et al., 2011). Therefore, transgenerational effects can generate links between parental and offspring environments and provide a mechanism to adapt during critical stages in the early life cycle.

Maternal effects in number and offspring size

Marine invertebrates exhibit a vast range of interspecific and intraspecific variation in offspring number and size; however, it has been particularly interesting that some species show intraspecific variation within and among broods from the same female (Marshall and Keough, 2007). Theoretically, offspring fitness will be optimal if, under constant conditions, offspring are produced with a uniform offspring size (Smith and Fretwell, 1974). However, conflicts among parents, siblings and environmental conditions produce size variations within and among populations. For example, variations within and among broods have been identified as bet-hedging strategies to deal with unpredictable environments (Marshall et al., 2008b).

Offspring size could be affected by different maternal influences such as: maternal size, maternal nutrition and maternal environmental conditions. Maternal size shows a positive correlation with offspring size in some species (Chaparro et al., 1999; Gianguzza et al., 2005; Parker and Begon, 1986; Valentinsson, 2002). Maternal nutrition is an important factor that can affect the offspring size because there is a direct relationship between maternal nutrition state and offspring quality. For example, starved female squid *Euprymna tasmanica* produced smaller clutch and eggs with high mortality rates. Also, the amount of lipids available for embryonic development was lower (Steer et al., 2004). On the contrary, in some species, females can improve offspring fitness. For example, females of the copepod *Calanus helgolandicus* exposed to a rich diet of diatoms showed an increase in the egg production rate. Total amino acid content was double in embryos from well-fed females than from wild females (Laabir et al., 1999). The same pattern was found in females of the bivalve *Mytilus edulis* exposed to high temperature and low or high rations of food before offspring release (Bayne et al., 1975). Starved females transferred more carbon (measured as labelled ^{14}C) to protect eggs from thermal stress than those females that were satiated. This transferred carbon was preferentially stored in the lipid fraction of the egg.

Maternal environmental conditions are another factor than can produce changes in the number and offspring size. Mothers can adjust the offspring size when they are exposed to seasonal variations (Atkinson et al., 2001), such as variations in temperature and nutrients. For example, in a field study conducted in gammarid amphipods it has been demonstrated that females showed a wide range of egg sizes depending on season. The egg volume in winter was 60% greater compared with summer. This variation in egg size was directly correlated with *in situ* temperature and may be a result of genetic variation within populations and between females to avoid energetic wastage (Sheader, 1996). Latitudinal thermal gradients also can impact fecundity, for example, Wehrtmann et al., (2012) found an impressive intraspecific variability of reproductive features in the intertidal decapod, *Petrolisthes armatus*. Females from a low latitude population exhibited higher

reproductive output (i.e. high egg production) than females from a high latitude population; however, this pattern has only been observed in decapod species.

1.3.2 Local adaptation

On the other hand, when populations are under strong selection and persistent environmental conditions (e.g. thermal gradient, upwelling events, hot spot, intertidal height) they can evolve physiological, morphological or behavioural adaptations to increase their fitness advantage under local conditions (Sanford and Kelly, 2011). Thus, across species' geographic distribution, species have evolved a mosaic of locally adapted populations in response to environmental fluctuations (Kuo and Sanford, 2009). Local adaptation, thus, can be defined as situations in which: "resident genotypes exhibit higher fitness in their native habitat than do foreign genotypes from other populations," (Kawecki and Ebert, 2004).

Historically, this phenomenon was thought to be restricted to species with low dispersal potential and low gene flow, like terrestrial and freshwater species (Castañeda et al., 2004; Lazzaro et al., 2008; Miller and Cummins, 2014; Postma and Ågren, 2016). Previously, marine environments were considered as open systems (i.e. without barriers), with high connectivity among populations (i.e. planktotrophic development) and high gene flow. However, research has shown that local adaptation can occur in marine environments even at a short distances of tens to meters (Sanford and Kelly, 2011). Studies conducted in marine species have demonstrated that populations are less genetically connected, exhibiting high genetic differentiation (Kuo and Sanford, 2009; Gleason and Burton, 2013). Local adaptation is particularly frequently in species inhabiting the coastline (Pardo and Johnson, 2005; Trussell, 2000; Zippay and Hofmann, 2010), because coastal systems are heterogeneous environments with high fluctuations in their physical parameters (e.g. temperature, pH, oxygen, salinity) and also biotic gradients (e.g. predation). Upwelling events, freshwater input from estuarine areas, and hypoxia are some of the coastal processes that produce strong and permanent gradients that have the potential to create spatially varying selection on marine species distributed over broad geographic ranges (Sanford and Kelly, 2011).

Local adaptation is expected to be strong in species with broad geographic distribution (i.e. higher heterogeneity), low connectivity and restricted gene flow among populations (Sanford and Kelly, 2011). Thus, species with direct development have greater potential for local adaptation than species with planktotrophic development (Beherens, 1989; Kuo and Sanford, 2009; Pardo and Johnson, 2005). For example, Gleason and Burton (2013) found that populations of the temperate gastropod *Chlorostoma funebralis* are locally adapted to natal environmental conditions even though *C. funebralis* has a short pelagic phase. Under common garden conditions, southern

populations showed lower mortality and high recovery after heat stress compared to northern populations. It was concluded that southern populations possessed genetic adaptations to tolerate heat stress. This study suggest that local adaptation could be more common among marine populations, even for species with planktotrophic development and high gene flow (Hedgecock, 1986).However, few studies have identified if the intraspecific differences observed among populations are due to phenotypic plasticity or local adaptation. Therefore, it is important to distinguish between these two phenomena because the effects of climate change (e.g. extinction and population vulnerabilities) will be different if species are locally adapted or they are exhibiting phenotypic plasticity. More studies are needed to advance our understanding of intraspecific differences and physiological adaptations across species' distribution.

1.4 Model of study: Gastropoda, Caenogastropoda

The Gastropoda are the largest and the most diverse class of phylum Mollusca, comprising almost three-quarters of the 110,000 or so species of molluscs known (Brown and Lydeard 2010). Gastropods inhabit diverse habitats especially associated with benthic and rocky shore environments providing important ecosystem services and supporting the 2 % of the molluscs fished in the world (Leiva and Castilla, 2001). According to the FAO (2018), in the past 40 years, worldwide marine gastropod catches increased from 50,000 tonnes in 1970 to 160,000 tons in 2016, indicating their important contribution to food security for humans. Most of them belong to the subclass Caenogastropoda (>23,000 living species; WoRMS database), previously known as Prosobranchia, including marine, freshwater and terrestrial species. Marine gastropods such as periwinkles, whelks, conches, slipper limpets, tuns and cones belong to this subclass (Ponder et al., 2008). The clade Caenogastropoda has been subdivided in three main groups: Architaenioglossa, Littorinimorpha and Neogastropoda, the latter being one of the most abundant of the clade (Osca et al., 2015; Ponder et al., 2008). The majority of caenogastropods are carnivores, although they exhibit a wide variety of dietary specialization (e.g. detritus feeders, suspension feeders, algal grazers). They have separate sex, males have a penis to copulate or to exchange spermatophores (Skewes 2005). Commonly, the life of cycle of a caenogastropod species includes early development associated with the benthos (i.e. intracapsular development), a swimming veliger larvae or a crawling juvenile that grow up until reaching maturity .

1.4.1 Reproduction in Caenogastropoda

One of the most distinctive characteristics of caenogastropods is the mode of development. Generally, they maintain embryos inside gelatinous egg masses or firm, leathery egg capsules for a period or during the whole early development (Pechenik, 1979). Development can be direct, i.e.

after the intracapsular development a crawling juvenile emerges from the egg mass or capsule (e.g. *Buccinum undatum*, Smith et al., 2013), or indirect or mixed; thus, after hatching swimming larvae emerge from egg capsules and subsequently spend days or weeks (depending on environmental temperatures) feeding and growing in the plankton before metamorphosing in the benthos (Pechenik, 1979). Generally, females deposit egg masses (i.e. a group of capsules) in a hard substrate; however in some species, egg capsules are brooded inside the female mantle cavity (e.g. *Crepipatella fecunda*, Mardones et al., 2013).

In the intertidal, egg masses are exposed daily to rapid and severe fluctuations in temperature, pH, oxygen availability and salinity (Helmuth and Hofmann, 2001; Przeslawski, 2004). As encapsulated embryos are unable to seek shelter during periods of low tide or when the environmental conditions are harsh, embryo encapsulation has been suggested as a protective barrier to face adverse conditions experienced in the coastal zone, such as osmotic challenges, desiccation, predation and UV radiation (Pechenik, 1982, 1983; Rawlings, 1999; Przeslawski, 2004, 2005). However, encapsulation has been considered as a barrier to the normal development of embryos as well (Segura et al., 2010). High temperatures and low oxygen availability present constraints for development, producing high mortalities in encapsulated embryos (Cancino et al., 2011). In the deep-sea, there are few developmental studies concerning the reproduction and biology of caenogastropods (Averbuj et al., 2018; Bouchet and Warén, 1993). Long reproductive season, slow development rates, non-planktonic development (i.e. direct), asynchronous development, high maternal provision resources (i.e. intracapsular fluid, nurse eggs or albumin-like) are the main features of caenogastropod species inhabiting the deep sea (Montgomery et al., 2016; Penchaszadeh et al., 2016).

Due to their great economic importance, ecological role in coastal systems and high diversity, prosobranch gastropods are an important group that must be studied under the potential impact of climate change. Global temperatures are likely to increase by 3 °C at the end of this century (IPCC 2013) producing negative impacts for species living in the intertidal zone (Helmuth et al., 2002). Many gastropods species are developing and growing in the intertidal zone; thus, it is relevant to understand how early life stages of different gastropod species will respond to the imminent increased temperature.

1.4.2 Thermal studies in intertidal Caenogastropoda

Few studies have been focused on understanding the thermal tolerance response of caenogastropods at different life-history stages and less specifically focused on latitudinal effects. Previous studies have measured the effects of high temperature in one stage of the life of cycle,

e.g. during the intracapsular development (Cancino et al., 2011) or juvenile stage (Hadfield, 1989), while others studies have been focused on the thermal sensitivities of adults and early stages (Truebano et al., 2018) and few of them have conducted comparative studies among populations (Zippay and Hofmann, 2010). Comparative studies of caenogastropod species can provide valuable information on the development of intraspecific adaptations to local environmental conditions. In the next section, temperature effects studies on maternal investment with emphasis on the effects of environmental temperatures on fecundity were summarised, as a good proxy to estimate reproductive potential. Then, temperature effects during the intracapsular development, as early stages are the most sensitive stage of the life of cycle of marine organisms, were discussed

Thermal studies in maternal investment

Fecundity is a life history trait that has received a central position in the life history theory because it is a good proxy to estimate the maternal reproductive potential and the future stock size of a given species or population (Pechenik et al., 2017; Torres et al., 2009). Fecundity is a highly plastic character that can vary within and between populations or even between individuals from the same population (Llodra, 2002). Fecundity can be affected by different abiotic and biotic variables such as temperature (Wehrtmann et al., 2012), salinity (Berger, 2009), oxygen availability (Fernández et al., 2007), and maternal nutrition (Steer et al., 2004).

In encapsulated species, fecundity can be directly estimated as the number of embryos per capsules per brood and/or female (Llodra, 2002). The number of embryos and capsules has a positive correlation with the length of female shell; fecundity increases as female shell length increases (Chaparro and Flores, 2002; Perron and Corpuz, 1982). However, there is scarce evidence of how latitudinal gradients or geographic origin can impact the fecundity patterns on encapsulated species. For example, Fernández et al. (2007) studied the latitudinal pattern of embryo packing in the temperate muricid gastropod *Concholepas concholepas*. They demonstrated that the number of embryos per unit of capsule depends on the site of origin; thus, local temperatures and oxygen conditions experienced by mothers before egg deposition determine fecundity. It was conclude that females from low latitudes laid fewer embryos per unit capsular area than females from high latitudes. However, it is not clear whether this is a general latitudinal pattern for encapsulated embryos or if maternal investment can also vary according regional environmental conditions.

Thermal studies in early stages

Early life stages are often considered more sensitive to thermal stresses than adults (Gosselin and Chia, 1995; Truebano et al., 2018). Early stages of marine invertebrates exhibit narrower thermal tolerance than adults (Gosselin and Chia, 1995). For example, Truebano et al. (2018) demonstrated that early stages of *Littorina obtusata* were less tolerant of acute exposure to high temperature than adults. They found that small changes in temperature could impact negatively, producing greater mortalities in early stages than in adults. Therefore, tolerance ranges of early stages can set up the abundance and limits of adult distribution (Zippay and Hofmann, 2010).

The encapsulation period is highly correlated with temperature, as temperature increases development rate increases (Roller and Stickle, 1989; Smith et al., 2013). However, temperatures outside of the thermal tolerance window can cause deleterious effects on encapsulated embryos including abnormalities, mortalities, and shell damage, among others (Cancino et al., 2011, Smith et al., 2013). Encapsulated embryos are more tolerant to low temperatures (Przeslawski, 2004); however, high temperatures affect the oxygen availability inside capsules, impacting the development of encapsulates embryos (Cancino et al., 2011).

Little attention has been focussed on the thermal tolerance response of embryos and larvae within a species' distribution range (Fernandez et al., 2007). Previous reports in gastropods egg masses have been focused on the effects of oxygen and temperature (Cancino et al., 2011; Fernández et al., 2006; Moran and Woods, 2007; Strathmann and Strathmann, 1995). However, few have explored whether regional environmental conditions can influence the intracapsular development or whether the embryonic physiological capacities vary according to geographic origin. The scarce latitudinal/comparative studies conducted to date in encapsulated gastropods have shown that thermal tolerance response might depend on the site of origin or parental environment (Fernández et al. 2007). For example, Zippay and Hofmann (2010) found that the acute thermal tolerance response of veliger larvae of the temperate *Nucella nostrina* significantly correlates with the latitude. Veligers from higher latitudes were less tolerant to heat stress, with high mortalities and narrower thermal tolerance than veligers from lower latitudes. It was concluded that slight changes in environmental temperatures can negatively impact early development, producing negative effects on survival and possible local extinction in high latitude population under warming. Therefore, understanding the physiological adaptations and the potential effects of geographic origin during the intracapsular development help us to predict the effects of global warming on the distribution of encapsulated species.

1.4.3 Study organisms: *Ocenebra erinaceus*

The temperate European sting winkle *Ocenebra erinaceus* (Linnaeus, 1758), commonly known as oyster driller, is a predatory gastropod from the Muricidae family, within the order Neogastropoda (WoRMS). The distribution of *O. erinaceus* extends from the Shetland Islands, north of Scotland, to Greece and the Azores (Fig. 1.3; OBIS 2020). The specie is, however, most commonly found on the intertidal and shallow subtidal of UK and French Atlantic coast.

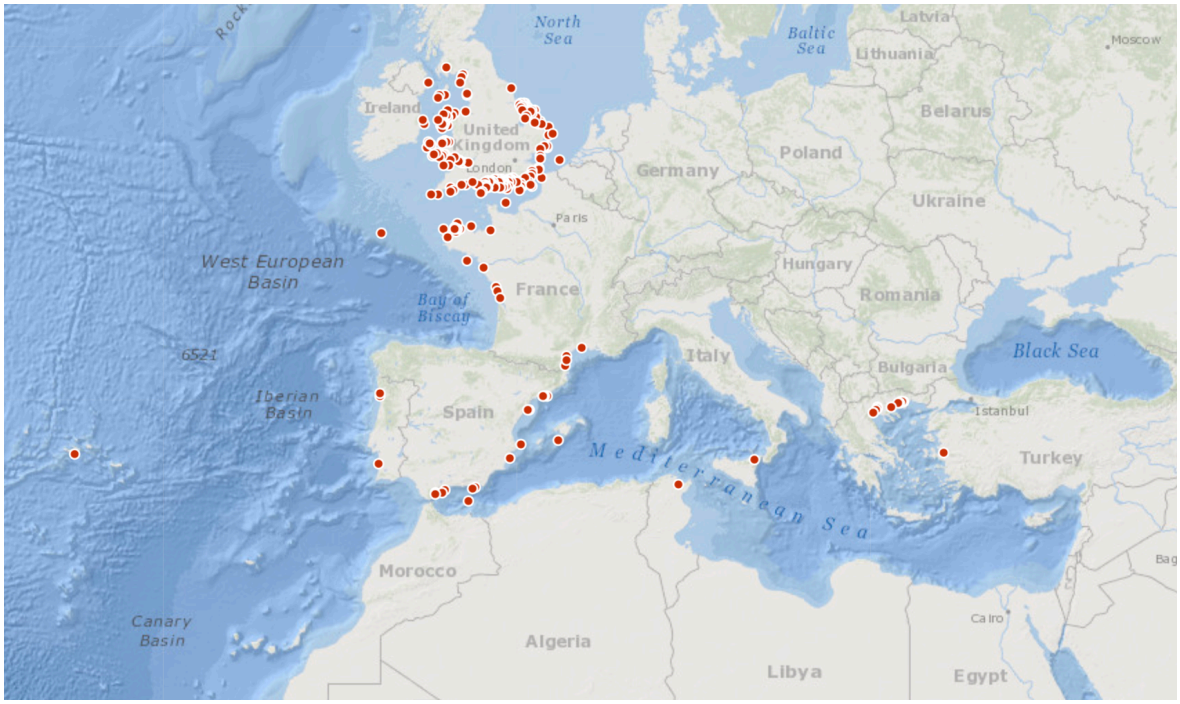

Figure 1.3. The global distribution of *Ocenebra erinaceus*, each red dot represents a population. Taken from the freely-available OBIS database: www.iobis.org (OBIS 2020, accessed on 16th March 2020).

Habitat and ecology

*O*cenebra *erinaceus* is predominantly sublittoral, occurring on rocks and under stones to a depth of 150 m, with a shell size between 8 and 65 mm (Fig. 1.4a; Skewes, 2005). It is a predator species that use their radula to drill a hole in the shell of their prey (Smith et al., 2013). In the past, it was considered as a pest for the shellfisheries, especially in oyster beds (Hancock, 1960). However, throughout 1970s-1990, populations of *O. erinaceus* were highly impacted by tributylin (TBT), which induce imposex in many gastropods (Gibbs 1996), producing populations declined. Gibbs in (1996) indicated that the proportion of sterile females (i.e. oviduct malformation) in South-west of England reached over 75% in heavily polluted areas.

Females exhibit one reproductive laying peak per year between April and March when temperatures reach up to 14 – 15 °C (The Solent, UK; Smith et al., 2015). *Ocenebra erinaceus* is direct developer; females enclose their embryos in capsules and leave them for up to two-three

months on rocky shores (Fig. 1.4b-c; Smith et al., 2015). During intracapsular development, the species develops through eight ontogenetic stages: egg, trochophore, early veliger, veliger, pediveliger and late-pediveliger stage (Fig. 1.4d-i). At hatching, swimming larvae emerge from the capsules (Fig.1.2i), spending a few days in the plankton before they settle as juveniles (Fig. 1.2j; Gibbs, 1996). However, Smith et al. in (2015) have reported dispersal polymorphisms, meaning that from the same egg mass, two different larval forms might hatch: swimming larvae and crawling juveniles. Smith et al. (2015) reported that a proportion hatched as swimming larvae whilst others as crawling juveniles; at 15 °C (experimental temperature), 14% of hatchlings were juveniles. Dispersal polymorphisms seem to be a very advantageous reproductive strategy, in terms of connectivity between populations.

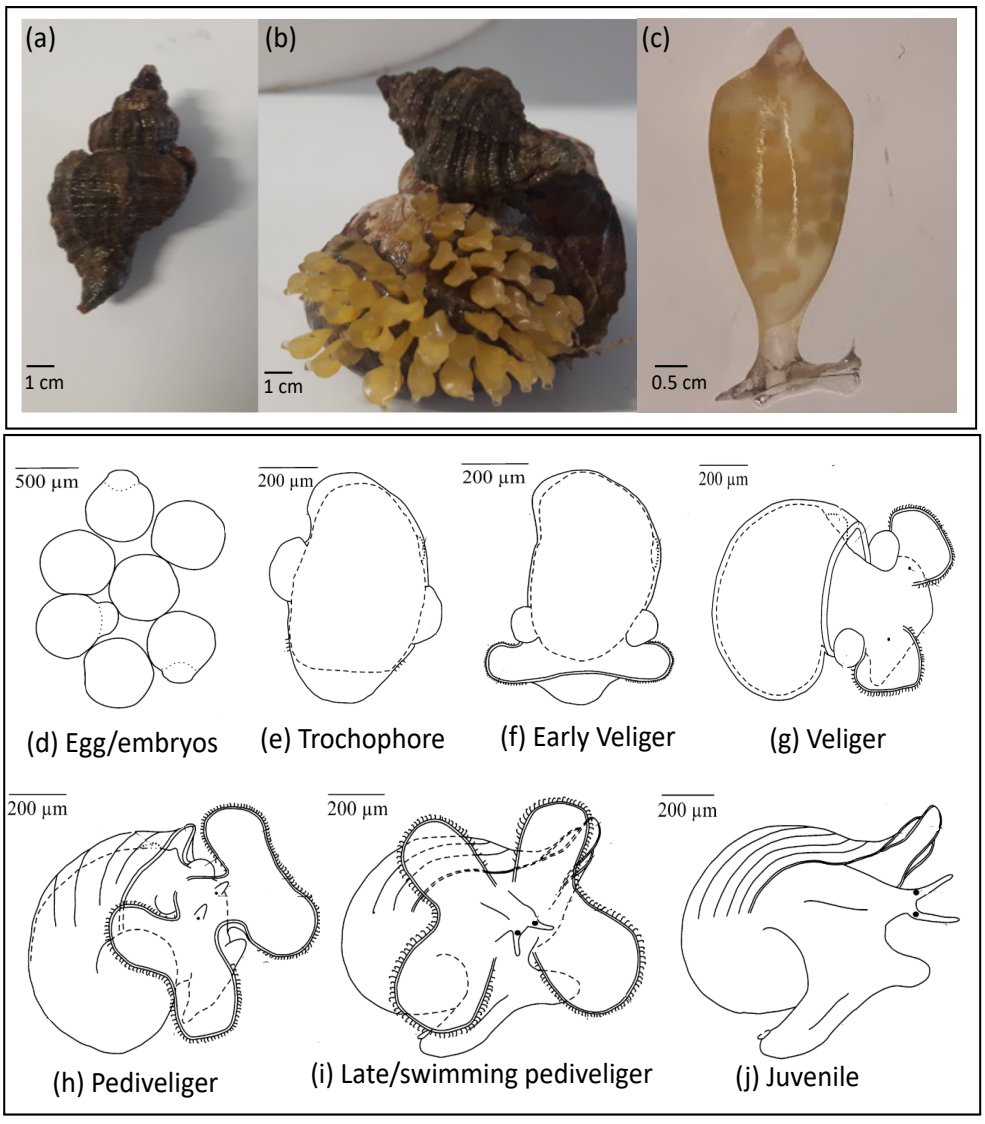

Figure 1.4 Life of cycle of *Ocenebra erinaceus*. **(a)** adult stage; **(b)** female laying egg capsules; **(c)** egg capsule; Ontogenetic stages during the intracapsular development **(d-i)**; at hatching **(i)** and juvenile or crawling stage **(j)**. Modified from Smith et al., 2015.

1.5 Thesis aims and objectives

Global temperatures are likely to increase by 3 °C at the end of this century; one of the main effects of increased temperature is the shift of the biogeographic ranges of marine organisms and local extinctions (Thomas et al., 2004). Many predictions assume that each population of a particular species has the same environmental niche; however, these predictions do not consider that across species geographic distribution, the species exhibit intraspecific differences among populations. This could be more pronounced for species that undergo direct development, with restricted connectivity and low gene flow. These constraints increase the potential for individuals to evolve local adaptations to native environmental conditions. Therefore, there is a fundamental gap in our understanding of whether intertidal encapsulated gastropod species exhibit intraspecific differences between populations; whether they are locally adapted, and how increased temperatures will affect the development in encapsulated embryos.

Ocenebra erinaceus was chosen as study species:

1) Embryos of *Ocenebra erinaceus* develop inside of capsules for up to 3 months at 15 °C (Smith et al., 2015). Therefore, encapsulated embryos are unable to seek shelter when the environmental conditions are harsh (i.e. as predicted in warming scenarios). Therefore, encapsulated embryos must rely on their physiological tolerance only.

2) Encapsulation has been described as a protective barrier for embryo development reducing mortality; however, elevated temperature and oxygen availability may represent a constraint for embryonic development. Therefore, under global warming scenarios, capsules might be a significant barrier for oxygen diffusion and the development of early stages in *O. erinaceus*.

3) *Ocenebra erinaceus* is a mixed developing species with a short pelagic phase of up to 5 days, thus, it is expected that this species has evolved intraspecific differences among populations according to their local conditions. Therefore, it is a good model to study physiological adaptations between different thermal environments.

The thesis has four main objectives:

1. Identify the thermal tolerance response of early stages of *Ocenebra erinaceus* from two populations with different thermal histories and determine whether the thermal tolerance is influenced by geographic origin (Chapter 2).

2. Investigate whether the aerobic response of encapsulated embryos are influenced by geographic origin and determine the possible impact of the physical characteristics of the capsule wall on the embryos' aerobic response (Chapter 3).

3. Determine whether the physiological differences in *Ocenebra erinaceus* are due to adaptation to local environmental conditions and whether these adaptations can persist during the early development of *O. erinaceus* (Chapter 4).

4. Investigate maternal effects and offspring performance from a cold-adapted population of *Ocenebra erinaceus*, in terms of physiology and molecular response to future ocean conditions according to the worst-case scenario RCP8.5 (Chapter 5).

Chapter 2 Variation in thermal tolerance response associate with geographic location during early development of the European sting winkle, *Ocenebra erinaceus* (Linnaeus, 1758)

Environmental temperature plays an important role in shaping the distribution and abundance of ectothermic organisms. As a general rule, larvae and juveniles are more sensitive to thermal stress than adults and, as a consequence, represent key life stages that determine in part the geographic range of a species. Identifying critical thermal limits during ontogeny allow the prediction of the potential impacts of climate change on the distribution of marine ectotherms. *Ocenebra erinaceus* is a gastropod species that inhabits the intertidal and shallow subtidal of UK and French Atlantic coasts. Females lay one clutch of capsules between April and May when temperatures reach up to 14-15 °C (UK). The aim of this study was compare the thermal tolerance response of two populations from the UK and France with an annual sea surface thermal difference of 3 °C. The results showed that thermal tolerance response was influenced by the geographic origin. Embryos from the relatively warm waters population (France) showed a eurythermal tolerance window with optimal temperatures between 12 and 18 °C. On the contrary, embryos from the cold waters population (UK) showed a narrow thermal tolerance window with optimal temperatures between 14 and 16 °C. In both populations, temperatures outside of thermal range caused lethal and sub-lethal effects. Importantly, dispersal polymorphism was not observed at hatching time in both populations. The results of this chapter showed that during early stages, embryos are adapted to local environmental conditions and they are living very close to their upper thermal limits; temperatures outside this range cause detrimental effects on intracapsular development of *O. erinaceus*. This study suggested that future warming could shift the geographical distribution range of *O. erinaceus* poleward.

2.1 Introduction

Temperature is the main environmental driver controlling the physiology, ecology and distribution patterns of terrestrial and marine species (Castañeda et al., 2004; Jones et al., 2009; Somero, 2005). As marine invertebrates are unable to regulate body temperatures independently from the marine environment (i.e. ectothermic), nearly all-physiological and behavioural traits are sensitive to temperature (e.g. metabolism, locomotion, immune function, foraging ability, rates of feeding and growth; Angilletta et al., 2002). Within an organism's thermal tolerance range, the performance

rate increases as temperature increases from a critical minimum to an optimum (Schulte et al., 2011). However, temperatures outside of the optimal thermal range cause a decrease in the physiological performance (e.g. aerobic metabolism) due to oxygen limitation (i.e. the mismatch between oxygen supply and demand), which leads to a shift in aerobic metabolism to an anaerobic mode, until the failure of the whole-organism (Pörtner, 2002, 2010). Physiological thermal capabilities of organisms define the thermal tolerance limits and are predictive of their distribution patterns (Somero, 2002). Hence, the identification of these thermal limits can provide important insight about limits in distribution ranges of marine species and at the same time, describe the potential impact of future warming on shifts in distribution ranges.

The thermal tolerance range in ectothermic organisms can vary across species geographic distribution and among populations of a single species distribution (Sunday et al., 2011). Latitudinal thermal gradients can influence the thermal tolerance response, especially in terrestrial ectotherms where thermal range increases as latitude (from tropical to polar) or environmental variation increases (Gaitan-Espitia et al., 2016; Yu et al., 2018). However, marine ectotherms show a lower rate of increase in thermal tolerance with latitude and a poleward decrease at both upper and lower thermal limits (Sunday et al., 2011). Persistent environmental variations (e.g. tidal regimes, wave action, local temperature) can promote physiological genetic adaptations within a species' distribution range, resulting in a mosaic of populations variously physiologically adapted (Kelly et al., 2012, Kuo and Sanford, 2009; Sanford and Kelly, 2011). Thus, it is important to understand whether the thermal tolerances can vary among populations across a species' geographic range to predict the potential impact of warming.

Global sea temperatures are rising, producing a significant shift in species' distributions and promoting extinction of more thermally-sensitive species (Burrows et al., 2011; Harley et al., 2006; Helmuth et al., 2002; Sunday et al., 2015). Early stages are considered as the bottleneck of the life of cycle of marine organisms as they are more thermally sensitive than adults from the same population (Anger et al., 2003; Sanford et al., 2006). This could be more evident for gastropods species that deposit their embryos in external structures (i.e. gelatinous mass or capsules) on the rocky shore, until they hatch as swimming larvae or juveniles (Pechenik, 1979). Encapsulation can confer protection during the intracapsular development against osmotic changes, UV exposure and predation (Przeslawski, 2004); however, previous reports have shown that high temperatures increase hypoxic conditions inside capsules producing high mortality and abnormalities (Fernández et al., 2006; Smith et al., 2013). Thus, if the external environmental conditions (e.g. heat waves) exceed the physiological limits, encapsulated embryos cannot move and find alternate suitable conditions; thus, encapsulated species can be at potential risk under warming. Little is known about

the thermal tolerances in encapsulated embryos and whether the thermal tolerances can vary among populations across a species' geographic range.

To study the thermal tolerance of an encapsulated species, the temperate caenogastropod mollusc *Ocenebra erinaceus* was chosen as a model of study. This species is a direct developer; females enclose their embryos in capsules and leave them for up to three months on rocky shores (Smith et al., 2015). During intracapsular development, the species develops through eight ontogenetic stages from egg to different veliger stages (Fig 1.3). At hatching, swimming larvae emerge from the capsules, spending a few days in the plankton before they settle as juvenile (Gibbs, 1996). However, Smith et al. in (2015) have reported dispersal polymorphisms, meaning that from the same egg mass, two different larval forms might hatch. Smith et al. (2015) reported that a proportion hatched as swimming larvae whilst others as crawling juveniles; at 15 °C (experimental temperature), 14% of hatchlings were swimming larvae (Smith et al., 2015). Dispersal polymorphism seems to be a very advantageous reproductive strategy, in terms of increasing the dispersal connectivity between populations and also increasing the possibilities to find more suitable environments.

This chapter aims to identify the thermal tolerances of early stages of *O. erinaceus* and to determine if the thermal tolerance is influenced by their geographical origin. To determine intraspecific differences, two populations from different thermal environment were compared: one from the middle and another from the south of the species' geographic range. It was predicted that embryos from relatively warm waters would exhibit wider thermal tolerance windows and high survival as a physiological adaptation to the warmer seawater temperatures that they experienced through the year. The findings of this study will contribute to our understanding of physiological adaptations to local environmental conditions, and provide insights in how early ontogenetic stages will be impacted by ocean warming.

2.2 Material and methods

2.2.1 Study sites and reproductive life of cycle of *Ocenebra erinaceus*

To study the intraspecific differences in the thermal tolerance response during early developmental stages of *Ocenebra erinaceus*, adult gastropods from two different populations were collected for reproductive experiments (Fig. 2.1): one from the middle of their geographic distribution, the Solent, Southampton, UK (50° 51' N, 001° 21' W) and the other towards the southern end of the geographic distribution: Arcachon, France (44° 41' N, 001° 11' W). In total, sixty individuals were collected from The Solent, UK and 150 individuals from Arcachon, France. The distribution of *O. erinaceus* extends from the British Isles to the Mediterranean, Madeira and the Azores (Skewes,

2005). In the Solent, the reproductive season is between April and May, when sea surface temperatures (SST) fluctuate between 12 and 16°C. Females typically enclose 48 eggs per capsule and each brood consists of 21 capsules on average, with a mean laying effort of 1012 eggs per female (Smith et al., 2015). The Solent population exhibits one reproductive laying peak per year between April and May. No information was found regarding the reproductive cycle of *O. erinaceus* in Arcachon, France. However, Martel et al. (2004) observed only one laying peak in May in Rivedoux, France, a site 250 km North from Arcachon. At the end of May, a unique recruitment event of juveniles of *O. erinaceus* was observed 3 weeks after laying (Martel et al., 2004). Martel et al. (2004) reported that Rivedoux females enclosed 74 eggs per capsule and each brood consisted on 24 capsules on average, with a mean laying effort of 1800 eggs per female.

2.2.2 Thermal data acquisition

The Solent is an estuarine system that experiences seasonal temperature variations, with surface water temperatures fluctuating between 7 and 10 °C in winter, and in summer between 17 and 20 °C. On the contrary, Arcachon is a coastal system in which temperature fluctuations throughout the year are less pronounced. Surface water temperatures range from 10 – 12 °C in winter and 19 – 22 °C in summer (Fig. 2.2). The annual averages of surface water temperature are 13 and 16 °C in the Solent and Arcachon waters, respectively.

Seawater temperature data for the Solent were collected from XAUK buoy (https://stormcentral.waterlog.com/public/XAUKBuoy) and Arcachon from REPHY dataset 1987-2016 (http://doi.org/10.17882/47248) for the interval 2014-2016. Monthly temperatures were obtained by averaging the daily temperatures per month (Fig. 2.2a). Seasonal temperatures for winter (December to February) and spring (March to May) were compared between populations (Fig. 2.2.b). These seasons were chosen due to their importance in the reproductive cycle of *O. erinaceus* (Martel et al., 2004). The increase in temperature between winter and spring induces the spawning in this species and the intracapsular development occurs during the spring months.

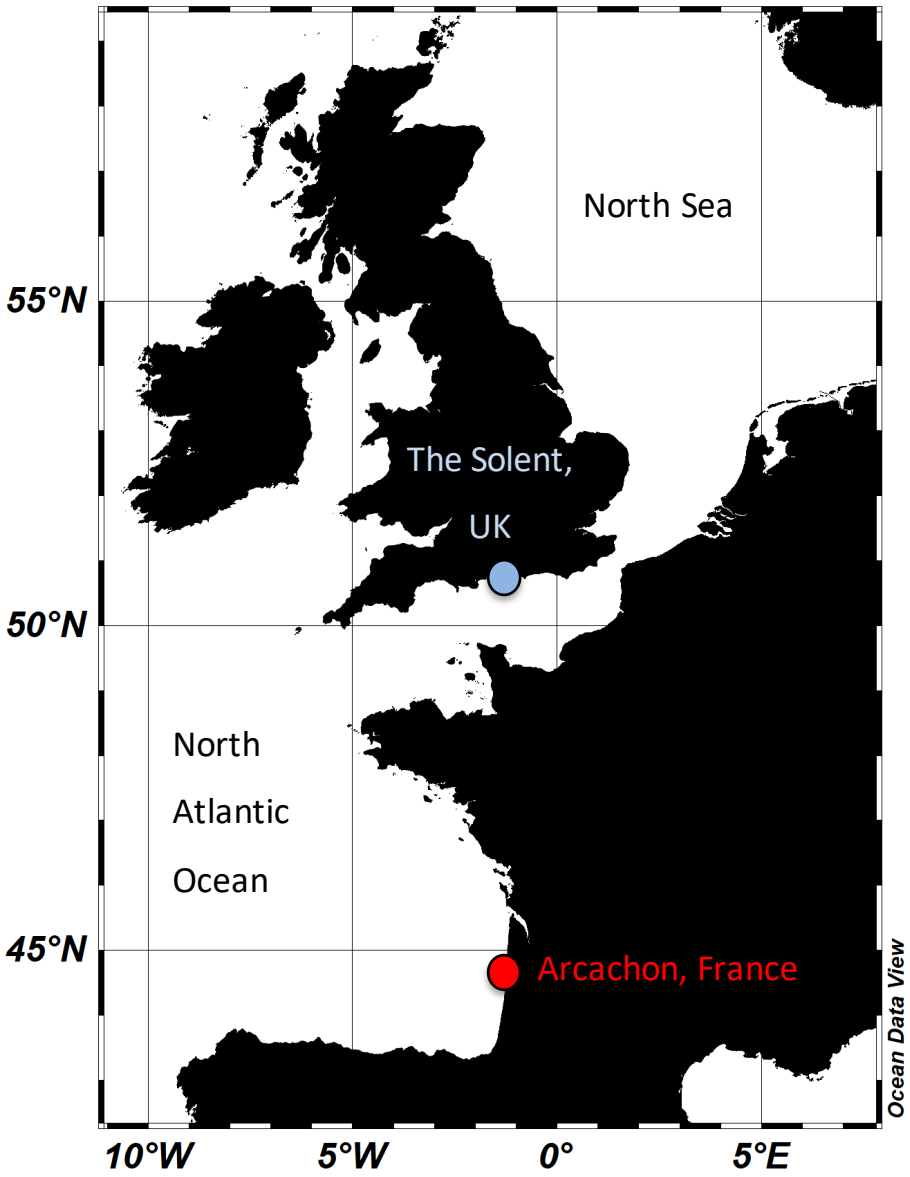

Figure 2.1 Sampling sites of *Ocenebra erinaceus* populations: blue spot represents the Solent, UK (50° 51' N, 001° 21' W) and red spot represent Arcachon, France (44° 41' N, 001° 11' W).

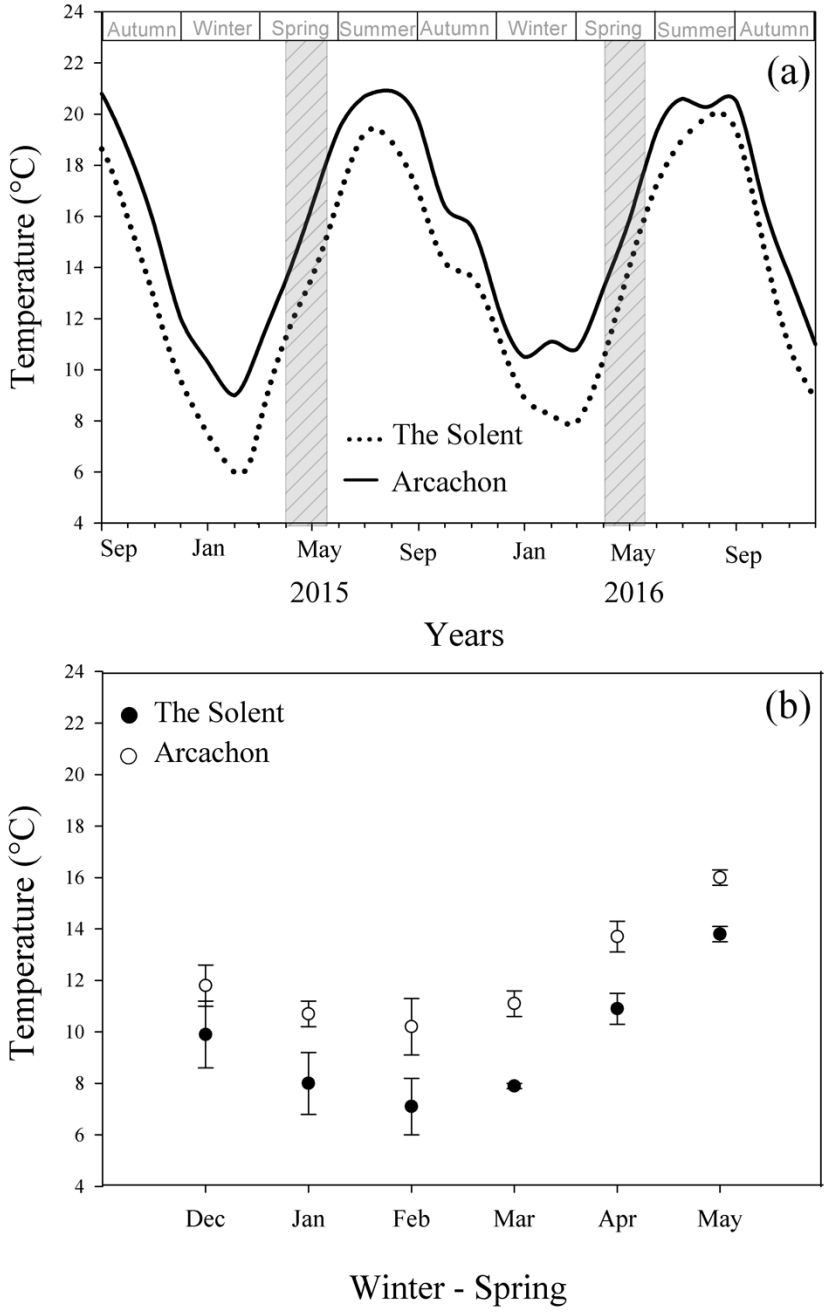

Figure 2.2 (a) Average monthly seawater temperatures for the Solent, UK and Arcachon, France from September 2014 to December 2016. Grey dashed bars indicate the time that embryos spend inside capsules in the field, according to Smith et al. (2015) and Martel et al. (2004). **(b)** Average surface seawater temperatures between 2014 and 2016 for winter (December to February) and spring (March to May) in the Solent and Arcachon. Values given are average ± standard error for the 3 consecutive years.

2.2.3 Female collection and maintenance

Females of *O. erinaceus* were collected via trawling from the Solent between February-March 2016 and from a commercial oyster bed in Arcachon in October 2017. During sampling, seawater temperature in the Solent ranged between 9 and 10 °C and between 14 and 16 °C in Arcachon. After sampling, females from both populations were transferred in thermally controlled boxes to the National Oceanography Centre, Southampton (NOCS), UK to aquarium conditions of continuously re-circulated seawater that originated from Empress Dock. Both populations were maintained under different regimes of temperature to ensure that the acclimation period in the aquarium was very similar to the temperatures that embryos experienced in the field. Average monthly temperatures were calculated for each population from annual databases available during three consecutive years 2014 – 2016 (Fig. 2.2). As specimens collected in the Solent were collected near the aquarium facilities, females were maintained in the laboratory for two months before the beginning of capsule laying process. For the Solent population, females were maintained at: March: 8 ± 1 °C, April: 11 ± 1 °C and May: 14 ± 0.3 °C. They started to deposit egg capsules between April and May 2016. On the contrary, the 150 individuals from Arcachon, France were collected during winter months and then transferred to the NOCS's laboratory facilities. To ensure that the females experienced conditions similar to the Arcachon, they were maintained as follows: October: 17 ± 1 °C; November: 15 ± 1 °C, December: 12 ± 1 °C; January: 10 ± 1 °C, February: 10 ± 1 °C; March: 11 ± 1 °C (Fig. 2.2b). They started to deposit capsules between February and March 2017. Individuals from both populations were feed *ad libitum* with mussel and oysters 3 times per week. In both populations, salinity was maintained constant with values ranging between 32.5 and 34.0 on average.

2.2.4 Egg mass collection and maintenance

After one or two days of egg laying, egg masses from identified females from the Solent and Arcachon populations were transferred to one of six experimental temperature treatments (10, 12, 14, 16, 18 and 20 °C). For the Solent population, high mortalities were observed at 18 °C; thus, embryos were not exposed to 20 °C. To acclimate to experimental conditions, the initial egg mass temperature (The Solent: 15 °C; Arcachon: 11-12 °C) was gradually increased or decreased one degree every 24 hours, depending on the required experimental temperature treatment. Egg masses were maintained individually in 1.8 L aquariums with filtered seawater (1 µm, 33-35 salinity) under constant aeration. 100% water changes were conducted 3 times per week. Temperature and salinity conditions were measured three times per week in each experimental treatment. Female size, number of capsules and rearing experimental conditions for both populations are summarized in Table 2.1.

Table 2.1 Summary of experimental conditions of egg masses assigned to temperatures between 10 and 20 °C from the Solent and Arcachon populations. 'Exp.T' indicates experimental treatments; 'FS' indicates female size (in mm); 'NC' indicates number of capsules per egg mass; 'T' indicates temperature in °C; 'S' indicates salinity. 'n.d.' indicates non identified female. Values are given as mean ± standard deviation.

Exp. T	**Solent, UK**				**Arcachon, France**			
	FS	**NC**	**T**	**S**	**FS**	**NC**	**T**	**S**
10 **°C**	40.0	38	10.2 ±0.7	31.1 ± 1.5	-	18	10.3 ± 0.4	33.4 ± 1.3
	28.0	30			-	14		
	31.4	28			-	28		
12 **°C**	35.7	28	12.4 ± 0.8	31.6 ± 1.2	38.3	32	12.7 ± 1.2	33.2 ± 1.0
	45.0	36			41.9	26		
	37.2	28			36.1	26		
14 **°C**	43.92	20	14.3 ± 0.4	32.3 ± 1.3	-	30	14.0 ± 0.6	33.4 ± 0.8
	28.99	20			30.3	15		
	28	22			40.1	33		
16 **°C**	n.d.	24	16.0 ± 0.5	33.1 ± 0.5	35.5	26	16.0 ± 0.2	33.4 ± 0.8
	n.d.	18			36.5	24		
	n.d.	16			36.5	23		
18 **°C**	38.9	25	18.2 ± 1.0	32.1 ± 2.1	40	45	18.0 ± 0.4	33.4 ± 1.1
	37.5	26			34.1	27		
	37.6	28			38	18		
20 **°C**					36.1	26	20.0 ± 0.5	33.7 ± 1.3
					39	26		
					39	15		

2.2.5 Experimental measurements during intracapsular development

Between 2-3 egg masses per population were exposed individually per temperature treatment to quantify the following traits: developmental rates, embryo size, capsular survival and proportion of abnormal embryos at hatching (n= 20-25 capsules per egg mass). Each week, 1-2 capsules per egg mass were dissected to identify the intracapsular ontogenetic stage, number and egg size, and capsular mortality. Intracapsular ontogenetic stage was determined according to Smith et al. (2015). Eight different ontogenetic stages were identified: egg, trochophore, early veliger, veliger, pediveliger, late pediveliger, swimming-late pediveliger and crawling juvenile. Capsular size was obtained measuring the capsule height excluding the peduncle under a microscope (LEICA MZ16). Number and embryo size was obtained throughout using photographs recorded with a digital camera attached to a microscope (x100 magnification; LEICA MZ16). The maximum shell length was measured using the image analysis software ImageJ. Capsular mortality was checked each week. A capsule was considered dead when capsules developed an external purple colour or when no larval activity was found. Dead capsules were dissected and the ontogenetic stage was recorded. Developmental abnormalities were identified when some of the embryos inside the capsule showed malformations in their shell morphology.

Until hatching, three capsules from each egg mass exposed to 14, 16 and 18 °C in the Solent and 14, 16, 18 and 20 °C in Arcachon population were maintained individually in small aquaria of 0.25l to observe the time to metamorphosis after hatching (i.e. the time that swimming veliger larvae spent in the water column until they settled as a crawling juvenile). Once the hatching process had begun, larvae were exposed to light for 15 minutes under a dissecting microscope (x40 magnification). If larvae exhibited pronounced velum lobes they were considered a swimming late-pediveliger and metamorphosis was deemed incomplete. However, if larvae showed crawling activity and the complete loss of the velum lobes they were considered to be crawling juveniles. Larvae that did not complete metamorphosis were maintained for additional days in the same aquarium until metamorphosis was completed. Every day, swimming late-pediveliger larvae were examined to see if they had reached metamorphosis. The number of days to reach complete metamorphosis was recorded. Also, the size of recently settled juveniles after the metamorphosis was recorded.

2.2.6 Statistical analysis

Two-way ANOVA's were used to compare the average monthly seawater temperatures between Arcachon and the Solent in winter (Dec – Feb) and spring (Mar – May) independently, for three

consecutive years (2014 - 2016) to establish if pre-spawned females and intracapsular embryos experienced different temperatures between populations.

One-way ANOVA was used to compare the effect of temperature on the survival and embryonic development followed by Tukey *post hoc* analysis in each population. Prior to analysis, the percent survival and abnormal capsules was arcsine transformed. Intracapsular development time was analysed by two-way ANOVA followed by Tukey *post hoc* analysis, with temperature and population as factors. Normality and homogeneity of variance were confirmed before respective analysis and statistical significance was identified at $p < 0.05$. Kruskal-Wallis analyses of variance by ranks, followed by Dunn's method *post hoc* analysis was used to compare the metamorphosis time in both populations.

2.3 Results

2.3.1 Thermal history of sampling sites

Significant differences in average monthly temperatures (SST) were observed between Arcachon and the Solent during winter months (Location: $F_{(1,16)}=26.34$, $p < 0.001$; Month: $F_{(2,16)}=7.569$, $p < 0.05$); however, the interaction between location and month was not significantly different ($F_{(2,16)}=0.55$, $p > 0.05$). The Solent experienced colder water temperatures than Arcachon during January and February, reaching values between 7 and 10 °C compared with Arcachon where SST ranged between 10 and 12 °C (Tukey *post hoc* analysis; $p < 0.05$). During spring months, a similar pattern was observed between Arcachon and the Solent (Location: $F_{(1,14)}=138.02$, $p < 0.001$; Month: $F_{(2,14)}=178.98$, $p < 0.001$), again with no significant interaction ($F_{(2,14)}=0.36$, $p > 0.05$). Tukey *post hoc* analysis showed colder spring SST in the Solent than in Arcachon with temperatures ranging between 10 and 14 °C and 13 – 17 °C, respectively (Fig. 2.2b).

2.3.2 Temperature effects on intracapsular development and embryo size

Temperature and geographic location produced a significant effect on the developmental time of *Ocenebra erinaceus* ($p < 0.05$; Two-way ANOVA, Fig. 2.4, Table 2.2). Intracapsular development was successful between 10 and 20 °C in Arcachon population, and between 12 and 18 °C in the Solent population. In general, as temperature increased the developmental time decreased. At low temperatures (10 – 12 °C), development was slower for Arcachon (i.e. southern population) than in the Solent population, taking approximately 140 days and 100 days, respectively (Tukey *post hoc* analysis). At high temperatures, development was similar and faster in embryos from both populations, taking approximately 45 and 30 days at 18 and 20 °C, respectively.

In the current study, *O. erinaceus* exhibited a mixed development with every embryo passing through a pelagic and benthic phase. During intracapsular development, *O. erinaceus* showed six ontogenetic stages: egg, trochophore, early veliger, veliger, pediveliger and late pediveliger stage (Fig. 2.3a-f). During hatching, larvae emerged as a swimming late-pediveliger (Fig. 2.3g) and depending on rearing temperatures, larvae spent hours or days in the plankton, showing active swimming and photosensitive behaviour. Dispersal polymorphism was not observed at hatching in the Solent or Arcachon population. 100% of the embryos hatched as swimming late-pediveliger larvae. After that, swimming larvae lost their velar lobes completely and then settled as crawling juveniles (Fig. 2.3h). Embryo sizes and developmental times per ontogenetic stages were summarized in table 2.3 and 2.4 for the Solent and Arcachon populations, respectively.

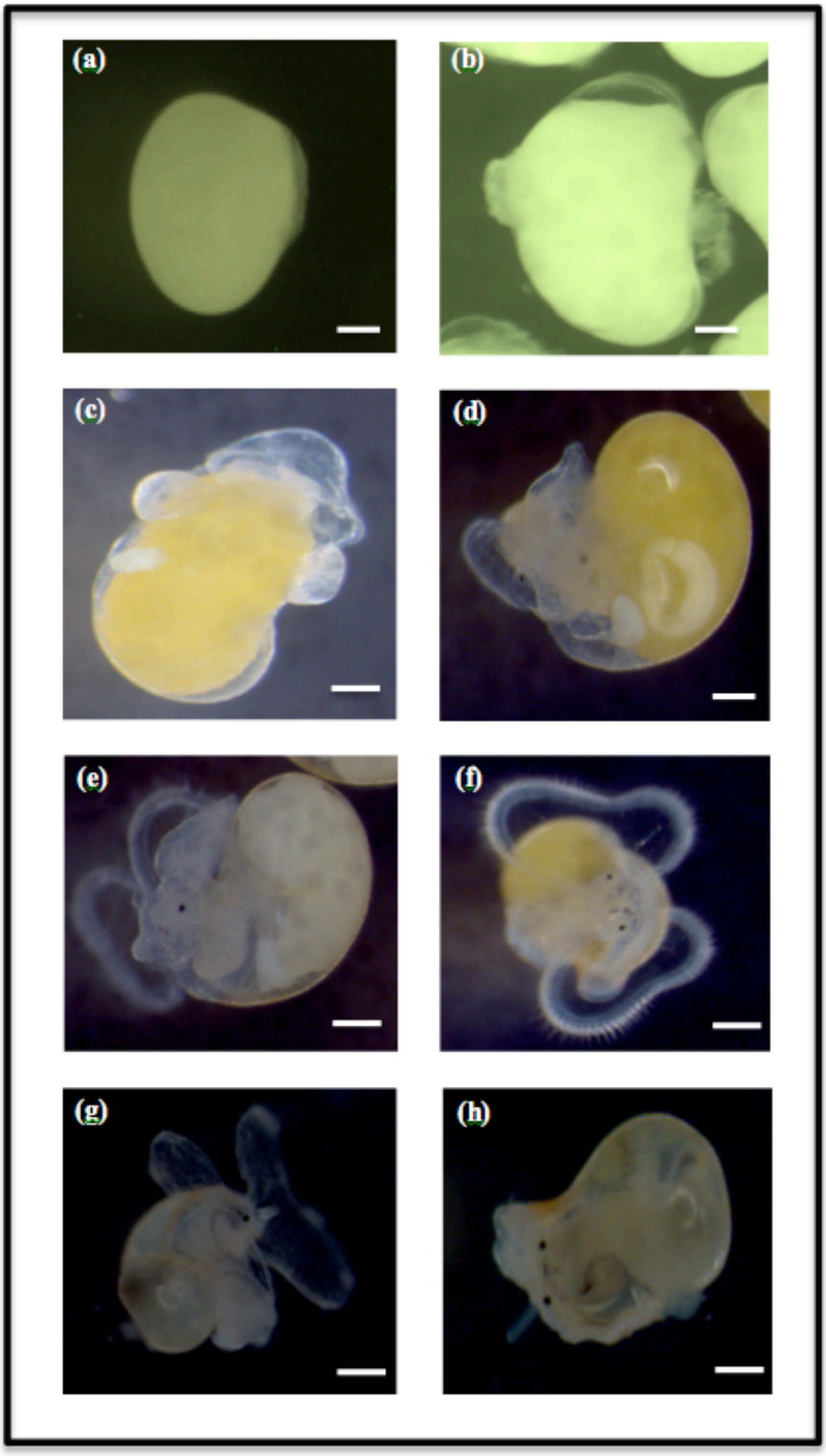

Figure 2.3 Ontogenetic stages during the intracapsular development of *Ocenebra erinaceus*. **(a)** Egg; **(b)** Trochophore; **(c)** Early veliger; **(d)** Veliger; **(e)** Pediveliger; **(f)** Late pediveliger; **(g)** Swimming late pediveliger; **(h)** Crawling juvenile. Scales bars: (a-g) = 100 µm, (h) = 250 µm.

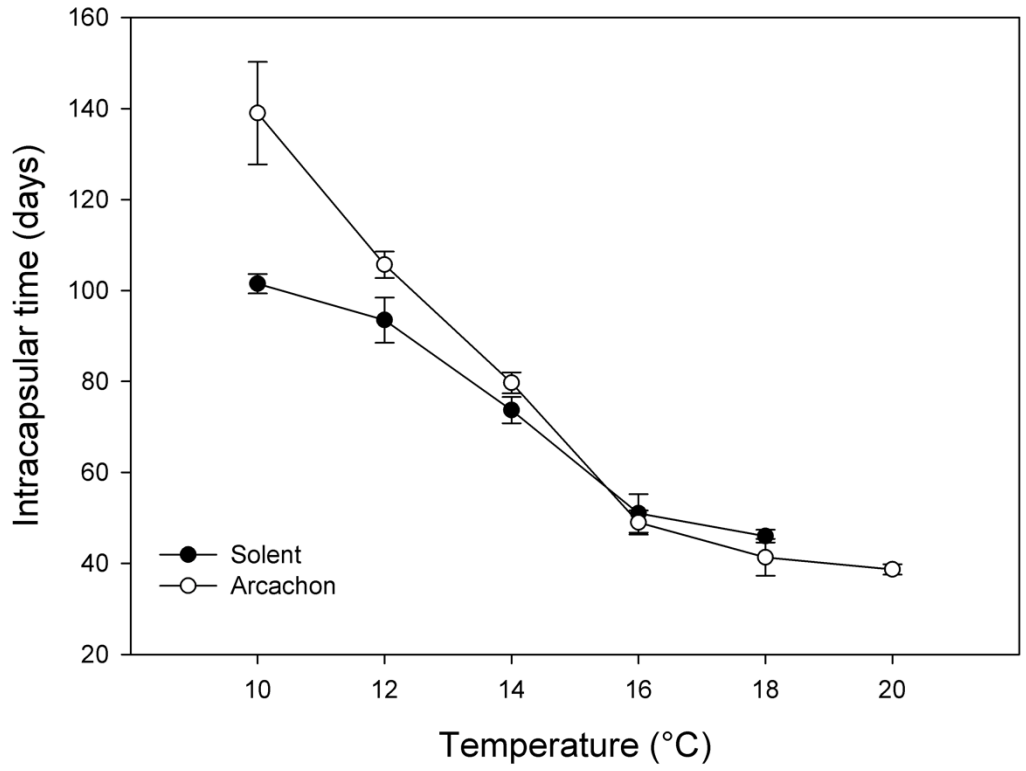

Figure 2.4 The effect of temperature on the intracapsular developmental time of embryos from the Solent and Arcachon populations. Embryos were exposed to experimental temperatures between 10 and 20 °C. In the Solent, development was not fully completed at 10 - 12 °C. Values are given as mean ± standard deviation (SD). n = 3 egg masses per temperature treatment.

Table 2.2 Results of two-way ANOVA using embryos from the Solent and Arcachon populations for the effect of temperature on the intracapsular developmental time.

Developmental time				
Source	DF	MS	F	P
Location	1	49.60	5.87	0.028
Temperature	3	3975.29	470.56	**<0.001**
L x T	3	88.37	10.46	**<0.001**
Error	23	538.08		

Table 2.3 Developmental time in days and embryo size (µm ± SD) in each ontogenetic stage during the intracapsular development of *Ocenebra erinaceus* from The Solent, UK (50° 47' N, 1° 15' W), at temperatures ranging 10 to 18 °C. (?) indicates no information because of high mortalities. Ontogenetic stages: eggs (E), trochophore (T), early veliger (EV), veliger (V), pediveliger (PV), late-pediveliger (LP), swimming late-pediveliger (SLP). n = 3-5 capsules (40 embryos approx.) per ontogenetic stage and temperature treatment.

	Developmental times (days) / Embryo Size (µm ± SD)				
Stage	**10 °C**	**12°C**	**14°C**	**16°C**	**18°C**
E	0 to 29	0 to 27	0 to 21	0 to 11	0 to 18
	(510 ± 31)	(520 ± 20)	(501 ± 30)	(497 ± 20)	(516 ± 27)
T	29 to 50	27 to 40	21 to 31	11 to 18	18 to 22
	(606 ± 71)	(623 ± 70)	(570 ± 50)	(585 ± 69)	(563 ± 83)
EV	50 to 53	40 to 58	31 to 38	18 to 21	22 to 25
	(639 ± 51)	(653 ± 31)	(646 ± 48)	(726 ± 10)	(714 ± 45)
V	53 to78	58 to 75	38 to 51	21 to 34	25 to 31
	(617 ± 62)	(672 ± 54)	(621 ± 67)	(630 ± 59)	(653 ± 64)
PV	78 to 102	75 to 94	51 to 63	34 to 41	31 to 39
	(785 ± 107)	(785 ± 32)	(812 ± 76)	(793 ± 39)	(801 ± 49)
LP	102 to ?	94 to ?	63 to 74	41 to 51	39 to 46
	(903 ± 38)	(919 ± 31)	(897 ± 32)	(849 ± 26)	(888 ± 44)
SLP/J	?	?	74 to 78	51 to 57	46 to 60
			(933 ± 46)	(857 ± 30)	(936 ± 19)

Table 2.4 Developmental time in days and embryo size (µm ± SD) in each ontogenetic stage during the intracapsular development of *Ocenebra erinaceus* from The Arcachon, France population (44° 41' N, 1° 12' W), at temperatures ranging 10 to 20 °C. Ontogenetic stages: eggs (E), trochophore (T), early veliger (EV), veliger (V), pediveliger (PV), late-pediveliger (LP), swimming late-pediveliger (SLP). n = 3-5 capsules (40 embryos approx.) per ontogenetic stage and temperature treatment.

	Developmental times (days) / Embryo Size (µm ± SD)					
Stage	**10 °C**	**12 °C**	**14 °C**	**16 °C**	**18 °C**	**20 °C**
E	0 to 30 (576 ± 26)	0 to 23 (538 ± 37)	0 to 21 (500 ± 30)	0 to 12 (539 ± 23)	0 to 11 (498 ± 52)	0 to 12 (514 ± 51)
T	30 to 41 (574 ± 21)	23 to 37 (565 ± 5)	21 to 28 (560 ± 46)	12 to 17 (595 ± 41)	11 to 15 (530 ± 64)	12 to 16 (581 ± 20)
EV	41 to 57 (665 ± 34)	37 to 47 (661 ± 27)	28 to 39 (681 ± 15)	17 to 22 (675 ± 19)	15 to 18 (665 ± 30)	16 to 21 (695 ± 32)
V	57 to 74 (579 ± 34)	47 to 63 (594 ± 22)	39 to 52 (643 ± 59)	22 to 29 (629 ± 29)	18 to 24 (590 ± 47)	21 to 27 (592 ± 44)
PV	74 to 113 (735 ± 58)	63 to 83 (698 ± 68)	52 to 67 (768 ± 49)	29 to 38 (725 ± 34)	24 to 33 (746 ± 68)	27 to 32 (768 ± 39)
LP	113 to 139 (834 ± 18)	83 to 106 (829 ± 70)	67 to 80 (848 ± 34)	38 to 49 (848 ± 28)	33 to 41 (848 ± 44)	32 to 39 (850 ± 22)
SLP/J	139 to 140 (885 ± 39)	106 to 107 (876 ± 47)	80 to 81 (881 ± 40)	49 to 50 (886 ± 40)	41 to 42 (905 ± 41)	39 to 41 (886 ± 39)

2.3.3 Temperature effects on survival and abnormalities in the development

Survival and percentage of normal development during intracapsular development differed between temperature treatments and populations. In the Solent population, embryo survival was significantly affected by temperature ($F_{(4,14)}$=24.85, $p < 0.05$; Fig. 2.5a). Tukey *post hoc* analysis showed differences at both ends of the thermal range; survival was lower at 10 and 18 °C with values of 50 – 40%, respectively. Optimal temperatures for intracapsular development were observed between 14 and 16 °C. On the contrary, temperature did not significantly affect survival in the Arcachon population; high survival was observed for all temperature treatments ($F_{(5,17)}$=1.77, $p > 0.05$; Fig. 2.5b).

The percentage of capsules with embryos with normal development was not significantly different between temperature treatments in the Solent ($F_{(4,14)}$=1.03, $p > 0.05$; Fig. 2.6a). On the contrary, Arcachon embryos exhibited ~20 – 25% of abnormalities (i.e. shell deformations) during the development at both extremes of the experimental range ($F_{(5,17)}$=6.24, $p < 0.05$; Fig. 2.6b). Tukey *post hoc* analysis showed that normal embryonic development was observed between 12 and 18 °C.

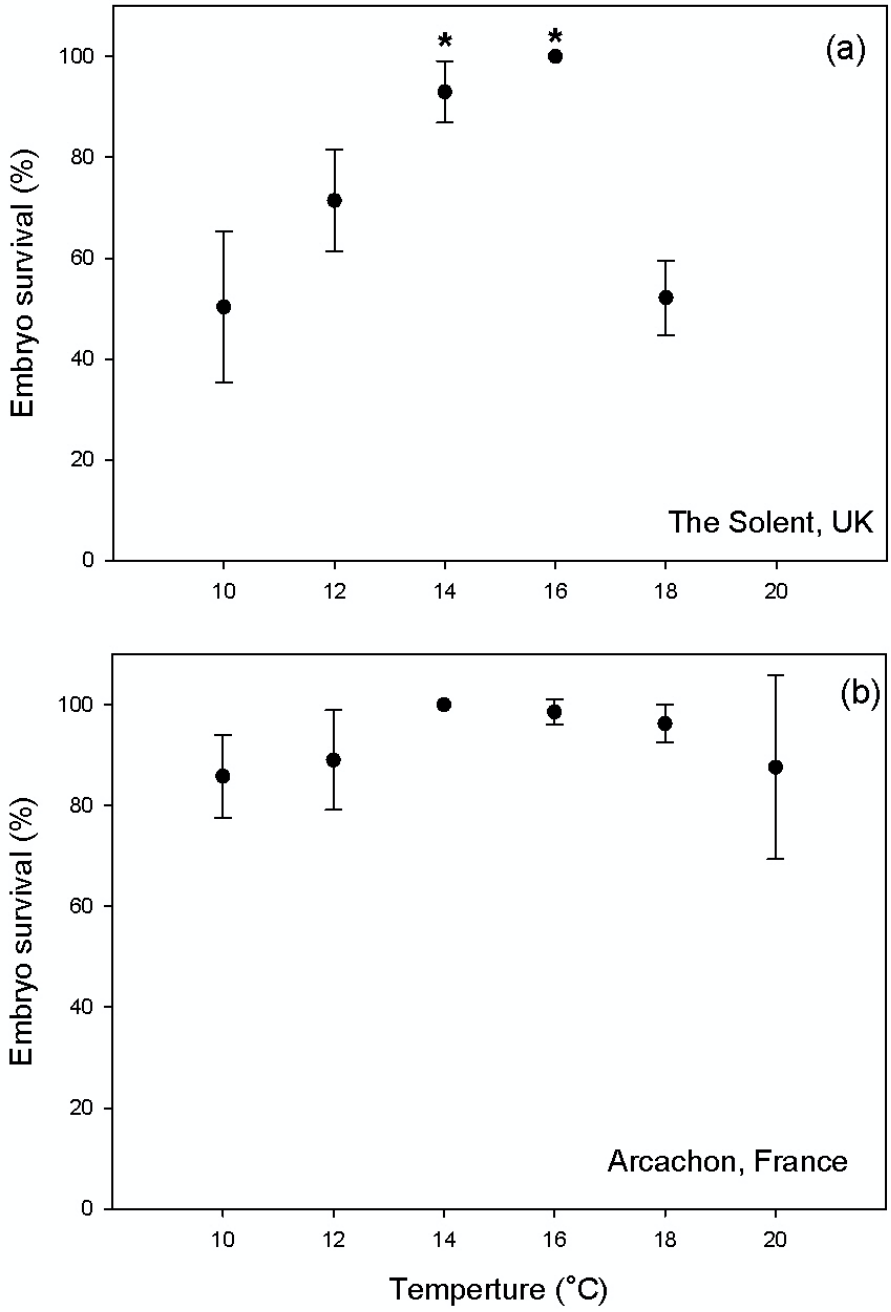

Figure 2.5 Effects of temperature on the percentage of embryo survival in: **(a)** the Solent and **(b)** Arcachon. (*) means significant differences between treatments. Values are given as mean ± standard deviation. n = 3 egg masses per temperature treatment

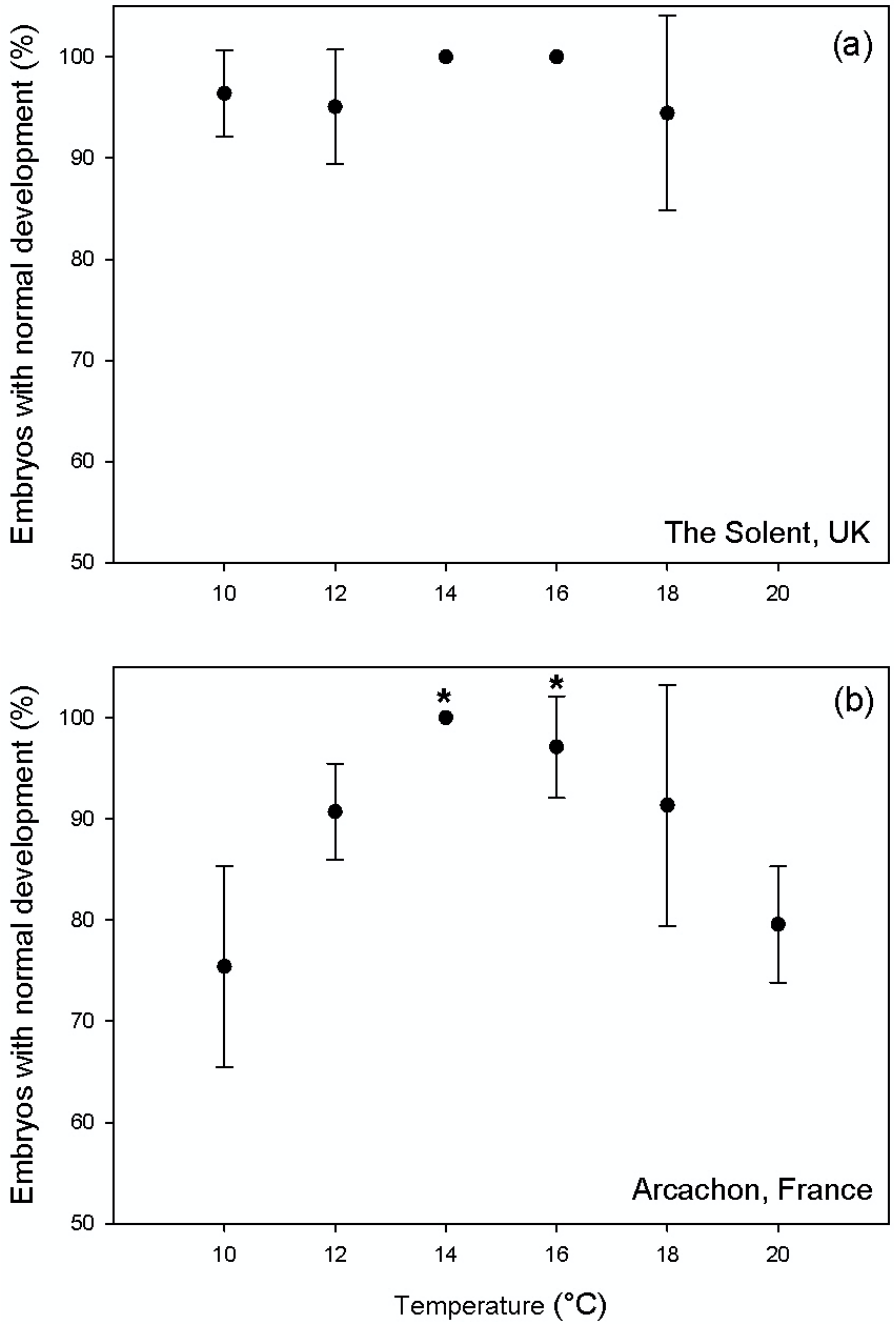

Figure 2.6 Effects of temperature on the percentage of embryos with normal development in: **(a)** the Solent and **(b)** Arcachon. (*) means significant differences between treatments. Values are given as mean ± standard deviation. n = 3 egg masses per temperature treatment.

2.3.4 Hatching

Time To Metamorphosis (TTM) (i.e. the time that swimming late-pediveliger larvae spent in the column of water until they metamorphosed into juvenile stage) was affected by increased temperature increase and geographic origin. In the Solent, TTM was significantly affected by temperature ($H= 11.18$, $p < 0.05$; Fig. 2.7a). Dunn's *post hoc* analysis showed that fifty percent of swimming late-pediveliger exposed to 16 or 18 °C spent more time in the pelagic environment compared with larvae maintained at 14 °C. Swimming late-pediveligers settled before 24 hours at 14 °C; however, at higher temperature treatments (16-18 °C) this took approximately 60 hours. On the contrary, the MMT of swimming larvae from Arcachon increases with increasing temperature but it is not significant ($H= 5.66$, $p > 0.05$; Fig. 2.7b). The time that larvae spent in the plankton was not affected by temperature treatments with values ranging between 24 and 48 hours (Dunn's *post hoc* analysis).

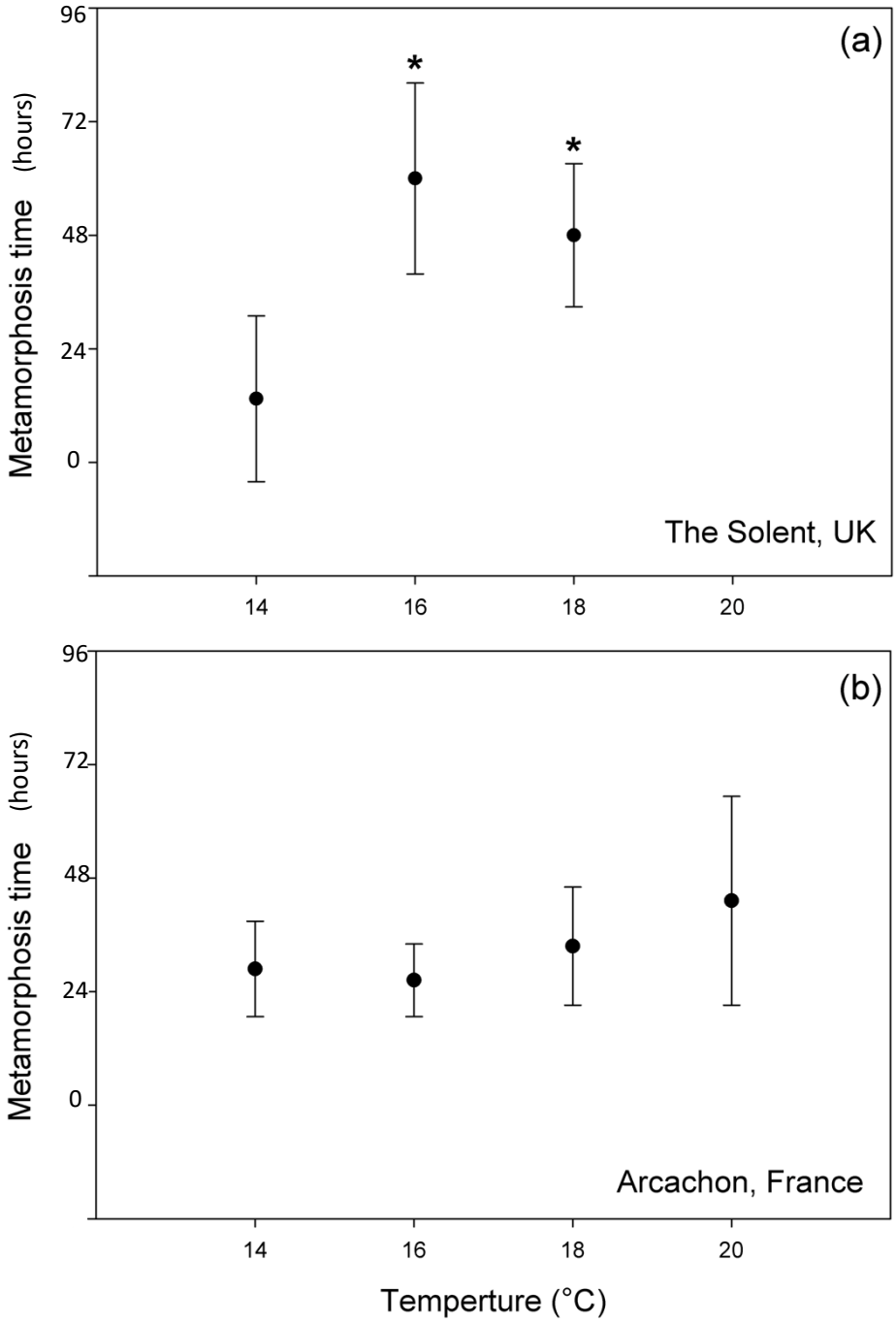

Figure 2.7 Effects of temperature on the metamorphosis time (hours) in: **(a)** the Solent and **(b)** Arcachon. (*) means significant differences between treatments. Values are given as mean ± standard deviation. n = 9 – 10 capsules (48 embryos approximately per capsule) per temperature treatment.

2.4 Discussion

The aim of this chapter was to identify whether geographic origin has an impact on the thermal tolerance response of encapsulated embryos of a shallow subtidal neogastropod species. The results showed that the thermal response of early stages of *Ocenebra erinaceus* was influenced by local thermal conditions that they experienced during the intracapsular development, which led to intraspecific differences in their thermal limits and optimum temperatures. These differences could be a result of physiological adaptions to local environmental conditions.

2.4.1 Temperature effects on development and dispersal polymorphism

In both populations, encapsulation period was highly correlated with the rearing temperatures (Fig. 2.4). At 10 – 12 °C, the intracapsular development was slower than at 18 – 20°C. Exposure to low temperatures produced similar results in other marine species. Strathmann and Chaffee (1984) showed that low environmental temperatures might decrease metabolic rates in embryos because the oxygen diffusion coefficients decreased and viscosity increased in the intracapsular fluid, limiting the transport of oxygen inside of capsules. In this study, the prolonged intracapsular development exhibited by embryos of *O. erinaceus* could be a response to a reduction in their metabolic rates and oxygen deficiency. At high temperatures (18 – 20 °C), development proceeds more quickly as was observed in this study and also it has been reported in other species such as *Buccinum undatum* (Smith et al., 2013) and *Thais haemastoma* (Roller and Stickle, 1989).

Ocenebra erinaceus did not exhibit dispersal polymorphism (i.e. crawling and swimming morphs at hatching) during the hatching process in both populations (Fig. 2.3). Six ontogenetic stages were observed during the intracapsular development (i.e. between egg to late-pediveliger stage). At the end of the intracapsular development, development was synchronous and larvae passed through a pelagic late pediveliger stage in all experimental conditions. This result contradicts observations made by Smith et al. (2015). At the same developmental time, Smith et al. (2015) observed an asynchronous development: larvae with pronounced velar lobes (i.e. swimming late-pediveliger) and larvae without velar lobes (considered as pre-crawling juveniles). In this study, after hours or days, depending of the rearing temperature, larvae lost their velar lobes and settled as crawling juveniles. Smith et al. (2015) reported that 14% of larvae hatch as swimming late-pediveliger and 86% of larvae hatch as crawling juveniles (i.e. dispersal polymorphism) in the Solent population. However, the result of this study agrees with Gibbs (1996) who reported that larvae of *O. erinaceus* hatch with a short planktonic phase of up to five days before settling as a juvenile. In other species from the same genus, direct development in *O. inornatus* (Martel et al., 2004) and mixed development (i.e. offspring undergo both a non-pelagic and pelagic stages during development) in

O. pulsoni (Amio, 1963) have been reported, but there are no previous reports that indicate the presence of dispersal polymorphism in this family. The differences between our study and observations by Smith et al. (2015) could be methodological. In this study, when swimming larvae started to hatch, they were exposed under light for approximately 15 minutes to observe how larvae display the velar lobes and start to swim. However, environmental maternal conditions can influence the presence of dispersal polymorphism (e.g. temperature; Clemens-Seely and Phillips 2011); thus, from this study, the results found by Smith et al. (2015) cannot be completely discounted.

2.4.2 Thermal tolerance of Solent and Arcachon populations

Thermal tolerance response during early stages was influenced by the geographic origin and showed an inverse relationship between thermal latitude range and thermal tolerance. Embryos from the lower latitude population (Arcachon, 44° 41'N) were physiological more warm-adapted than embryos from the higher latitude (Solent, 50° 51' N; Fig. 2.5; 2.6). In the Arcachon population, the experimental range of temperatures between 10 and 20 °C did not affect the survival of embryos; which is correlated with temperatures that they experienced in the field between 12 and 18 °C. However, temperatures outside of the natural thermal range (10 and 20 °C) produced sub-lethal effects. Abnormalities (i.e. shell malformations) during development increased at both ends of the thermal tolerance range (10 – 20 °C), which established a thermal tolerance window of this population between 12 and 18 °C. Abnormalities during development have been associated with elevated temperatures (e.g. shell malformations and lysis in embryos from the giant clam *Tridacna gigas,* Enricuso et al., 2019) and this is particularly evident in encapsulated species where temperature and oxygen are critical for development. Low oxygen diffusion at extreme temperatures (i.e. low and upper thermal limits) affects the diffusion of oxygen through the capsule wall causing abnormalities during the development or the presence of empty shells (Fernández et al., 2006; Smith et al., 2013).

On the contrary, extreme temperatures reduced the survival by ~50% at both ends of the temperature range (10 – 18 °C) in the Solent embryos. No abnormalities were observed during the intracapsular development because embryos could not survive at extreme temperatures. In the field, Solent embryos experience colder temperatures than the Arcachon population with values ranging between 13 and 16 °C (Fig. 2.2). Therefore, the high survival observed between temperatures 14 and 16 °C is coincident with the temperatures that they normally experienced. However, temperatures outside of the natural thermal range produced deleterious effects, which establish a very narrow tolerance window with thermal limits between 14 and 16 °C. This result is in contradiction to the climate variability hypothesis (CVH), which states that thermal adaptation is

proportional to the magnitude of the environmental variation; thus, individuals from a species inhabiting at high latitudes requires broader tolerance ranges than individuals inhabiting lower latitudes (Yu et al., 2018, Gaitan-Espitia et al., 2016). However, previous reports have suggested that thermal tolerance variation among populations can be a result of genetic adaptations to local thermal conditions (Gleason and Burton, 2013; Jansen et al., 2007). For example, Reitzel et al. (2013) conducted a common garden experiment and they evidenced that adult and developmental stages of the temperate sea anemone *Nemastotella vectensis* were locally adapted. They found that low latitude populations have higher thermal tolerance as a consequence of the warm temperatures that they experienced. Zippay and Hoffmann (2010) found that veligers of the gastropod *Nucella ostrina* collected in the North Hemisphere from lower latitude populations were more tolerant to heat stress than higher latitude populations as an adaptation to the heat events that they experienced in the south. Thus, the results obtained in this study suggest that embryos from the Arcachon population (low-latitude) might possess genetic adaptations to tolerate warm temperatures; whilst embryos from the Solent population (high-latitude) are less adapted to such conditions. Further studies are necessary to elucidate whether the differences are due to physiological adaptions of encapsulated embryos (i.e. metabolic adjustment) to local environmental conditions.

In addition, embryos from the Solent and Arcachon populations are living in environments that are very close to the upper tolerance limits. An increase of one or two degrees from the maximal temperatures that embryos normally experienced in the field (i.e. 18 and 20 °C for Arcachon and Solent, respectively) increased the mortality and abnormalities during early development. Previous studies have reported that warm-adapted species are living at temperatures that are very close to their upper thermal limits in comparison with cold-adapted species (Reitzel et al., 2013; Tomanek, 2008; Zippay and Hofmann, 2010). For example, Somero et al. (2005) studied the thermal tolerance response of subtidal and intertidal porcelain crabs (genus *Petrolisthes*) collected from temperate and tropical locations. They found that warm-adapted crabs from the intertidal zone showed low capacity to acclimate to heat events than subtidal species, because intertidal crabs are living very close to the upper thermal limits of the species. The upper thermal limit for heart function was 31.5 °C and in the field they experienced temperatures between 31 and 32 °C; thus, an increase in temperature caused lethal effects. Because early stages *of O. erinaceus* from Arcachon and the Solent populations are living very close to their upper limits, they might be at potential risk for extinction in scenarios of ocean warming (i.e. heat wave events).

At the end of the intracapsular development, temperature did not significantly affect the metamorphosis time in the Arcachon population, swimming larvae spent between 24 and 48 hours in the water column. However, fifty percent of Solent larvae at 16 and 18 °C delayed their

metamorphosis for approximately 60 hours. These results completely disagree with previous report for gastropods. For example, larvae of *Crepidula fornicata (L)* metamorphosed faster at 24 °C rather than 18 °C temperatures (Pechenik, 1984). Pechenik (1984) concluded that delayed metamorphosis was related to developmental regimes: if larval development was fast, then metamorphosis will be reached sooner. Larvae of the congener *Crepidula plana* maintained at 29 °C were shown to be able to metamorphose more quickly than larvae maintained at low temperature 20°C (Zimmerman and Pechenik, 1991). The increased time to metamorphosis observed in Solent larvae could be a result of high energetic demands and low larval nutritional reserves. It has been identified that exposure to high temperatures can produce high energetic cost for embryos (Cancino et al., 2011). Increases in metabolic rates could produce a drop in the larval energy reserves and produce deleterious effects on the metamorphosis. Indeed, 4% of the Solent-hatched larvae at 18 °C were not able to metamorphose and after 10 days died as a swimming late-pediveliger. However, the bioenergetics condition of the embryos was not evaluated. Further study is required to understand this response of delayed in the metamorphosis at high temperatures in *O. erinaceus*.

2.5 Conclusion

In conclusion, this chapter showed that the geographic origin can influence the thermal tolerance of early stages of *Ocenebra erinaceus*. The southern population exhibited a wide, eurythermal tolerance between 12 and 18 °C, temperatures outside of this range caused sub-lethal effects. On the contrary, the Solent population showed a narrow, tolerance range between 14 and 16 °C, and temperatures outside of the natural range caused lethal effects. Possibly, these differences are due to physiological adaptions to local conditions; however, further studies are needed to address this question. Both populations are living near to their upper tolerance limits; thus, they can be at potential risk to acute/short heat wave events. Warming will negatively impact marine organisms, shifting species distribution and promoting future extinctions. In this context, *O. erinaceus* populations could be predicted to shift their geographic distribution range to poleward.

Chapter 3 The effects of environmental temperatures on the aerobic response of encapsulated embryos of *Ocenebra erinaceus* (Linnaeus, 1758)

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Climate warming can affect the developmental rate and embryonic survival of ectothermic species. In chapter 2, it was established that embryos of *Ocenebra erinaceus* from the Solent, UK and Arcachon, France populations exhibited contrasting thermal tolerance ranges. However, it is unknown whether the thermal tolerance differences are due to physiological adaptations to natal thermal conditions. Therefore, the effects of temperature on respiration rates and oxygen content of the intracapsular fluid were studied during the intracapsular development of *O. erinaceus*. In this chapter, embryos were exposed to temperatures in the range of 14 - 20 °C. The encapsulation period for both populations was shorter at higher temperatures and intracapsular oxygen availability decreased as development progressed. However, the embryonic aerobic response differed between populations. Encapsulated embryos from the southern population (Arcachon) showed higher respiration rates and metabolic adjustment to elevated temperatures; however, encapsulated embryos from the Solent showed no metabolic adjustment, high capsular mortalities and limited acclimation to high temperatures. The results suggest that aerobic response of encapsulated embryos is locally adapted to the temperature history of their natal environment, and therefore can explain the intraspecific differences observed in thermal tolerances response of *O. erinaceus*. The thermal response of metabolic rate depends on physiological adaptations of embryos to the local environmental conditions.

3.1 Introduction

Temperature plays an important role regulating multiple levels of biological organization, from limiting physiological and biochemical processes, to shaping the distribution of species across latitudes (Jones et al., 2009; Somero, 2005; Weiss et al., 2009). The thermal tolerance response of ectothermic organisms is partly a function of their capacity for metabolic adjustment to cope with environmental fluctuations (Sokolova and Pörtner, 2003). One indicator of such metabolic adjustment in ectotherms can be made through measurements of aerobic metabolism, which reflects the energetic cost of adaptation to a particular environment (Clarke, 2003; Schulte, 2015). Aerobic metabolism is highly influenced by environmental temperatures; if temperature increases, aerobic metabolism increases. However, if ambient temperatures surpasse an optimal threshold, then oxygen deficiency and insufficient energy supply within cells occurs, which causes a decrease in aerobic response and a transition to an anaerobic mode producing a systemic failure (Pörtner, 2002).

Temperature and oxygen availability are limiting factors for encapsulated species (Fernández et al., 2006; Strathmann and Strathmann, 1995), impacting development rates, embryonic shell calcification, the number of developed embryos, abnormalities, development synchrony, and clutch size (Cancino et al., 2003; Lardies and Fernández, 2002). Whilst the oxygen demand of developing embryos within capsules increases throughout development (Cumplido et al., 2011), an increase in temperature and the low oxygen diffusion through the capsule wall reduces the oxygen availability within capsules, which can adversely impact embryonic development (Cancino et al., 2003; Fernández et al., 2006). Few studies have been aimed at understanding the effects of elevated temperatures on the aerobic response of encapsulated embryos, although Cancino et al., (2011) demonstrated high respiration rates, high mortality and increased abnormalities in encapsulated embryos of *Chorus giganteus* exposed to high temperatures, from 9 to 15 °C. However, it still remains unclear whether the metabolic response of encapsulated embryos is influenced by local conditions and whether embryos from different thermal regimes exhibit different aerobic adjustments to increased temperature.

Local adaptation has been shown in marine species that exhibit low dispersal potential in response to selection imposed by strong gradients (e.g. coastal systems; Sanford and Kelly, 2011). Species with direct development often exhibit low gene flow and restricted connectivity between populations, increasing the potential for local adaptation (Beherens Yamada, 1989; Parsons, 1998). Most studies however have been conducted on adult populations and little is known about early stages and whether the encapsulated stages are locally adapted to their natal environments (Sanford and Kelly, 2011). For example, Zippay and Hofmann (2010) showed a latitudinal effect on

the thermal tolerance response of encapsulated veligers of *Nucella ostrina*. Larvae from northern latitudes were less tolerant of heat stress than those from central latitudes. Thus, larvae displayed different thermal tolerance limits as a function of latitude at which they were collected. The local temperatures experienced by encapsulated embryos may determine the embryos' thermal tolerance. However, the degree to which local conditions could influence the thermal tolerance response of early ontogenetic stages in encapsulated species has not yet been explored.

This chapter seeks to identify the role of temperature warming on embryonic aerobic response and development of the European sting winkle *Ocenebra erinaceus*. This species is a mixed developer commonly found on the intertidal and shallow subtidal of UK and French Atlantic coasts. Females enclose their embryos in capsules and leave them on the rocky substrate for up to 2-3 months until they hatch as swimming larvae with a short planktonic phase of up to 5 days (Gibbs, 1996). After this period, and depending on ambient temperatures, larvae metamorphose into crawling juveniles. The aim of this chapter was: first, to understand whether the intraspecific differences between the Solent and Arcachon populations observed in Chapter 2 are due to physiological adaptations to local environmental conditions. Second, to contribute understanding of the aerobic response of encapsulated embryos at elevated temperatures and whether it is influenced by local conditions. Third, to understand the possible impact of the physical characteristics of the capsule wall on the embryos' aerobic response. It was hypothesized that there would be an effect of geographical origin in the thermal tolerance response of encapsulated embryos of *O. erinaceus*. Embryos from two widely geographically separated locations would exhibit differences in their ability to acclimate to temperatures outside of the natural thermal range. Considering that we are facing an increase in global temperatures, comparative studies between populations during early ontogenetic stages provide important insights about intraspecific adaptations and how populations may respond in the future.

3.2 Material and methods

3.2.1 Study sites and thermal data acquisition

To study the intraspecific differences in the aerobic response of encapsulated embryos, females were collected from two populations: one from the middle of their geographic distribution, the Solent, UK and the other from the south, Arcachon, France. Please refer to section 2.2.1 and 2.2.2 (Chapter 2) for information about study sites, reproductive information and thermal data acquisition.

3.2.2 Female collection and egg maintenance

Female collection, laying times are described in section 2.2.3 (Chapter 2). Egg collection and maintenance were described in section 2.2.4 (Chapter 2); however, to quantify the aerobic response to temperature, embryos were only exposed to 14, 16, 18 and 20 °C during intracapsular development. This period of intracapsular development was considered sufficient to test whether embryos exhibit intraspecific differences due to geographic origin; however, potential maternal effects cannot be completely discounted. After one or two days of laying at maternal rearing temperatures, 2 – 3 egg masses (n = 20-35 capsules per egg mass) were transferred individually to one of three common temperature treatments in the Solent (14, 16 and 18 °C) and four temperature treatments in Arcachon (14, 16, 18 and 20 °C) in 1.8 L aquaria with filtered seawater (1 µm) and constant aeration. Embryos from the Solent were not exposed to 20 °C because of high mortalities observed at 18 °C. The methodologies for measuring intracapsular time, embryo survival and abnormalities during the intracapsular development are described in section 2.2.5 (Chapter 2). Developmental abnormalities were identified when some of the embryos inside the capsule showed malformations in their shell morphology (Fig. 3.1).

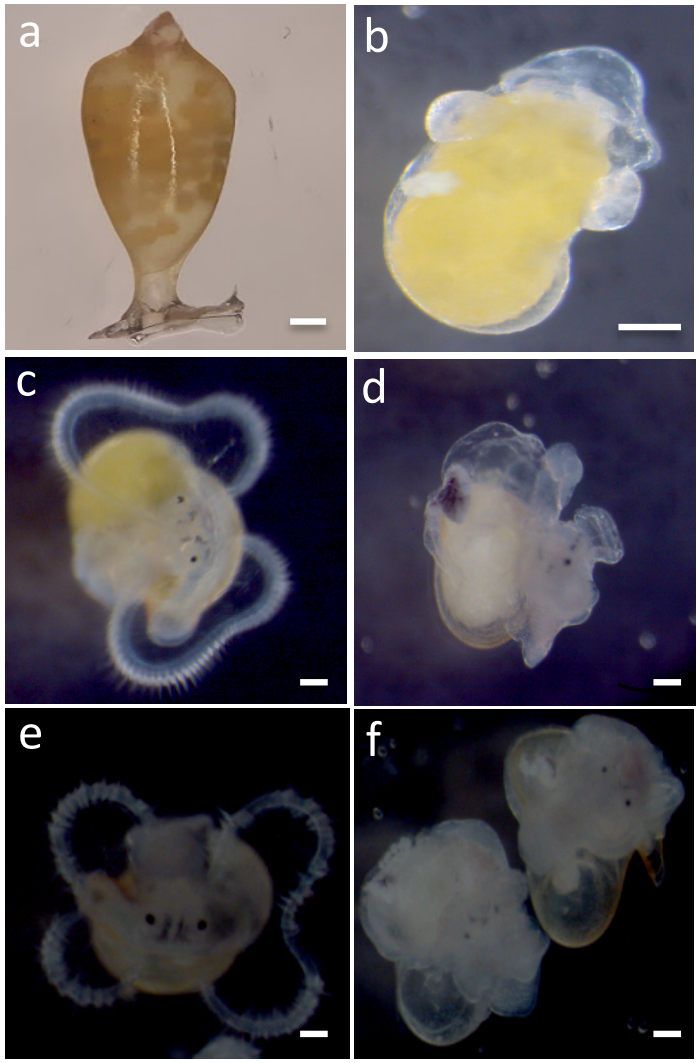

Figure 3.1 Ontogenetic stages of *O. erinaceus* during the intracapsular development used in the experiments. **(a)** egg capsule; **(b)** early veliger stage (EV); **(c)** pediveliger stage (PV); **(d)** abnormal embryo in pediveliger stage; **(e)** late pediveliger stage (LP); **(f)** Abnormal embryos in late pediveliger stage. Scale bars: (a) = 1 cm, (b-f) = 100 µm.

3.2.3 Oxygen concentration in the intracapsular fluid

Oxygen concentration inside capsules was determined according to Cancino et al., (2011), with minor modifications. Briefly, capsules in pediveliger (i.e. intermediate stage: 790 – 810 µm size) and late pediveliger stage (i.e. advanced stage: 880 – 890 µm size) were placed in 1.8 l aquarium filled with filtered seawater (1 µm; salinity 33) at 100% air saturation with stirring. A pre-calibrated microelectrode (Microx TX3 PreSens, calibrated with $NaSO_3$, according to manufacturer's protocol) was inserted through the capsule plug using a dissecting microscope to ensure that the embryos inside were not damaged. Oxygen concentrations were measured for 30 minutes; however, the first five minutes were not considered to avoid the disturbance produced by the microelectrode. Between 9 and 10 capsules per temperature treatment were measured in both populations. The

oxygen concentration of seawater in the aquarium was recorded with values ranging between 9 and 10 mg O_2 L^{-1} in all temperature treatments, with salinities ranging between 33 and 34. After the measurements, capsule size, number of eggs and stage of development were quantified using a dissecting microscope (LEICA MZ16).

Capsule size and number of embryos per capsule was different between populations (Table 3.1). In the Solent, capsule size was 7 ± 0.3 mm and the mean number of embryos per capsule laid was 45. For the Arcachon population, mean capsule size was 9 ± 0.4 mm and the number of embryos per capsule was 75. The oxygen volume inside capsules was estimated using the relation given by Pechenik (1983), whereby the egg mass volume (i.e. all eggs inside of one capsule) was subtracted from the capsule volume. The ratio of the oxygen volume available per egg mass inside the capsule was constant between populations with values ranging between 5.49 ± 1.5 and 5.53 ± 1.7 in Solent and Arcachon, respectively. Thus, the oxygen availability was similar and comparable between populations, despite the different capsule sizes and number of embryos per capsule.

3.2.4 Oxygen consumption rate (OCR) in encapsulated and excapsulated embryos

In order to understand the effect of temperature on the OCR in encapsulated and excapsulated embryos (i.e. embryos removed from the capsule chamber), capsules and embryos were kept in hermetically sealed vials filled with 2 ml of filtered seawater (0.5 µm and salinity 33 ppt), saturated at 100% of oxygen and at the same temperature used during their development. In each vial, the oxygen concentration was measured at the start of the study and after three hours. As controls, oxygen concentration was determined in three vials per measurement, with the same physical conditions but without animals. After three hours, the oxygen content of vials with embryos was subtracted from the mean oxygen concentration of the control vials to determine respiration rate. For the Solent population, measurements for pediveliger and late pediveliger stage were recorded and for the Arcachon population for early veliger, pediveliger and late pediveliger stage were measured (Table 3.1). To estimate the OCR, after each measurement, the number of embryos, stage of development and capsular development was recorded under a microscope. OCR was expressed as mg O_2 h^{-1}embryo^{-1} per capsule.

Table 3.1 Summary of capsule size and number of embryos per capsule exposed to temperatures (14, 16, 18 and 20 °C) used in the oxygen availability and oxygen consumption rate (OCR) experiments in the Solent and Arcachon. 'n.d.' represents no data collected under that treatment. Values are given as mean ± standard deviation.

		The Solent		
Experiments	**14 °C**	**16 °C**	**18 °C**	**20 °C**
Oxygen availability				
Capsule size	7.2 ± 1.0	6.7 ± 0.5	6.7 ± 0.5	n.d
N° of eggs	51 ± 9	47 ± 5	46 ± 6	
OCR				
Capsule size	7.0 ± 0.7	6.6 ± 0.6	7.0 ± 0.5	n.d
N° of eggs	41 ± 11	45 ± 6	46 ± 7	
		Arcachon		
Oxygen availability				
Capsule size	9.2 ± 0.8	8.9 ± 0.6	9.0 ± 0.8	9.8 ± 0.8
N° of eggs	72 ± 11	69 ± 8	70 ± 12	81 ± 11
OCR				
Capsule size	9.3 ± 0.9	9.1 ± 0.7	9.2 ± 0.8	9.9 ± 1.0
N° of eggs	75 ± 17.3	74 ± 11.8	74 ± 16.6	82 ± 19.7

3.2.5 Statistical analysis

To analyse the effects of temperature on the different variables measured in this study, the Arcachon and the Solent populations were treated independently due to different thermal regimes that females were maintained in the laboratory. Developmental rates were analysed with Kruskal-Wallis analysis of variance by ranks, followed by Tukey *post hoc* analysis. Capsular survival and abnormalities were analysed by one-way ANOVA followed by Tukey *post hoc* analysis. Prior to analysis, the percent survival and abnormal capsules was arcsine transformed. Intracapsular oxygen concentration in the intracapsular fluid was analysed by two-way ANOVA followed by Tukey *post hoc*, with temperature and ontogenetic stage of development as factors. Oxygen consumption rates were analysed by three-way ANOVA followed by Holm-Sidak multiple comparisons, with ontogenetic stage of development, temperature and embryo condition (i.e. excapsulated and encapsulated embryos) as factors. Normality and homogeneity of variance were confirmed before respective analysis and statistical significance was identified at $p < 0.05$. Statistical analysis was performed with SigmaStat Software 12.5.

3.3 Results

3.3.1 Intracapsular time, survival and embryos abnormalities

The intracapsular time decreased significantly with high temperatures in both populations (Table 3.2). In the Solent, development at 14 °C took approx. 74 days, whilst in capsules exposed to 18 °C the intracapsular time decreased by 38% ($p < 0.05$). Similar developmental rates were observed in Arcachon with faster development at high temperatures ($p < 0.05$). Survival throughout the intracapsular development period showed a different pattern between populations (Table 3.2). Egg masses from the Solent exposed to elevated temperatures showed poor acclimation to high temperatures; only half of the capsules containing developing embryos survived in each egg mass at 18 °C (Tukey *post hoc* analysis, $p < 0.05$; Table 3.2). On the contrary, survival was not affected by the increase of temperature for Arcachon embryos ($p > 0.05$). The percentage of capsules with embryos with normal development was not significantly different between temperature treatments in the Solent (Table 3.2; $p > 0.05$). On the contrary, 20% of capsules had abnormal embryos when egg masses were exposed to 20 °C for the Arcachon population ($p < 0.05$). From these data, it is clear that whilst extreme temperature impacts embryo survival in the Solent population, extreme temperature resulted in developmental abnormality in embryos from Arcachon, showing a differential effect of temperature – depending on population origin.

Table 3.2 Temperature effects on intracapsular time (IT), the percentage of embryo survival per egg mass (ES) and in the percentage of capsules with embryos with normal development (N) of *Ocenebra erinaceus*. Values given are mean ± standard deviation, n = 2-3 egg masses in the Solent and n = 3 egg masses in Arcachon per temperature treatment. Statistical analysis: Kruskal-Wallis Test for IT and one-way ANOVA for ES and N.

Location	T (°C)	IT (days)	ES (%)	N (%)
Solent	14	73.3 ± 2.9	93.0 ± 3.5	100 ± 0.0
	16	49.5 ± 2.1	100 ± 0.0	100 ± 0.0
	18	45.5 ± 0.7	52.2 ± 4.3	94.4 ± 5.6
	H = 5.455, **p < 0.05**		$F_{(2,8)}$ = 33.074, **p < 0.05**	$F_{(2,8)}$ = 1.0, p > 0.05
Arcachon	14	79.7 ± 2.3	100 ± 0.0	100 ± 0.0
	16	49.0 ± 2.6	98.9 ± 1.1	97.1 ± 2.5
	18	41.3 ± 4.0	93.9 ± 2.8	91.4 ± 4.7
	20	38.7 ± 1.2	89.9 ± 7.8	79.6 ± 3.3
	H = 9.528, **p < 0.05**		$F_{(3,14)}$= 1.875, p > 0.05	$F_{(3,11)}$ = 5.08, **p < 0.05**

3.3.2 Oxygen concentration in the intracapsular fluid

Intracapsular fluid oxygen concentration was significantly affected by the stage of development and increases in temperature for both populations, respectively. In the Solent, there was a significant interaction between temperature and ontogenetic stage of development ($F_{(2,1)}$=4.754, p < 0.05; Fig. 3.2a). The oxygen concentration in capsules containing embryos in the late pediveliger stage decreased compared to capsules in the pediveliger stage. Temperature significantly affected the oxygen concentration in the late pediveliger stage from 6 - 7 mg O_2 L^{-1} at 14 °C to 3 – 2 mg O_2 L^{-1} at 16 and 18 °C, respectively. Similarly, in the Arcachon population, oxygen concentration decreased significantly with increasing temperature and ontogenetic stage of development ($F_{(3,1)}$= 5.937, p < 0.05, Fig. 3.2b). Capsules containing pediveliger stage embryos exposed to 14°C showed relatively high oxygen concentrations (5 mg O_2 L^{-1}) in comparison with the other temperature treatments, where values ranged between 3-4 mg O_2 L^{-1}. In contrast, the oxygen concentration in capsules containing late pediveliger embryos was reduced at higher temperatures, from 2.5 – 3.5 mg O_2 L^{-1} at 14 – 16 °C to 1.3-1.9 mg O_2 L^{-1} at 18- 20 °C.

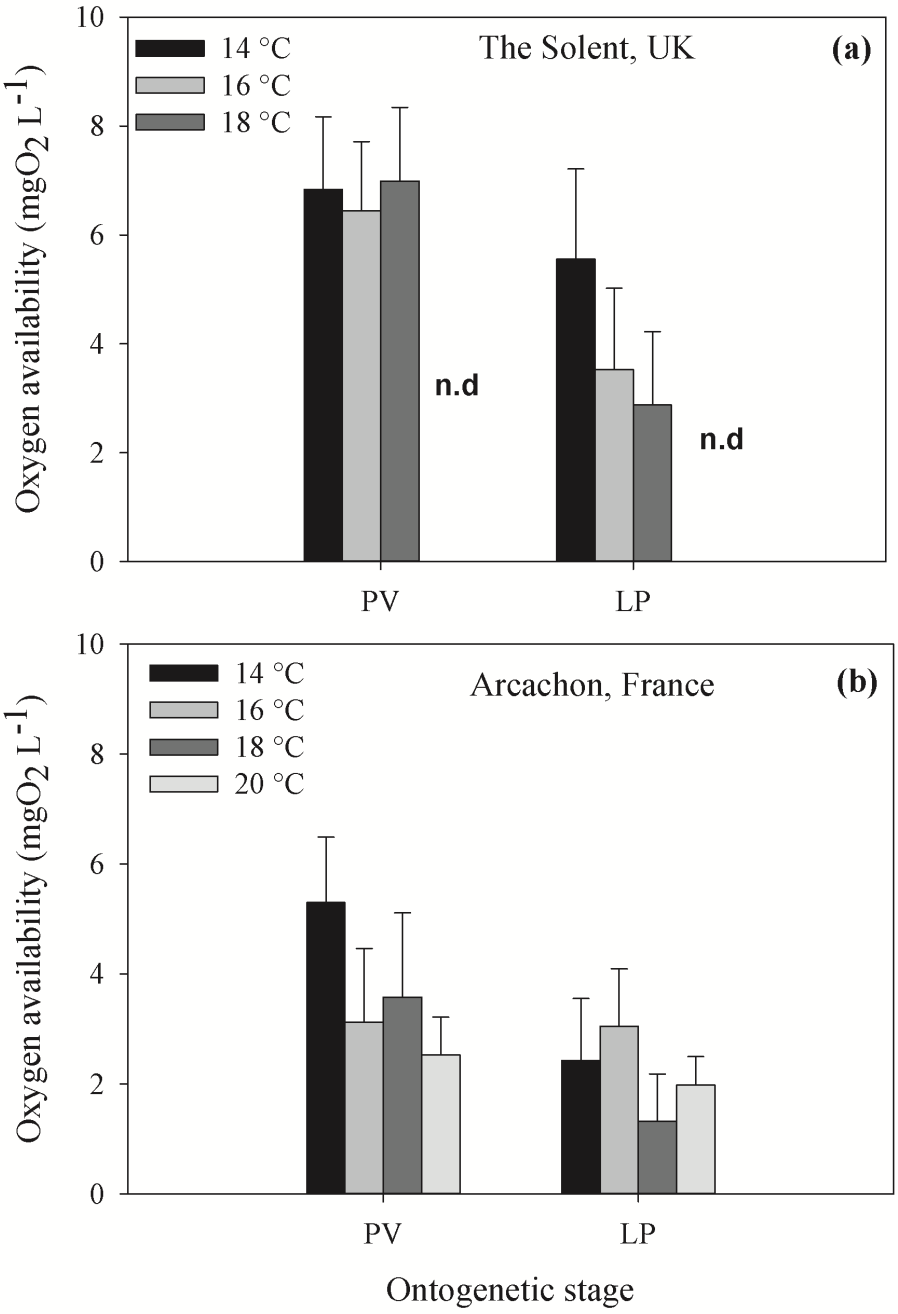

Figure 3.2 Temperature effects on the intracapsular oxygen availability of capsules with embryos in pediveliger (PV) and late pediveliger (LP) stages. **(a)** The Solent (Two-way ANOVA: T (°C) x Stage: $F_{(2,1)}$=4.754, $p < 0.05$; T (°C) = $p < 0.05$; Stage = $p < 0.05$; and **(b)** Arcachon (Two-way ANOVA: T (°C) x Stage: $F_{(3,1)}$= 5.937, $p < 0.05$; T (°C) = $p < 0.05$; Stage = $p < 0.05$). The 'n.d.' represents no data for the temperature treatment. Values given are mean ± standard deviation, n = 9 – 10 capsules were measured per temperature treatment in each population.

3.3.3 Oxygen consumption rate (OCR) in encapsulated and excapsulated embryos

The OCR changed as a function of increased temperature, ontogenetic stage of development, embryonic condition (i.e. encapsulated and excapsulated embryos), and population (Fig. 3.3; table 3.3). In both populations, the OCR increased as development progressed; however, excapsulated embryos consumed more oxygen than encapsulated embryos in advanced stages (Holm-Sidak; $p < 0.001$). Thus, the capsule wall restricted the OCR in encapsulated embryos (Fig. 3.3).

The OCR of encapsulated embryos in the Solent increased as the development progressed, from pediveliger to late pediveliger stage (Fig. 3.3 a-b; Table 3.3). However, increased temperature did not affect the oxygen consumption in encapsulated late pediveliger embryos ($p > 0.05$), demonstrating low acclimation to high temperatures. Interestingly, excapsulated embryos in the same ontogenetic stage showed an increase in their OCR when exposed to 18 °C ($p < 0.05$). Contrasting results were observed in Arcachon, where increased temperatures produced a flexible metabolic response (Fig. 3.3 c-d; Table 3.3). Embryos in advanced ontogenetic stages (encapsulated and excapsulated embryos) consumed more oxygen than early stages. Moreover, high temperatures increased the OCR in each ontogenetic stage, especially in embryos exposed to 18 and 20 °C ($p < 0.05$).

Table 3.3 Results of three-way ANOVAs using OCR of encapsulated and excapsulated embryos from two populations at pediveliger and late pediveliger stage in The Solent and early veliger, pediveliger and late pediveliger stage in Arcachon. (*) Means p values < 0.05 and (**) means p value of < 0.001. Factors: condition: excapsulated and encapsulated (C); Temperature (T°); developmental stage (S).

Factor	**The Solent**					**Arcachon**				
	df	SS	MS	F	*P*	df	SS	MS	F	*P*
C	1	1.5×10^{-5}	1.5×10^{-5}	45.3	**	1	3.4×10^{-4}	3.4×10^{-4}	445.1	**
T°	2	0.2×10^{-5}	0.1×10^{-5}	3.4	0.1	3	3.9×10^{-5}	1.3×10^{-5}	16.9	**
S	1	6.8×10^{-5}	6.7×10^{-5}	204.6	**	2	1.9×10^{-4}	9.7×10^{-5}	127.7	**
C x S	2	0.7×10^{-5}	0.3×10^{-5}	11.06	**	3	2.7×10^{-5}	0.9×10^{-5}	12.2	**
C x T°	1	1.4×10^{-5}	1.4×10^{-5}	42.9	**	2	9.4×10^{-5}	4.7×10^{-5}	62.0	**
T° x S	2	0.3×10^{-5}	0.1×10^{-5}	5.7	**	6	1.1×10^{-5}	1.8×10^{-6}	2.377	*
C x T° x S	2	0.5×10^{-7}	0.2×10^{-7}	0.1	0.9	6	0.9×10^{-6}	1.7×10^{-6}	2.165	0.1
Error	107	3.5×10^{-5}	0.3×10^{-6}			196	1.4×10^{-4}	0.7×10^{-6}		

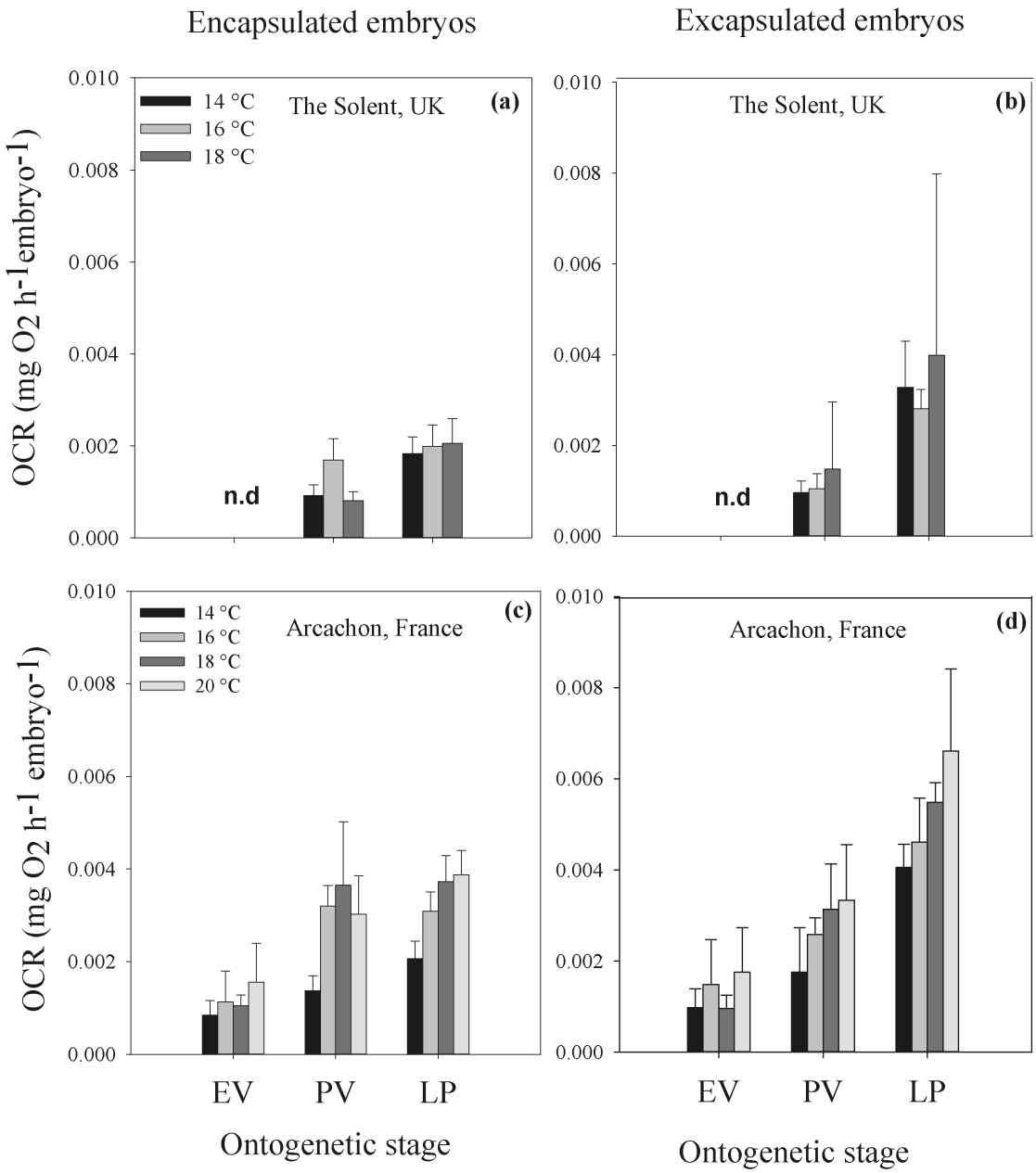

Figure 3.3 Temperature effects on oxygen consumption rate (OCR) on encapsulated and excapsulated embryos in early veliger (EV), pediveliger (PV) and late pediveliger stage (LP) from the Solent **(a,b)** and Arcachon **(c,d)** populations. The 'n.d.' represents no data for the temperature treatment. Values given are mean ± standard deviation, n = 8 – 10 eggs capsules per temperature treatment.

3.4 Discussion

The early life stages of marine organisms are thought to be more vulnerable to extreme temperatures, exhibiting narrow thermal tolerance windows in comparison with juvenile or adult stages (Byrne, 2012). This could be particularly pronounced for encapsulated species where temperature and oxygen availability are critical variables for tissue oxygenation and embryo survival inside of egg masses (Cancino et al., 2011). The aim of this study was to understand the effects of temperature on the development and aerobic response of embryos to determine whether the intraspecific differences observed in Chapter 2 are due to physiological adaptations of the embryos to local environmental conditions. The upper thermal tolerance limits of encapsulated embryos of *O. erinaceus* from the Solent and Arcachon could be explained in part by the physiological effects of temperature, but also adaptation to local environmental conditions.

The duration of intracapsular development decreased with increasing temperatures in both populations. Intracapsular development took 70 days at 14 °C but only 40 days at 18 °C for both populations (Table 3.2). Indeed, it is well known that increased temperatures affect the development rate of encapsulated species (Cancino et al., 2003; Przeslawski, 2004; Smith et al., 2013; Smith et al., 2015; Spight et al., 1974; Roller and Stickle, 1989). However, despite the fact that developmental rates were similar between populations, the aerobic response of encapsulated embryos varied according to the source population. Thus, the aerobic response of encapsulated embryos of *O. erinaceus* to increasing temperature appears to be locally adapted, as a function of temperature, oxygen availability and embryonic oxygen demands (Fig. 3.3; Table 3.3).

The respiration rate of encapsulated embryos from Arcachon increased during development (i.e. from pediveliger to late pediveliger stage) and with temperature, demonstrating a high metabolic acclimation to temperatures between 14 and 20 °C. The highest respiration rates were observed in advanced embryos exposed to elevated temperatures (18 - 20 °C). High temperature has been shown to cause an increase in the metabolic rates in ectothermic species. Indeed, positive correlations have been found between oxygen consumption and increased temperature in other encapsulated species (Brante, 2006; Cancino et al., 2011; Moran and Woods, 2007).

As development progressed in the Arcachon population, embryos consumed more oxygen and the oxygen concentration inside the capsules decreased, with values ranging between 5 and 6 mg O_2 L^{-1} in pediveliger stages to 3 and 2 mg O_2 L^{-1} in late pediveliger stages (Fig. 3.3). This situation was more pronounced in advanced stages exposed to high temperatures as they consumed more oxygen, which resulted in hypoxic conditions inside the capsules (1-1.8 mg O_2 L^{-1} at 20 °C). Similar findings have been observed in other prosobranch gastropods, where the oxygen content within capsules decreases as embryonic development progresses and temperature increases (Cancino et

al., 2011; Cumplido et al., 2011; Lardies and Fernández 2002). Hypoxic threshold has been established around ~ 2 mg O_2 L^{-1} for shallow-water habitats, when marine organisms exhibit abnormal behaviour; however, they can widely differ between taxa, ontogenetic stages, or habitats (Peruzza, 2017). Therefore, a hypoxic threshold should be established when in stressful conditions, organisms reach the critical oxygen level in their tissues, leading to an anaerobic mode or metabolic suppression, which cannot be energetically maintained (Peruzza, 2017). In this chapter, values between 1 and 1.8 mg O_2 L^{-1} caused embryo abnormalities and high mortalities, which set the hypoxic conditions for *O. erinaceus* embryos. However, further studies are necessary to elucidate the critical oxygen levels and define the hypoxic threshold in *O*. *erinaceus*.

The thermal acclimation to higher temperatures observed in the aerobic response of the Arcachon embryos could explain the high survival rates (between 90 and 100%) observed in all temperature treatments. Normally, subtidal Arcachon embryos are exposed to temperatures ranging between 13 and 18 °C during intracapsular development in the field (Fig. 3.4), thus the experimental range between 14 and 18 °C did not affect embryonic survival. Nonetheless, temperatures outside of the normal temperature range had a sub-lethal cost on embryonic fitness; 20 % of capsules from each egg mass showed abnormal embryos at 20 °C. We conclude that in order to tolerate high temperatures, embryos incur high energetic demands, which result in development abnormalities. Previous reports have shown that high temperatures and low oxygen conditions negatively impact embryonic development, increasing the number of abnormal embryos (Fernández et al., 2006), or the number of empty shells (Smith et al., 2013).

In contrast, encapsulated embryos from the Solent population showed limited metabolic acclimation to high temperatures. For this population, oxygen consumption increased as development progressed and oxygen content within capsules decreased (Fig. 3.3a). However, for the Solent population, an increase in temperature did not affect embryonic oxygen consumption. It has been argued that the increase in metabolism with temperature represents a significant metabolic energetic challenge for organisms and some populations show temperature-insensitive metabolism as a strategy to survive in fluctuating environments (Verbeck et al., 2016). The lack of acclimation observed in Solent embryos might be because they were thermally insensitive to temperatures outside of the normal range due to the high energetic demands. These high energetic demands were coincident with high embryo mortality observed at elevated temperatures. Only half of the embryos from each egg mass exposed to 18 °C reached the juvenile stage, which demonstrates that embryos were not able to tolerate temperatures above 18 °C. Usually, Solent embryos are exposed to temperatures between 12 and 16 °C in the subtidal during intracapsular development (Fig. 3.4) and therefore, 18 °C represents a critical point for development in this

population. Please refer to the graphical summary for the aerobic response of late-pediveliger embryos in both populations (Fig.3.4).

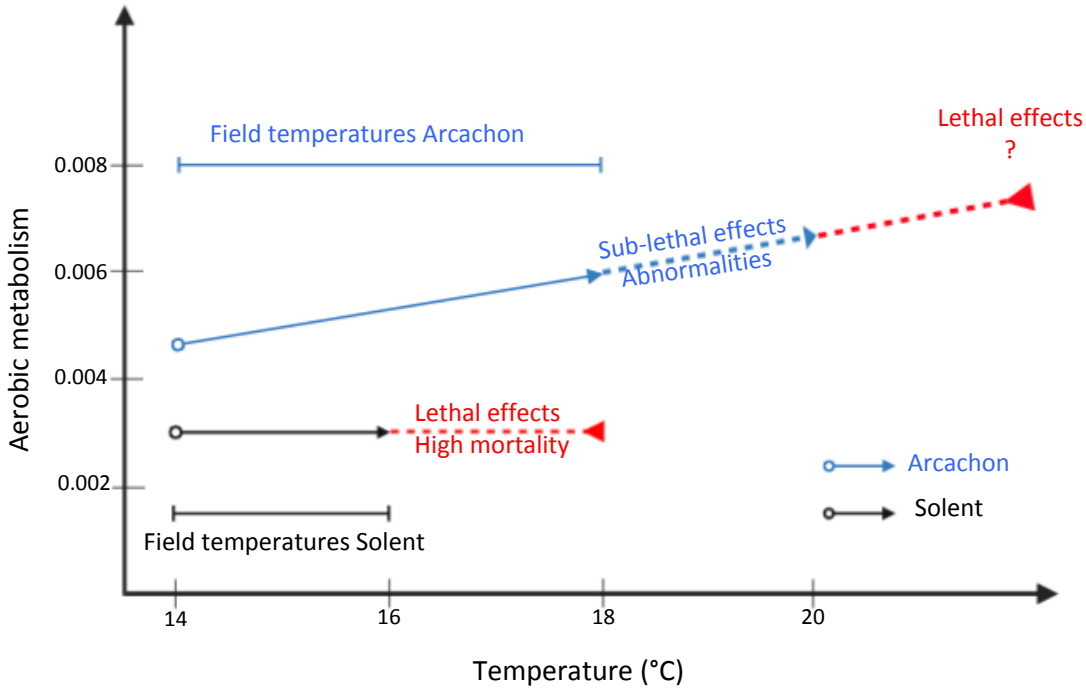

Figure 3.4 Graphical summary of the aerobic response of late-pediveliger embryos from the Solent and Arcachon populations exposed to high temperatures. Aerobic response increased as temperature increased in Arcachon embryos; however, temperatures outside of the normal range leaded to sub-lethal effects. On the contrary, the aerobic response of Solent embryos remained constant as temperature increased with no metabolic adjustment; lethal effects were observed at temperatures outside of the normal range.

As such, encapsulation seems to be a maladaptive strategy to warming temperatures. In this study, the capsule wall acted as a barrier for the oxygen diffusion and hence, impacted embryonic oxygen demands (see also: Segura et al., 2010). Embryos removed from their capsule chambers showed higher respiration rates compared to encapsulated embryos in both populations (Fig. 3.3; Table 3.3, factor: embryonic condition). For example, at the same developmental stage the respiration rate of excapsulated embryos was 100% higher than encapsulated embryos. Unexpectedly, when excapsulated embryos from the Solent population were exposed to aerated filtered seawater (100% saturation), they then showed metabolic adjustment to increased temperature. Therefore, the capsule wall restricted oxygen diffusion and, combined with elevated temperatures, reduced the oxygen availability which affected the respiration rate of advanced encapsulated embryos. As Segura et al. (2010) have shown, the capsule wall may act as a barrier for the diffusion of oxygen. The oxygen diffusion coefficient of the capsule wall of *Crepipatella dilatata* was lower than the oxygen diffusion coefficient in pure water, which impacted the oxygen availability inside the

capsules (Segura et al., 2010). To increase oxygen availability, the capsule wall of *C. dilatata* became thinner as the development progressed increasing its permeability. However, even though the permeability increased, it was insufficient to supply the embryonic oxygen demands at the end of the intracapsular development. The result of this chapter supports the idea that the capsule wall could act as a barrier for oxygen diffusion, significantly reducing oxygen availability inside the capsule (Cancino et al., 2011) and this oxygen reduction can be intensified under warming temperatures, ultimately impacting embryonic survival.

Local adaptation is likely to be a reasonable explanation for the differences observed in the aerobic response of these two populations. Previous studies have shown that geographically separated populations (i.e. reproductively isolated) have evolved different thermal tolerances to their local environments (Kelly et al., 2012). Theoretically, populations from low latitudes will be the most thermally tolerant due to local adaptation to warmer conditions (Kelly et al., 2012); however, thermal tolerance limits can vary not only latitudinally but also among different habitats and geographic location (Kuo and Sanford 2009). For example, Kuo and Sanford (2009) found that the upper thermal tolerance limit in juveniles of the gastropod *Nucella caniculata* was locally adapted throughout the northeastern Pacific coast. They identified that regional differences, such as tidal regimes, can act as selective forces resulting in populations that are physiologically adapted to local environmental conditions. Thus, in this study, the local environmental conditions experienced by embryos influenced the aerobic response and limited the thermal tolerance response. However, it must be stressed that – from this work – it was not possible to distinguish between phenotypic plasticity and genetic differentiation (i.e. local adaptation). The potential influence of maternal effects or non-genetic developmental plasticity could not be eliminated from these results. Female thermal history before spawning can induce phenotypic carry-over effects on the offspring and, therefore, can influence the thermal tolerance response of embryos (Shama et al., 2014). In this study, the colder temperatures experienced by Solent females could have influenced the thermal tolerance of embryos, resulting in poor acclimation to temperatures outside of their normal thermal range.

3.5 Conclusion

This study is among the first to report intraspecific differences in the aerobic response of encapsulated species. We demonstrated that early stages of *Ocenebra erinaceus* are vulnerable to high temperatures that extend beyond their normal range of temperature. The aerobic response of embryos was constrained by local environmental conditions. Moreover, we demonstrated that encapsulation might be maladaptive for developing embryos of *O. erinacea* at high temperatures. These results can explain the intraspecific differences found in the thermal tolerance response of

Solent and Arcachon population; encapsulated embryos experienced warmer environment were physiologically adapted to cope increased temperature. However, further studies are necessary to determine whether or not these differences are due to adaptations to local environmental conditions. With a projected increase of +2.73 °C (± 0.72) in sea surface temperature by the end of this century (Bopp et al., 2013) it is likely that the survival of *O. erinaceus*, especially during early stages when they are more sensitive to thermal stress, will be affected by climate warming – but this will be differentially expressed (mortality vs. abnormality) in populations from different thermal environments.

Chapter 4 Intraspecific variability in reproductive traits and early development of *Ocenebra erinaceus* (Linnaeus, 1758): A transplant study

Marine organisms exhibit high intraspecific differences among and within species in response to natural selection; this variability can be explained, in part, by phenotypic plasticity but also by trans-generational adaptation to strong and persistent local environmental conditions (e.g. thermal gradients). *Ocenebra erinaceus* is a temperate muricid gastropod species that shows high intraspecific variability in maternal investment (i.e. fecundity), which could suggest physiological adaptations to local environmental conditions. Indeed, during early stages of development, the thermal tolerance response seems to be locally adapted, with embryos from a warm-water population (France) more physiologically adapted to increased temperatures than embryos from a cold-water population (UK; Chapter 2-3). The aim of this chapter is to determine whether the physiological differences observed between these two populations are due to adaptations to local environmental conditions and whether these adaptations can persist during the early development of *O. erinaceus*. The results showed that both populations of *O. erinaceus* have different adaptive costs to live under their natal environmental conditions, resulting in trade-offs between reproductive traits. The poor performance of transplanted embryos (Arcachon into Solent climatic conditions) during development suggests that the intraspecific differences in the thermal tolerance observed between these two populations are due to adaptations to warm temperatures.

4.1 Introduction

Marine organisms have evolved intraspecific variation among and within species in response to strong selection pressures such as predation (Pardo and Johnson, 2005) or the physical environment (Hadfield, 1989; Ramajo et al., 2016). Indeed, in Chapter 2-3 it was observed that local environmental temperatures caused intraspecific variation in the thermal tolerance response of encapsulated embryos. Coastlines are often characterized by strong fluctuations of temperature, pH, nutrients and oxygen availability that can vary not only latitudinally but also according to local conditions such as tides, substrate heterogeneity, heat or upwelling events (Feely et al., 2008; Helmuth et al., 2006; Schoch et al., 2006). As a result, coastal species exhibit intraspecific differences in physiological traits such as metabolism or growth across a species' geographic distribution (Kuo and Sanford, 2009, Osores et al., 2017; Pardo and Johnson, 2005; Zippay and Hofmann, 2010).

Two mechanisms underlie the intraspecific variability within species: phenotypic plasticity and genetic adaptation (Thomas et al., 2018). Phenotypic plasticity facilitates for example an organism's physiological acclimation to a range of environmental conditions (Yu et al., 2018). However, when there is a persistent environmental gradient imposing divergent selection, species can evolve morphological, physiological and behavioural adaptations that can be passed on to the next generations (Kuo and Sanford, 2009). Indeed, growing evidence has demonstrated that local selective gradients such as tides, wave action or local temperatures can also lead to local adaptation resulting in populations with high fitness in their native locations compared to populations from a more distant location (Kawecki and Ebert, 2004). Thus, understanding the intraspecific variability among populations is relevant for predicting, for example, whether increasing temperature will promote extinction and/or range shift of species.

Ocenebra erinaceus is mixed developer gastropod (i.e. with a short planktonic phase at hatching), which inhabits the intertidal and shallow subtidal zones of UK and French Atlantic coasts. Appreciable intraspecific differences in maternal investment have been reported for *O. erinaceus*. For example, fecundity has been estimated at 1012, 476 and 2356 eggs per egg mass/female per year in the Solent, Plymouth, and Falmouth, UK respectively (Gibbs, 1996; Hancock, 1960; Smith et al., 2015). However, from Rivedoux, France, an average of 1830 eggs was estimated (Martel et al., 2004). This variability could be the result of maternal effects, such as maternal nutritional history (Qian and Chia, 1991; Steer et al., 2004; Tamburi and Martín, 2011), maternal size (Chaparro et al., 1999; Gianguzza et al., 2005; Parker and Begon, 1986), maternal environmental conditions (Atkinson et al., 2001) but also to local adaptations to regional environmental conditions (Bas et al., 2007; Wehrtmann et al., 2012). Local adaptation often results in differential trade-offs, because the adaptation to one environmental constraint results in a cost of adaptation to other constraints (Hereford, 2009); thus, these differences might represent differential adaptive cost (i.e. trade-offs) that affect the maternal investment in response to local conditions.

Moreover, during early development, *O. erinaceus* has shown physiological adaptations to local environmental temperatures (Chapter 2-3). Embryos from the warm-adapted population (Arcachon) showed wide thermal tolerances, high survival and metabolic adjustment to elevated temperatures. However, embryos from the cold-adapted population (Solent) showed a very narrow thermal tolerance, with high mortalities at both ends of the thermal range and no metabolic adjustment to elevated temperatures. It is unclear whether these intraspecific differences between these two geographically separated populations are due to adaptations to local environmental conditions.

It is expected that species with direct development, low gene flow, high spatial environmental heterogeneity and restricted connectivity result in high levels of genetic differentiation between populations (Beherens, 1989; Parsons, 1998; Sanford and Kelly, 2011). Therefore, it could be expected that *O. erinaceus* evolved in populations adapted to local environmental conditions, as this species is a mixed developer species, inhabiting heterogenous habitats (i.e. intertidal and subtidal), with restricted connectivity and gen flow (i.e. adults have limited dispersal capability).

The aim of this chapter is to determine whether the physiological differences observed in the thermal tolerance and metabolic response of embryos from the Solent and Arcachon are due to adaptation to local environmental conditions and whether these adaptations can persist during the early development of *O. erinaceus*. The possibility of local adaptation was evaluated by "local versus foreign" criteria according to Kawecki and Ebert, (2004); individuals are transferred from their native environments to a new foreign environment. When the performance of local genotypes exceeds the foreign genotypes, local adaptation is likely. To test whether *O. erinaceus* exhibits local adaptation, females from Arcachon were transplanted into the Solent climatic conditions for 10 months to study maternal investment (i.e. fecundity) as a direct measure of fitness. Then, to determine whether local adaptation persists during the early development, encapsulated embryos were exposed to the same range of temperatures used in Chapter 2. It was hypothesized that *O. erinaceus* will exhibit high fitness in their local habitats but not when transplanted into foreign environmental conditions, which will provide evidence for local adaptation.

4.2 Material and methods

4.2.1 Study sites and thermal acquisition

To study the intraspecific variability in maternal investment, females of *Ocenebra erinaceus* were collected from two populations: one from the middle of their geographic distribution, the Solent, UK and the other from the south, Arcachon, France. Please refer to section 2.2.1 and 2.2.2 (Chapter 2) for information about study sites, reproductive information and thermal data acquisition.

4.2.2 Female collection and maintenance

Please refer to section 2.2.3 (Chapter 2) for information about female collection and laboratory maintenance.

4.2.3 Non-native population: Transplant experiment

A transplant experiment was conducted to test whether *O. erinaceus* populations are adapted to local environmental conditions and whether this adaptation persists during early development. Individuals from Arcachon were collected in October 2017 and they were maintained from October 2017 to June 2018 under Arcachon field temperatures (Section 2.2.3, Chapter 2). Then, the same individuals were transferred to Solent seawater thermal conditions from July 2018 to April 2019 in aquaria conditions of continuously circulated water directly from the Solent. Individuals were transferred at the end of June 2018 to ensure that females had finished the laying process and they started a new reproductive cycle (Fig. 4.1a). Individuals experienced the seawater temperatures in the Solent before females started the laying process (Fig. 4.1b). Females were fed *ad libitum* with mussel and oysters three times per week. Fifteen transplanted females started to lay egg masses between March and April 2019, when temperatures ranged between 12 and 14 °C.

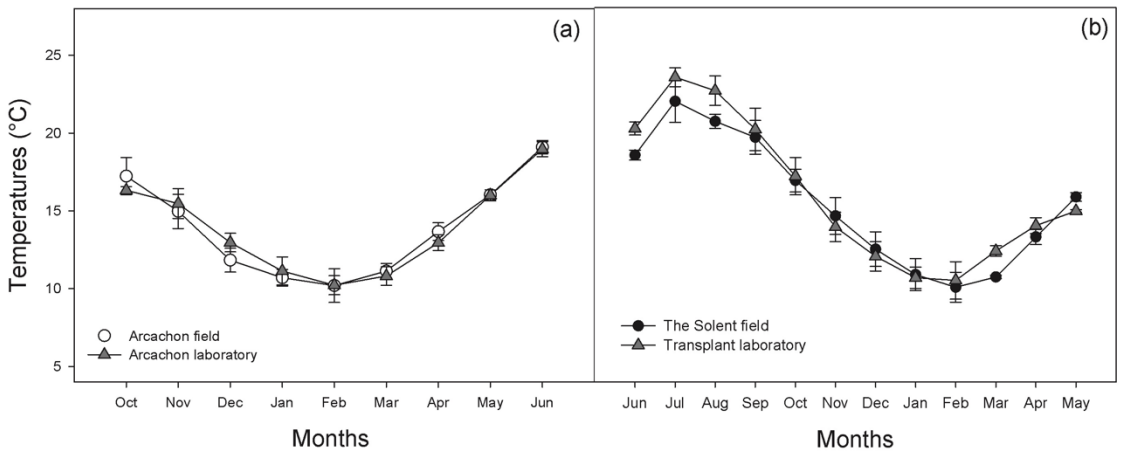

Figure 4.1 Summary of laboratory conditions (NOCS facilities) for Arcachon females collected in October 2017 until June 2018 and transferred to Solent climatic conditions when they ceased reproduction from July 2018 to May 2019. **(a)** Arcachon field and laboratory temperatures for Arcachon females until females stopped laying capsules. **(b)** Solent field and laboratory temperatures for Arcachon transferred females from July 2018 to May 2019, when females started to lay egg masses. Field temperatures are collected from databases from 2014 to 2016 (please refers to section 2.2.2 in Chapter 2). White and dark circle dots represent field temperatures from Arcachon and Solent, respectively. Grey triangles represent laboratory temperatures where females were maintained.

4.2.4 Reproductive traits

After 1 or 2 days of laying, three capsules per egg mass were dissected under microscope (LEICA MZ16) to measure different reproductive traits: fecundity (i.e. total eggs per egg mass), number of eggs per capsule, capsule size, number of capsules per egg mass, and embryo size. Between 15 and

16 egg masses were analysed from the Solent and Arcachon populations and during the transplant experiment. The number of eggs per egg mass was estimated from the average of number of eggs of three capsules per egg mass. To estimate the relationship between capsule size and number of eggs, a group of capsules with a wide range of sizes was collected from each location (i.e. Solent and Arcachon populations and transplant experiment).

4.2.5 Egg mass collection and maintenance

After the laying process, egg masses from transplanted females were transferred to one of the six experimental temperature treatments (10, 12, 14, 16, 18 and 20 °C). Egg masses were maintained in the laboratory under the same conditions as the Solent and Arcachon capsules (please refer to section 2.3.4). Female size, number of capsules and rearing experimental conditions for the transplant experiment are summarized in Table 4.1. During the intracapsular development, embryo size, embryo survival, abnormalities and metamorphosis at hatching were measured to compare with Arcachon and Solent populations, please refer to section 2.2.4 for the methodology (Chapter 2). The presence of 'soft capsules' (i.e. capsules with abnormal wall consistency) was observed in all temperature treatments, they were considered dead as embryos escaped from capsules before hatching.

4.2.6 Statistical analysis

Kruskal Wallis one-way ANOVA analysis followed by Dunn's *post hoc* analysis was used to compare female size (i.e. females laying capsules) between the Solent and Arcachon populations and the transplant experiment. Fecundity data (i.e. number of eggs, number of capsules, capsule size, egg size and female size) was analysed by standard linear regression. All data were $\log_{(x)}$-transformed before analysis. Linear regressions were compared using ANCOVA followed by Tukey *post hoc* analysis. Multiple comparisons of means (MCM) were used to compare the intercept fitted values. Software used was R platform 3.3.3 (r Development Core Team, 2017).

Developmental data was analysed with one-way ANOVA to compare the effect of temperature on embryo survival and embryo development followed by Tukey *post hoc* analysis. Prior to analysis, the percent survival and abnormal capsules were arcsine transformed. Juvenile size was analysed by two-way ANOVA followed by Tukey *post hoc* analysis to compare between the Solent and Arcachon populations and the transplant experiment, with temperature and population as factors. Normality and homogeneity of variance were confirmed before respective analysis and statistical significance was identified at $p < 0.05$. A kruskal-Wallis analysis of variance by ranks, followed by

Dunn's method post hoc analysis was used to compare the metamorphosis time in the transplant experiment.

Table 4.1 Summary of experimental conditions of egg masses assigned to temperatures between 10 and 20 °C from transplanted females. 'Exp. T' indicates experimental temperature treatments; 'FS' indicates female size (in mm); 'NC' indicates number of capsule per egg mass; 'FE' indicates fecundity or total eggs per egg mass; 'T' indicates temperature in (°C); 'S' indicates salinity. Values are given as mean ± standard deviation.

Exp. T	**Transplant experiment (Arcachon egg masses under Solent conditions)**				
	FS	NC	FE	T	S
10 **°C**	38.1	19	937	10.2 ± 0.3	33.2 ± 0.5
	37.8	11	550		
	24.5	10	440		
12 **°C**	28.6	22	843	12.7 ± 0.5	33.0 ± 0.6
	36	13	468		
	29.9	16	1163		
14 **°C**	34	17	708	14.2 ± 0.8	33.5 ± 0.6
	37.1	14	644		
	38	15	630		
16 **°C**	36.9	15	525	16.1 ± 0.5	33.5 ± 0.4
	35	12	512		
	n.d	12			
18 **°C**	n.d	22		18.2 ± 0.3	33.3 ± 1.2
	n.d	28			
	n.d	25			
20 **°C**	37.4	19	675	19.9 ± 0.5	33.5 ± 0.4
	34.1	11	293		
	n.d	16			

4.3 Results

4.3.1 Female size range

There was no significant difference between the size of females used in the first comparison between Arcachon and the Solent populations; however, transplanted females (i.e. Arcachon females) laying capsules in Solent climatic conditions were smaller than Arcachon females ($H = 7.818$, $p < 0.05$; Fig. 4.2). In the Solent, a total of 14 females and their egg masses were analysed, female average shell length was 34.6 ± 6.0 mm. In Arcachon, sixteen females were analysed with an average shell size of 37.7 ± 6.0 mm. On the contrary, transplant females laying capsules had smaller sizes than Arcachon females; a total of fifteen females were analysed, with an average female shell size of 33.3 ± 5.0.

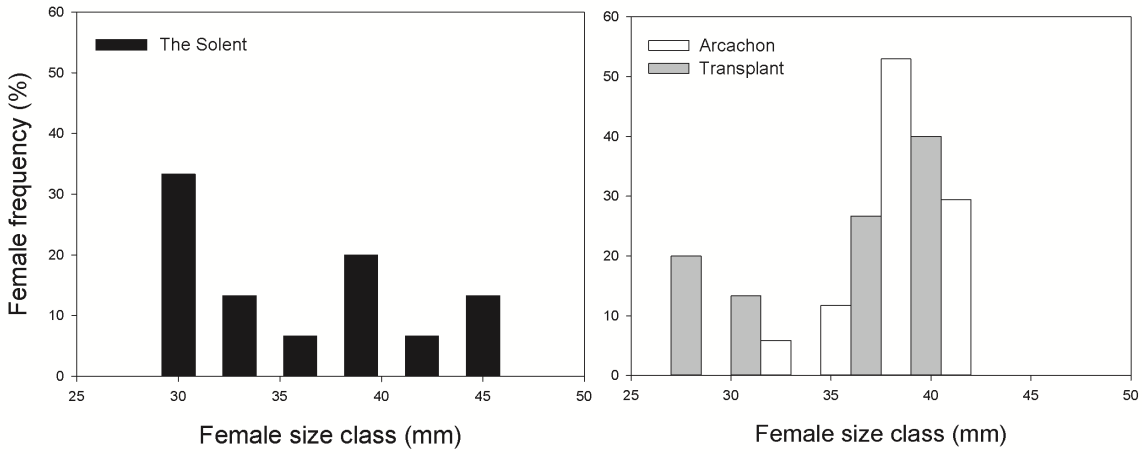

Figure 4.2 Shell size frequency distribution of laying females from the Solent and Arcachon populations and transplant experiment. Class width (3 mm). n = 15 females in the Solent; n = 16 females in Arcachon and n = 15 females in transplant experiment.

4.3.2 Reproductive traits in *Ocenebra erinaceus*

Fecundity (i.e. the total number of eggs per egg mass) was highly correlated with female size in Arcachon and the Solent; larger females produced more eggs per egg mass than smaller females. However, in the transplant experiment, fecundity was not correlated with female size (Fig. 4.3, Table 4.2). The slope and intercepts of the lines relating female shell size and fecundity were significantly different among populations ($p < 0.05$, Table 4.3). *A posteriori* MCM analysis showed that Arcachon and Solent females had similar fecundities but that they were significantly greater

than those of the Arcachon transplant females. On average, the fecundity was 1402 ± 657 and 2076 ± 641 eggs per egg mass in the Solent and Arcachon, respectively. In the transplant experiment, fecundity was lower with 613 ± 230 eggs per egg mass. Similar results were observed in the number of eggs per capsule size among populations; larger capsules had more eggs developing inside of them (Fig. 4.4, Table 4.2). Slopes and intercepts correlating capsular size with number of eggs were significantly different among populations ($p < 0.05$; Table 4.3). *A posteriori* MCM analysis showed more eggs developing inside capsules from Arcachon and the Solent populations than in the transplant experiment.

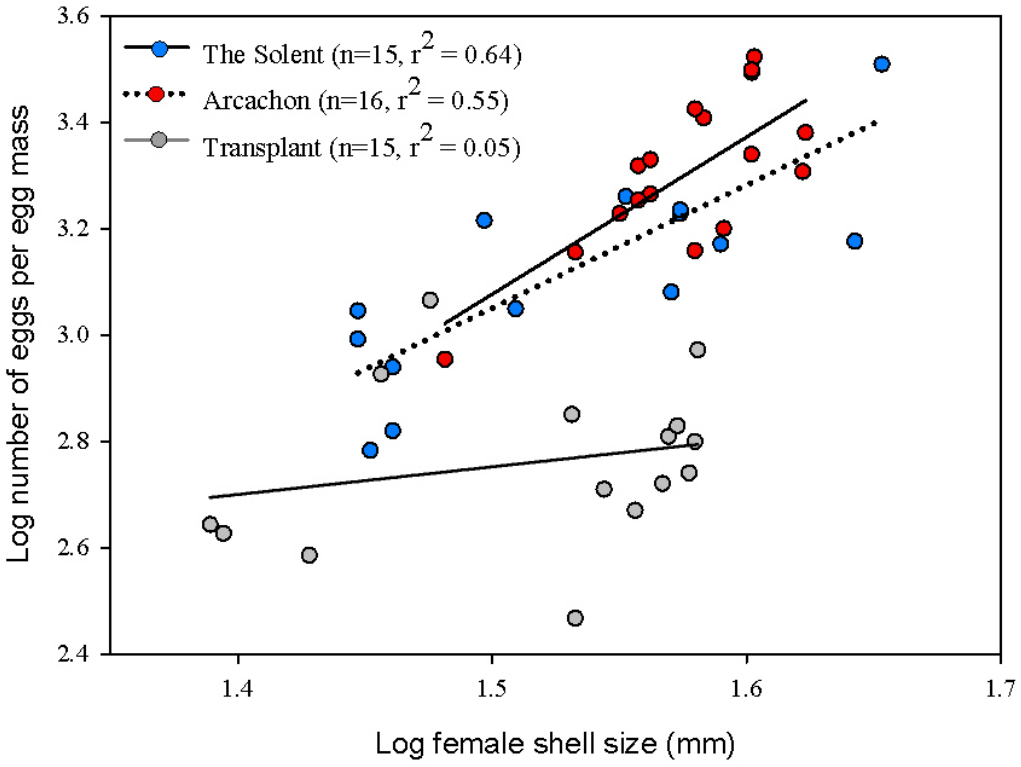

Figure 4.3 The effects of location on the relationship between number of embryos per egg mass and female size in *Ocenebra erinaceus*. All data were log-transformed. Each dot represents the average number of eggs per egg mass.

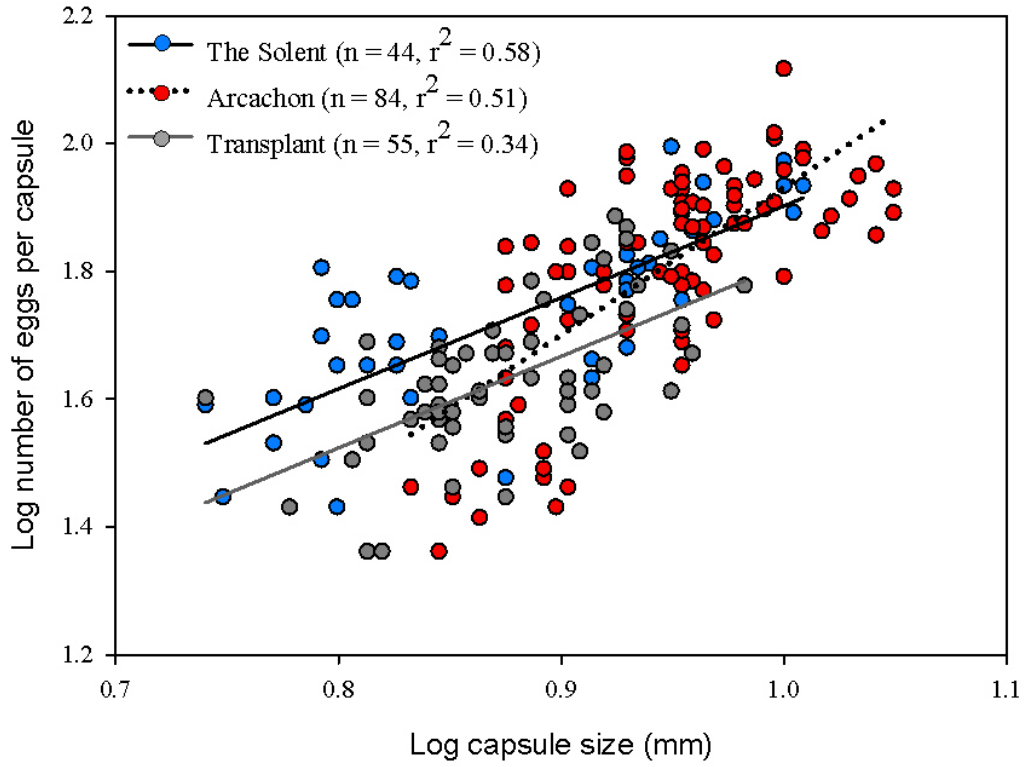

Figure 4.4 The effects of location on the relationship between number of eggs per capsule and capsular size in *Ocenebra erinaceus*; all data were log-transformed.

Egg size was not correlated with female size between populations; larger females did not lay larger eggs (Fig. 4.5; Table 4.2). The slope and intercepts of the lines relating female shell size and egg size were significantly different among populations ($p < 0.05$; Table 4.3). *A posteriori* MCM analysis showed that transplant females laid smaller eggs than Arcachon and Solent females. On average, embryo size ranged between 500 and 550 µm in the Solent and Arcachon. However, females from transplant experiment laid eggs with sizes of 440 µm on average.

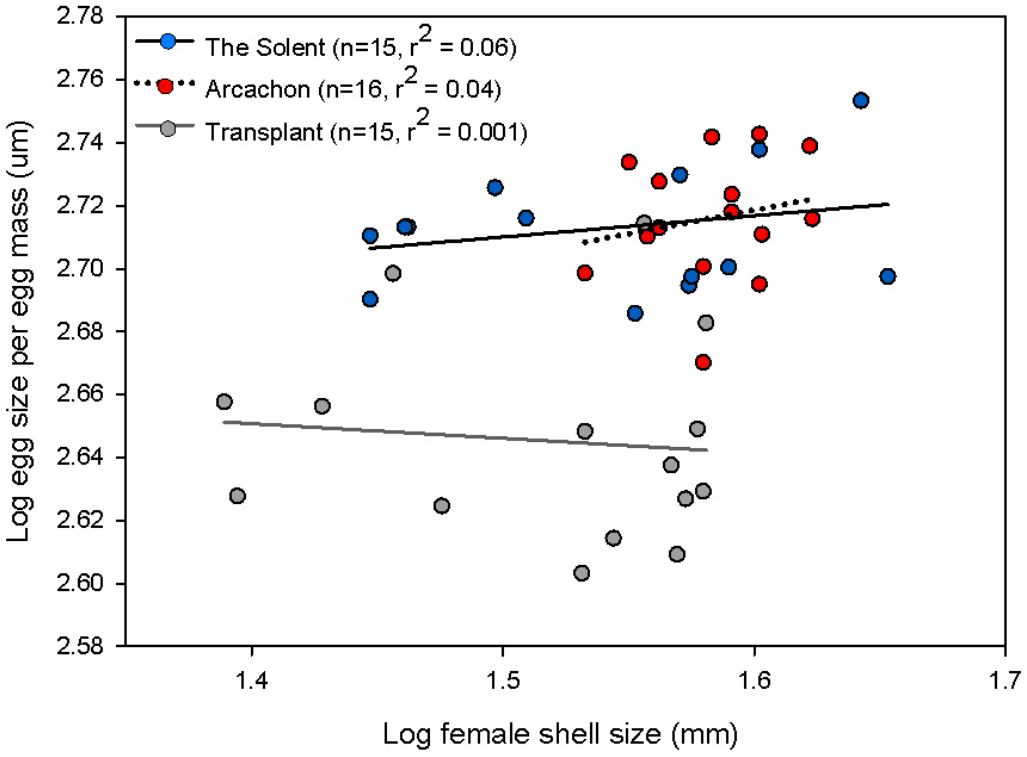

Figure 4.5 The effects of location on the relationship between egg size per egg mass and female size in *Ocenebra erinaceus*. All data were log-transformed. Each spot represents the average egg size per egg mass.

A positive correlation was found between capsule size and female shell length among populations, larger females deposited larger capsules than smaller females (Fig. 4.6, Table 4.2). Whilst the slopes of these relationships did not differ between populations, i.e. female investment in capsule size, scales consistently with female size, irrespective of origin; the intercepts were significantly different ($p < 0.05$, Table 4.3). MCM analyses showed that Arcachon females deposited larger capsules than females from the Solent population and were also larger than those used in the transplant experiment. On the contrary, the number of capsules was not correlated with female size (Fig. 4.7, Table 4.2). The regression slope relating the number of capsules with female shell length were not significantly different between populations ($p > 0.05$, table 4.3). Thus, larger females did not lay significantly more egg capsules per egg mass than smaller individuals in all populations studied. However, the intercept did differ significantly between populations (Table 4.3), with the Solent and Arcachon females producing more capsules than transplant females. On average, the number of capsules was similar in the Solent and Arcachon populations, with 26 capsules on average per egg mass; on the contrary, transplant females deposit 14 capsules on average.

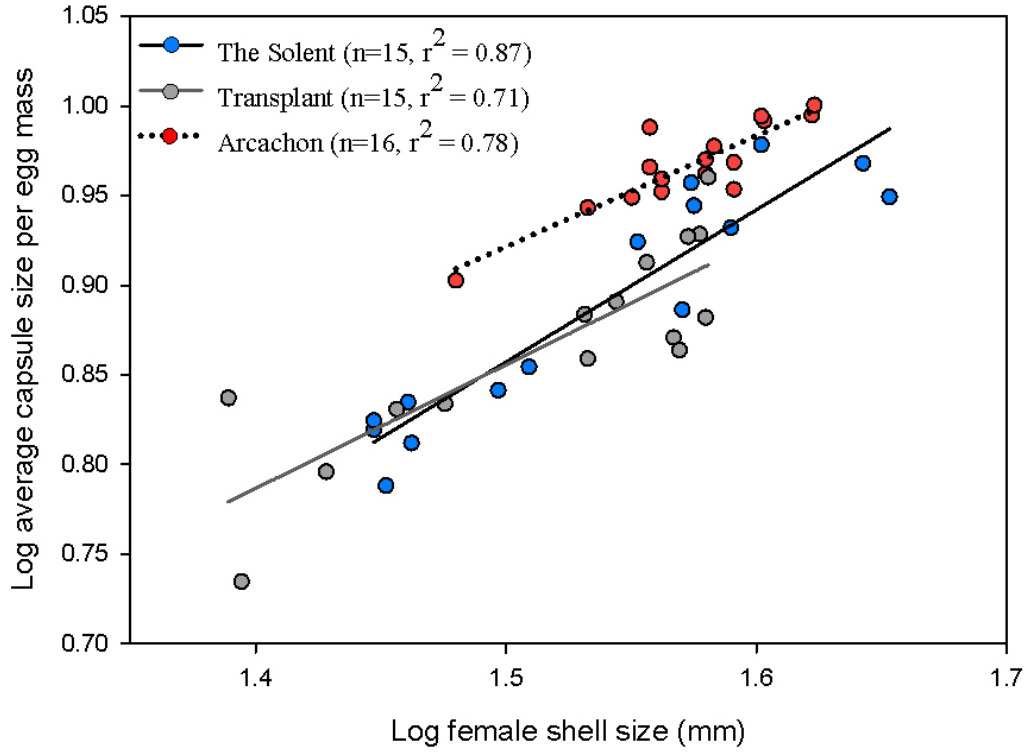

Figure 4.6 The influence of sampling location on the average capsule size average per egg mass in females of *O. erinaceus*. All data were log-transformed. Each dot represents the average capsule size per egg mass.

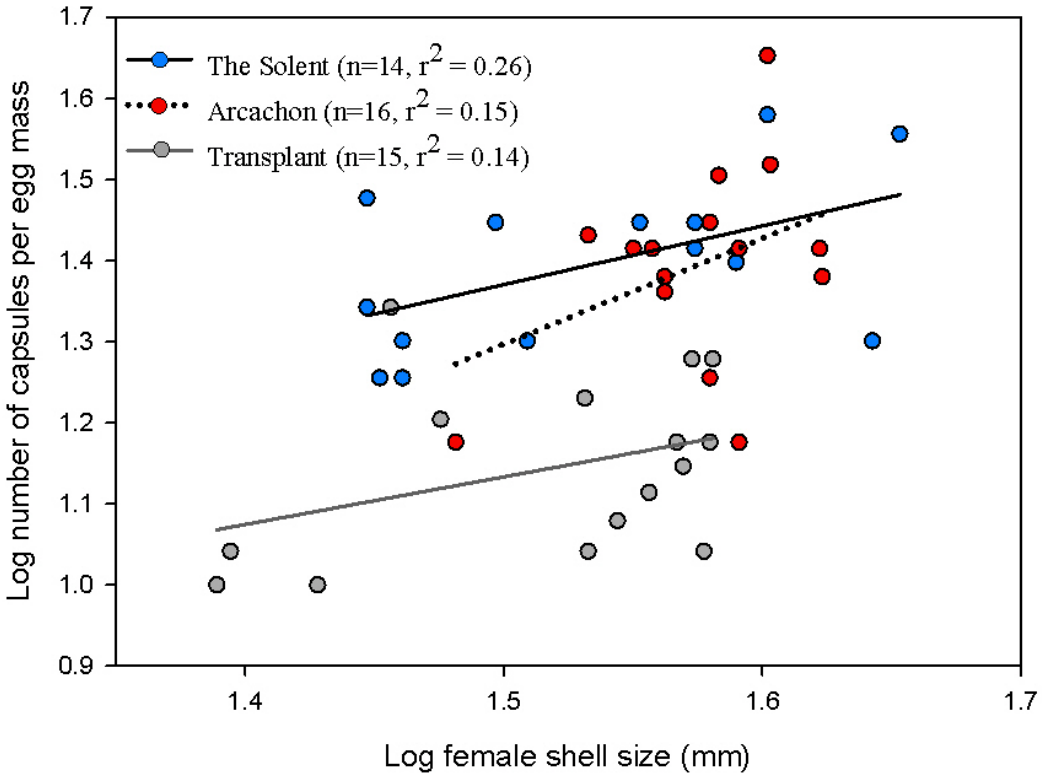

Figure 4.7 The effect of location on the relationship between female size and number of capsules per egg mass. All data was log-transformed.

Table 4.2 Linear regressions and analysis of variance comparison of the mean conducted in the experimental variables measured in the Solent (SO), Arcachon (AR) populations and transplant (TR) experiment. 'CI' means confidence interval. All data were log-transformed before to conduct the analysis.

Variable	Population	Equation	n	CI	R^2	F	*p*-value
(a) Number of eggs per egg mass							
	SO	$y = -0.42 + 2.31^X$	15	2.95 ± 1.53	0.64	22.97	**p < 0.05**
	AR	$y = -1.35 + 2.95^X$	16	2.31 ± 1.04	0.55	17.24	**p < 0.05**
	TR	$y = 1.97 + 0.52^X$	15	0.52 ± 1.33	0.05	0.73	p = 0.41
(b) Number of eggs per capsule size							
	SO	$y = 0.47 + 1.43^X$	44	1.43 ± 0.37	0.58	60.79	**p < 0.001**
	AR	$y = -0.38 + 2.31^X$	84	2.31 ± 0.50	0.51	85.53	**p < 0.001**
	TR	$y = 0.38 + 1.44^X$	56	1.44 ± 0.53	0.34	48.80	**p < 0.001**
(c) Egg size per egg mass							
	SO	$y = 2.61 + 0.07^X$	15	0.07 ± 0.16	0.01	0.80	p = 0.39
	AR	$y = 2.48 + 0.15^X$	16	0.15 ± 0.45	0.04	0.53	p = 0.48
	TR	$y = 2.72 + 0.04^X$	15	-0.04 ± 0.45	0.001	0.13	p = 0.72
(d) Capsule size per egg mass							
	SO	$y = -0.41 + 0.85^X$	15	0.85 ± 0.20	0.87	88.85	**p < 0.001**
	AR	$y = -0.01 + 0.62^X$	16	0.69 ± 0.26	0.78	48.80	**p < 0.001**
	TR	$y = -0.18 + 0.69^X$	15	0.62 ± 0.26	0.71	31.68	**p < 0.001**
(e) Number of capsules per egg mass							
	SO	$y = 0.28 + 0.72^X$	14	0.72 ± 0.77	0.26	4.25	p = 0.06
	AR	$y = -0.66 + 1.30^X$	16	1.31 ± 1.69	0.15	2.69	p = 0.12
	TR	$y = 0.25 + 0.59^X$	15	0.59 ± 0.88	0.14	2.11	p = 0.17

Table 4.3 Parameter estimation using regression analysis for comparing slopes and intercepts describing the scaling relationships (log-transformed data) between number of eggs, capsule size and egg size variables and female size (covariate variable); and number of eggs variable and capsule size (covariate variable) in the Solent and Arcachon populations, and transplant experiment. Below each parameter is showed the ANCOVA summary (F_{value}, p-value) testing for equal slopes and homogeneous intercepts. Multiple comparisons of means (MCM) were used to compare the intercept fitted values. Identical letters means no significant differences. SO' indicates The Solent populations; 'AR' indicates Arcachon population; 'TR' indicates transplant experiment.

Variable	Parameters estimation		
Source	Slope ± SE	Intercept ± SE	MCM
(a) Number of eggs per egg mass			
SO	-0.41 ± 0.13	0.62 ± 0.54	a
AR	-0.93 ± 0.31	0.09 ± 0.05	a
TR	2.39 ± 0.19	-0.34 ± 0.05	b
	$F_{(2,42)}$ = 4.44; **p = 0.02**	$F_{(2,42)}$= 36.86; **p < 0.001**	
(b) Number of eggs per capsule size			
SO	0.47 ± 0.17	0.20 ± 0.12	a
AR	-0.84 ± 0.20	-0.03 ± 0.02	a
TR	-0.01 ± 0.28	-0.09 ± 0.02	b
	$F_{(3,186)}$= 4.81; **p = 0.01**	$F_{(2,186)}$= 23.01 ;**p < 0.001**	
(c) Egg size per egg mass			
SO	-0.42 ± 0.74	0.62 ± 0.54	a
AR	-0.93 ± 1.65	0.09 ± 0.05	a
TR	2.39 ± 1.07	-0.34 ± 0.05	b
	$F_{(2,42)}$ = 4.42; **p = 0.02**	$F_{(2,42)}$= 36.86 ;**p < 0.001**	
(d) Capsule size per egg mass			
SO	-0.41 ± 0.13	-0.27 ± 0.09	a
AR	0.84 ± 0.31	-0.22 ± 0.01	b
TR	0.24 ± 0.19	-0.25 ± 0.01	a
	$F_{(2,42)}$ = 1.12; p = 0.34	$F_{(2,42)}$= 23.01; **p < 0.001**	
(e) Number of capsules per egg mass			
SO	0.28 ± 0.13	0.26 ± 0.39	a
AR	-0.94 ± 0.31	-0.03 ± 0.04	a
TR	-0.03 ± 0.19	-0.24 ± 0.04	b
	$F_{(2,42)}$ =0.36 ; p = 0.70	$F_{(2,42)}$= 22.58; **p < 0.001**	

4.3.3 Local adaptation in early stages

Transplanted embryos (i.e. Arcachon embryos under the Solent conditions) exhibited low survival at both ends of the experimental temperature range ($F_{(5,17)}$=3.96; $p < 0.05$; Fig. 4.8a). At extreme temperatures 10 and 20 °C, the survival was significantly lower with values between 50 and 30%, respectively (Tukey *post hoc* analysis). The presence of 'soft capsules' was observed in all temperature treatments. The maximal survival was observed at 14 – 16 °C, which is coincident with the optimal temperatures for development observed in the Solent. The development of transplanted embryos was not affected by temperature ($F_{(5,17)}$=4.45, $p > 0.05$), embryos showed normal development at all experimental temperature treatments (Fig. 4.8b). Temperature significantly affected the metamorphosis time in transplanted swimming larvae (H= 12.23, $p < 0.05$; Fig. 4.8c). Larvae exposed to high temperatures spent between 24 and 48 hours in the water column compared to larvae exposed to 14 °C where larvae spent only 24 hours (Dunn's *post hoc* analysis).

The size of juveniles was significantly affected by geographic origin and temperature ($p < 0.05$; Table 4.4; Fig. 4.9); juveniles were significantly larger for the Solent and Arcachon population compared to the transplant experiment (Tukey *post hoc* analysis). On average, juvenile size was 0.90 mm for the Solent and Arcachon; however, the average size of juveniles from transplant experiment was 0.81 mm. Temperature also affected the juvenile's size ($p < 0.05$; Fig. 4.9). In the Solent, larger juveniles were observed at 16 °C. In the Arcachon population and transplant experiment, high temperatures did not affect the juvenile size.

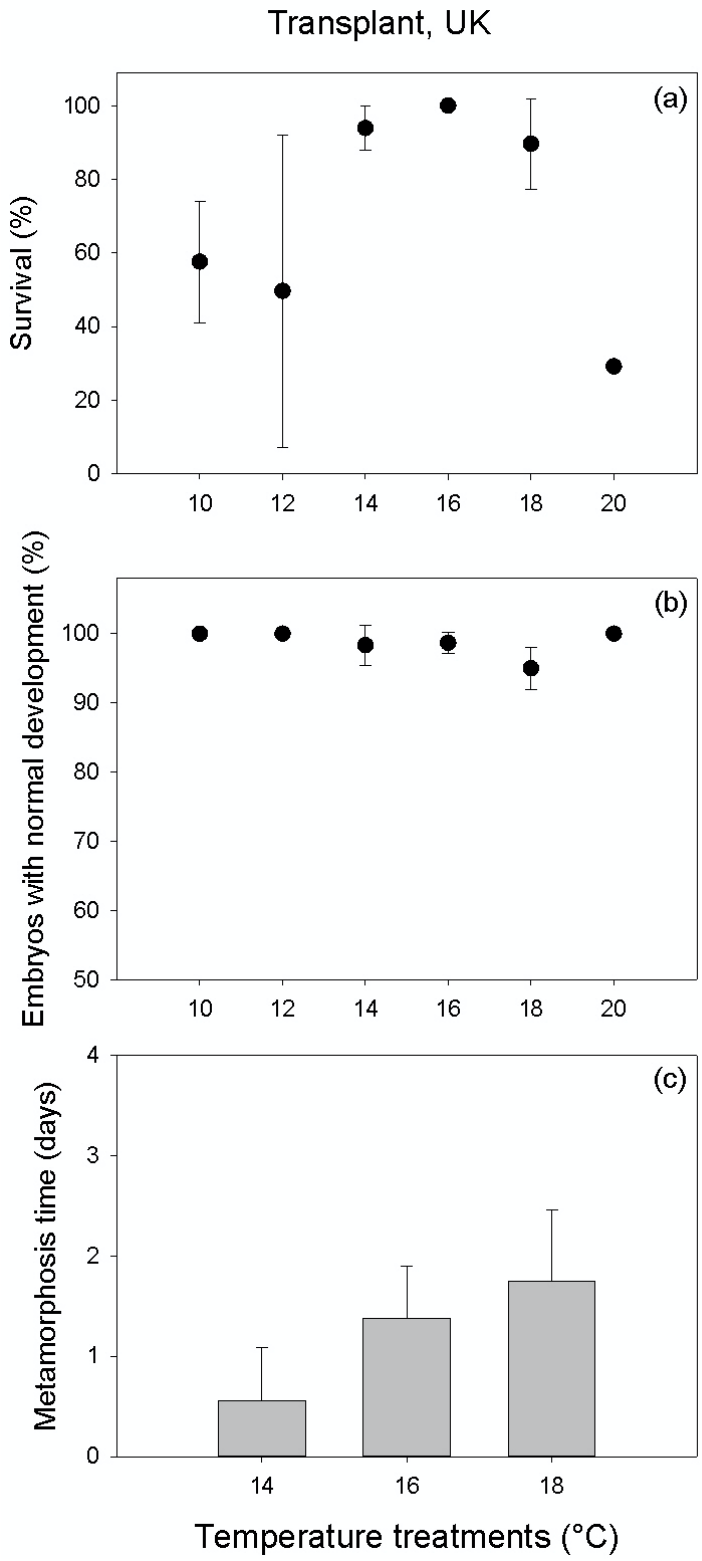

Figure 4.8 Effects of temperature on **(a)** embryo survival, **(b)** embryo abnormalities and **(c)** metamorphosis time in transplanted embryos from Arcachon under Solent climatic conditions. Values are given as mean ± standard deviation. n = 3 egg masses per temperature treatment in (a) and (b), respectively; n = 9 – 10 capsules (48 embryos approximately per capsule) per temperature treatment in (c).

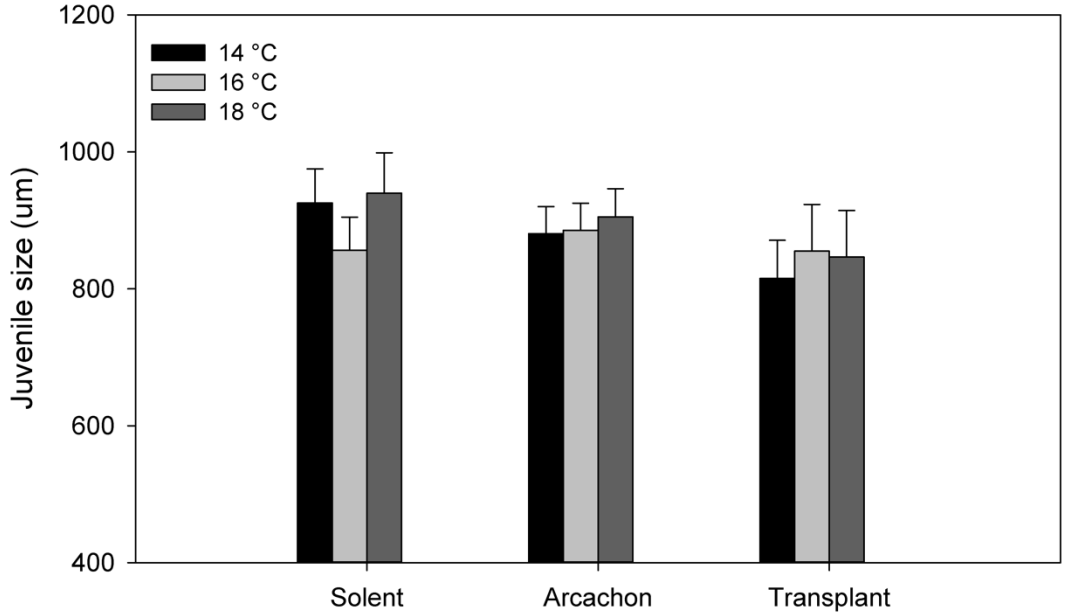

Figure 4.9 Effects of temperature on the size of recently settled juveniles of *Ocenebra erinaceus* from the Solent and Arcachon populations and transplant experiment (i.e. Arcachon embryos under Solent climatic conditions). Values given are mean ± standard deviation. n = 35 – 40 juveniles per temperature treatments.

Table 4.4 Results of two-way ANOVA using recently hatched juveniles from the Solent and Arcachon populations, and transplant experiment for the effect of temperature on juvenile size.

		Juvenile size		
Source	DF	MS	F	P
Location	2	129635.23	44.016	**<0.001**
Temperature	2	29878.34	10.145	**<0.001**
L x T	4	38430.41	13.049	**<0.001**
Error	343	4223.05		

4.4 Discussion

Natural selection acts on marine organisms leading to different trade-offs among life history traits, which gives rise to variable and complex reproductive strategies (Llodra, 2002). This is more evident in direct developing species that exhibit restricted connectivity and low gene flow among populations (Beherens, 1989; Parsons, 1998), which results in a mosaic of locally adapted populations (Kawecki and Ebert, 2004). In this study, it was observed that females from each population of *Ocenebra erinaceus* exhibited similar fitness (i.e. maternal investment); however, they have different adaptive costs resulting in trade-offs between reproductive traits. The decrease in the reproductive output observed in the transplant experiment (i.e. Arcachon females into the Solent climatic conditions) suggests that reproductive traits exhibit, to some degree, local adaptation. Furthermore, this maladaptation persisted during the early development of transplanted embryos. Solent climatic conditions influenced the thermal tolerance range of transplanted embryos in a similar way than embryos from the Solent; however, high mortalities, presence of soft capsules (i.e. low quality capsules) and reduction in juvenile size, demonstrated that transplanted embryos were not adapted to live under this new environment.

4.4.1 Local adaptation in reproductive traits

The laying time of *O. erinaceus* was highly correlated with environmental temperatures. Only one reproductive peak was observed during the year, when temperatures started to increase from winter to spring. In the Solent, the egg capsule laying time was in April and May, when temperatures increased from 11 to 14 °C; which is coincident with the laying time observed by Smith et al. (2015) at the same location. In contrast, Arcachon females experienced warmer temperatures throughout the year. Egg capsule laying took place between February and March when temperatures started to rise from 10 to 11 °C.

Regardless of the temperatures that females of both populations experienced in the field (Fig. 2.2), no differences were observed in maternal investment (i.e. total number of eggs per egg mass) between Arcachon and Solent females (Fig. 4.3). Egg production depends on maternal size in gastropods species (Chaparro and Flores, 2002; Chung et al., 2013; Gianguzza et al., 2005; Spight et al., 1974) and as observed in this study, where fecundity was correlated positively with female size. Egg production and size was similar in both populations and within the range of previous estimates (Gibbs, 1996; Martel et al., 2004). The results suggest that natural selection favoured the same reproductive potential for both populations in their native conditions. However, there were adaptive costs to live under those local conditions, leading to a trade-off between capsule size and number of eggs.

Capsules deposited by Arcachon females (i.e. warm temperatures) were larger and with more eggs than for Solent and transplant females (Fig. 4.6). This difference in size could be explained as a combined effect of temperature and oxygen. In prosobranch gastropods, encapsulation can be constrained by the increase of temperature and oxygen availability (Lardies and Fernández, 2002; Cancino et al., 2011). Females deposit eggs inside capsules with the same oxygen concentration that they experienced in the field, usually with values between 8 - 9 mg O_2 L^{-1} (Cancino et al., 2011; Cumplido et al., 2011). There is minimal diffusion of oxygen through the capsule wall from the external environment (Segura et al., 2010). Therefore, the oxygen at the beginning of the development depends on the environmental temperature and oxygen saturation that the mother experienced at the time of laying. In this study, Arcachon females and their offspring experienced warmer temperatures in the field than in the Solent (Fig. 2.2); thus, it can be concluded that Arcachon females allocated more resources in laying larger capsules to allow more oxygen availability per embryo which could impact positively on the embryo survival at high temperatures.

4.4.2 "Local versus foreign" criteria: transplant experiment

Environmental temperatures influenced the laying time of transplanted females after 9-months of exposure under Solent climatic conditions; females began laying capsules between March and April, as observed in the Solent population. However, even though the experimental female group from Arcachon was the same in the transplant experiment (i.e. same group of gastropods collected from Arcachon, France), the average size of the spawning transplanted females was smaller than females observed in the Arcachon population (Fig. 4.2). The decrease in size impacted the fecundity of transplanted females reducing egg production by 70% in comparison with Arcachon females. Therefore, Solent thermal conditions affected the laying process of bigger females from Arcachon population. Moreover, although the spawning transplanted females were of similar size to Solent females, the fecundity of transplanted females was lower than those from the Solent, indicating a poor adaptation to the foreign environment (i.e. colder conditions). In addition, transplanted females laid smaller capsules and eggs than females from Arcachon and the Solent; which means that females under a new environment reduced the maternal provisioning per egg. Please see the graphical summary (Fig. 4.10).

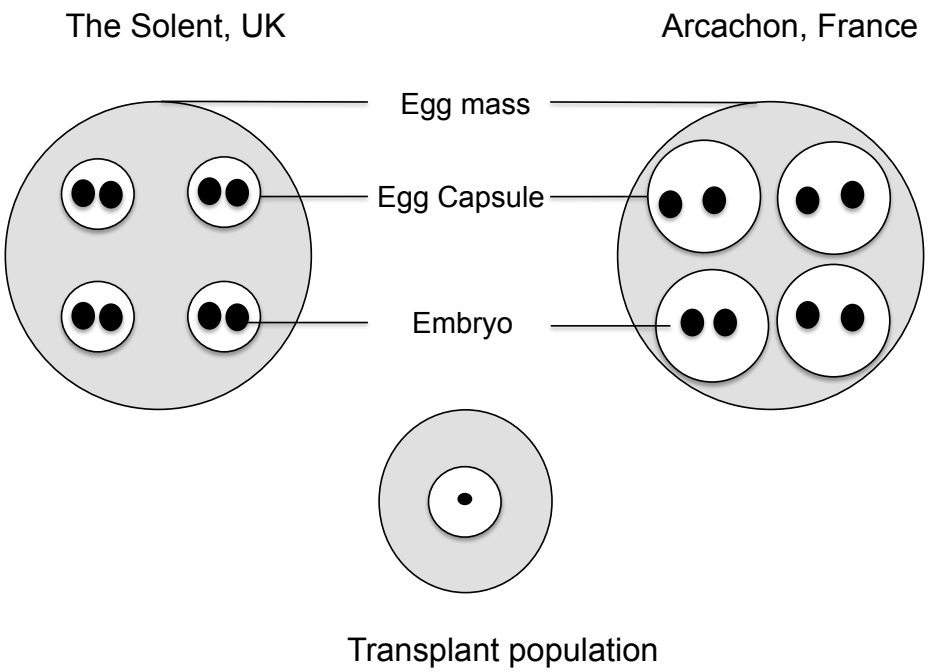

Figure 4.10 Graphical summary of reproductive traits in Solent and Arcachon populations, and transplant experiment. Females from the Solent and Arcachon have similar reproductive output (egg masses size, number of capsules per egg mass, number of eggs and egg size); however, capsules were larger in Arcachon than in the Solent egg masses. On the contrary, transplanted females (i.e. Arcachon females under the Solent thermal conditions) laid a few number of capsules (i.e. small egg mass), smaller capsules, small eggs and low number of eggs.

The low reproductive fitness observed in Arcachon females under the foreign environment indicates that the environmental conditions in the Solent imposed a strong selection against Arcachon female genotype. Even though transplanted females were fed *ad-libitum*, the reduction in number and egg size suggests that Arcachon females were not adapted to experience colder temperatures (i.e. Solent environmental conditions). Previous reports in terrestrial (e.g. plants and insects) and marine environments have shown that reproductive traits can be strongly influenced by thermal environmental conditions leading to genetic changes among populations (Lazzaro et al., 2008; Miller and Cummins, 2014; Postma and Ågren, 2016). For example in terrestrial environments, a population of *Drosophila melanogaster* from a tropical environment showed decreased fecundity and low resistance to bacterial infection in comparison with a temperate population when they were exposed to low temperatures. Lazzaro et al., (2008) suggested that these differences could be attributable to genetic adaptations to local abiotic environment. The same pattern was observed in plants, Miller and Cummins (2014) showed that the geographical

origin (i.e. altitude) influenced the reproductive performance of the grassland *Nardus stricta*. Plants growing in their native location showed home-site advantages over foreign plants.

In marine environments, Gleason and Burton (2013) studied whether temperate populations of the marine gastropod *Chlorostoma funebralis* (Veligastropoda), a species with a similar development of *O. erinaceus* (i.e. short larvae pelagic phase), were adapted to their local environmental conditions. They found that although *C. funebralis* has a short pelagic phase, the species evolved in locally adapted populations. Under common garden conditions (i.e. similar conditions for all individuals), southern populations showed lower mortality and high recovery following heat stress compared to northern populations. They concluded that southern populations possess genetic adaptations to tolerate the extreme heat stress they experience.

In all these examples, 'local and foreign' criteria showed that local genotypes have higher fitness than foreign genotypes. In this study, it was shown that the differences observed between these two populations are due to local adaptations to natal environmental conditions. Solent and Arcachon genotypes showed higher fitness in their native environments; however, when Arcachon genotype was exposed to the Solent environmental conditions, females performed poorly, reducing their reproductive fitness.

4.4.3 Persistence of local adaptation during early development

Early stages can play an important role in local adaptation; however, little is known about the potential impact on the species genetic divergence between populations (Postma and Ågren et al., 2016). Natural selection can be severe during the first part of the life of cycle when organisms are vulnerable and suffer high mortalities, which can lead to selecting some genotypes over others producing cascading effects on later life stages (Postma and Ågren et al., 2016). In this chapter, it was observed that local temperatures influenced the development of transplanted embryos, showing a similar thermal response to those of Solent embryos (i.e. mortality and hatching time). However, mortality was more pronounced in the transplant experiment at both ends of the experimental thermal range (Fig. 4.8). In addition, the presence of 'soft capsules' (i.e. capsules with abnormal wall) was only observed in the transplant experiment, which demonstrates a high energetic cost of living in a new thermal environment. Indeed, the acclimation to the new environment (i.e. Solent) had a high cost in the size of transplanted juveniles (Fig. 4.9). The size of settled juveniles was smaller than Solent and Arcachon juveniles, which could suggest that they were not genetically adapted to live under the new thermal conditions.

Studies of local adaptation during early stages in terrestrial environments have shown that early stages can be locally adapted. For example, Postma and Ågren (2016) demonstrated strong

selection against non-local genotypes during the seed establishment phase of the annual plant *Arabidopsis thaliana*. They conducted a reciprocal transplant experiment with seeds from two locally adapted populations. The results showed that natural selection strongly favoured the local genotypes; local alleles were favoured against non-local; thus, they suggest that genetic differentiation may be strong in early stages and may contribute to genetic differences. On the contrary, few attempts have been made to determine local adaptation during early life stages in marine environments. Kuo and Sanford (2009) studied the influence of geographic origin in the upper thermal limits of newly hatched juveniles of the temperate gastropod *Nucella canaliculata*, a species with the same development of *O. erinaceus*. Individuals were reared in common garden conditions over two generations to avoid the potential impact of acclimatization and non-genetic effects. They demonstrated that the differences observed in the upper thermal limits of newly hatched juveniles are likely to be genetic. Individuals from populations inhabiting hot spots had greater thermal tolerance at high temperatures than those from less thermally stressful sites. They concluded that populations of *N. canaliculata* were genetically differentiated along a latitudinal gradient (Kuo and Sanford 2009).

The results obtained from transplanted embryos and their poor performance under Solent climatic conditions supports the idea that early stages of *O. erinaceus* are locally adapted to regional thermal conditions. However, there are some limitations in this study: (1) maternal effects can affect the phenotypic response of the offspring, limiting and imitating local adaptations (Kawecki and Ebert 2004). In this study, transplanted females were maintained for 9-months into the Solent environmental conditions, to reduce the effects of environmental history; however, in some cases, maternal influences can persist, as shown by Shama et al. (2014) in the marine stickleback fish *Gasterosteus aculeatus*. Further analysis over more generations are necessary to elucidate whether or not the intraspecific differences observed are due to genetic differentiation. (2) In this study, the transplant experiment was only conducted in one way, i.e. Arcachon individuals under Solent environmental conditions. Even though, the results obtained from the transplant experiment showed evidence for local adaptation; to understand the adaptive cost to live under each native location is necessary to do a fully reciprocal transplant experiment.

4.5 Conclusion

This chapter showed that Arcachon and Solent females have a similar reproductive effort under different regimes of environmental temperatures; however, the capsule size in Arcachon females was larger than in the Solent, indicating trade-offs between number of eggs and capsule size in response to high temperatures and low oxygen saturation. Warmer environments appear to select for larger females depositing larger capsules to improve oxygen availability to a greater quantity of

eggs per capsule. On the contrary, colder environments (i.e. Solent) could be selecting for smaller female sizes laying smaller capsules. According to the "local and foreign" criteria, Solent and Arcachon genotypes showed higher fitness in their native environments; however, when Arcachon genotype was exposed to Solent environmental conditions, females performed poorly reducing their fitness. During the early development, the transplant experiment showed that the thermal response of embryos is plastic and highly correlated with the environmental temperatures; however, the high energetic costs of surviving in a new environment (i.e. presence of soft capsules, high mortality and juvenile size reduction) were identified. Thus, this study showed that *Ocenebra erinaceus* is locally adapted to natal environmental conditions. In response to global warming, extinction risk and potential distribution shifts of marine species are generally expected; therefore, understanding that species exhibit physiological variability throughout their geographical distributions range (i.e. local adaptation) will improve our ability to predict the effects of increased temperatures on species extinctions.

Chapter 5 The impact of simultaneous exposure to high temperature and pCO_2 on the physiological and molecular response *Ocenebra erinaceus* (Linnaeus, 1978)

Global average temperatures and ocean acidification have increased at a great rate from preindustrial times due to the ocean uptake of atmospheric carbon dioxide (CO_2). The combined effect of temperature and CO_2 on marine organisms is still controversial because increased temperatures could either compensate or aggravate the effects of high CO_2. Few studies have assessed whether organisms can acclimate and adapt to rapid environmental changes expected by climate change. In this chapter was investigated the interactive effect of global warming (+3 °C) and ocean acidification (~ 900 µatm CO_2) on maternal effects and the physiological and molecular response during short (0-50 days) and long-term exposure (135 days) in *Ocenebra erinaceus* from the Solent, UK population. During early stages, this population is very sensitive to increased temperature (Chapter 2-3); thus, it is important to determine whether maternal effects can improve offspring performance. The results showed that after 10 months, reproduction ceased in females exposed to climate change conditions, thus, maternal effects were not evident. However, high T°/pCO_2 did not significantly affect the physiological and molecular responses (i.e. *hsp*70 expression) of adults after 132 days of exposure; though, there was a slight impact on oxygen consumption and weight rates and at the beginning of the experimental exposure. During the first fifty days, oxygen consumption rate increased and a reduction in weight was observed in gastropods exposed to high T°/pCO_2. However, after ninety-five days of exposure, stressed gastropods increased food intake; thus, gastropods relied on food resources to fully acclimate to the experimental condition (high T°/pCO_2). Therefore, *O. erinaceus* adults from the Solent population appear to be incapable of full compensation against the high energetic requirements imposed by potential climate conditions predicted for the end of this century if food resources are limited. Indeed, the negative impact on reproduction is an indicator that adults only survived under the experimental climate change conditions. Thus, future ocean conditions may well impact the maintenance of this species in the Solent.

5.1 Introduction

Anthropogenic greenhouse gas emissions (i.e. carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) have risen to unprecedented levels (Gattuso et al., 2015) producing changes in the weather, air and sea-surface temperatures, sea level and ocean pH, amongst others (IPCC 2013). Carbon dioxide is one of the most important greenhouse gases in terms of its effects on climate change (e.g. regulating Earth's surface temperature) and in regulating the chemistry of the global ocean. To date, CO_2 emissions into Earth's atmosphere has accelerated from 280 ppm in the pre-industrial period to 410.27 ppm at November 2019, according to the Mauna Loa Observatory CO_2 record (NOAA). The oceans have absorbed ~30% of the atmospheric CO_2 into: carbonic acid (H_2CO_3), bicarbonate ions (HCO_3^-) and carbonate ions (CO_3^{2-}); however, this process also releases free hydrogen molecules [H+] (Doney et al., 2009; Feely et al., 2004). The increase of H+ concentration produces a drop in the oceanic pH, a process that has been termed 'ocean acidification' (OA). By the end of the twenty-first century, it has been predicted that the sea surface pH will decrease by 0.33 (± 0.003) units (Bopp et al., 2013; Orr et al., 2005).

To date, it is well known that OA produces negative effects in marine animals, especially in calcifying organisms, reducing growth, survival, reproduction and shell mineralization, amongst others (Barton et al., 2012; Dupont et al., 2013; Kroeker et al., 2010; Parker et al., 2013; Talmage and Gobler, 2010). In addition, and due to the atmospheric CO_2 level increase, global sea temperatures are also rising, with an expected increase of 2.73 (± 0.72) °C at the end of this century (Bopp et al., 2013; Meinshausen et al., 2011). The increase of temperature-outside of the thermal tolerance range affects the metabolic rate of marine organisms, causing a rapid deterioration of cellular process and affecting the physiology of whole-organism (Pörtner, 2008). However, the combined effect of temperature and pCO_2 on marine organisms has been difficult to predict because, at the metabolic level, increased temperatures produce an increase in metabolism and pH/hypercapnia induces metabolic depression (Michaelidis et al., 2005; Kroeker et al., 2013). Thus, when both stressors temperature and alkalinity act together, the effects can be synergistic or antagonistic (Crain et al., 2008; Harvey et al., 2013; Kroeker et al., 2013).

Marine organisms largely depend on how fast they can acclimate and adapt to environmental changes (Hofmann and Todgham, 2010). Phenotypic plasticity has been identified as one of the most powerful mechanisms to adjust to climate change (Nagelkerken and Munday, 2016). Over short-term exposure to stressful conditions, phenotypic plasticity facilitates the persistence of a population, long-term exposure can lead to genetic adaptation (Foo et al., 2012; Kelly et al., 2012). Maternal effects are a class of phenotypic effects than can influence the offspring phenotype (Bernando, 1996); therefore, maternal effects can provide a strong mechanism for species to

acclimatise and adapt to new environmental conditions and moderate the negative effects of climate change on the offspring (Marshall and Uller, 2007; Munday, 2014). Several studies have been published to elucidate maternal effects on broadcasting species such as sea urchins, marine fishes, polychaete (Dupont et al., 2013; Chakravarti et al., 2016; Parker et al., 2012; Shama et al., 2014). For example, adults of the temperate Sydney rock oyster *Saccostrea glomerata* which were exposed to elevated CO_2 concentrations during reproductive conditioning had positive carry-over effects on growth, rate of development and survival in the larvae spawned (Parker et al., 2012). Chakravarti et al. (2016) exposed parents and their offspring of the temperate polychaete *Ophryyotrocha labronica* to high temperature and pCO_2; they found that the effects on metabolic response, juvenile growth and egg volume were ameliorated when the exposure was transgenerational. However, few studies have considered maternal effects on species with direct development and encapsulation as a reproductive strategy.

The principal aim of this study was to measure maternal effects and offspring performance in females of a direct developing species exposed to future ocean conditions expected for the end of this century according to RCP8.5 IPPC 2013 (+3 °C, ~ 900 µatm CO_2). To date, no studies have been conducted on the effects of high temperature and pCO_2 on females and the potential carry-over effects on early stages in *Ocenebra erinaceus*. However, Lardies et al. (2014) studied the effect of OA in juveniles of the gastropods *Concholepas concholepas (*a species from the same family of *O. erinaceus*), showing negative effects on the juvenile physiology (e.g. increases in metabolic rate). In Chapter 2, it was shown that during early stages, the thermal tolerance response of *O. erinaceus* is locally adapted. Early stages from the Solent, UK population (i.e. from the middle of the geographic distribution) showed a narrow thermal tolerance and a limited metabolic adjustment to high temperatures. Therefore, it would be important to determine whether Solent females will have a role in the acclimation of early stages to future ocean conditions (temperature and pCO_2). However, after ten months (from October 2017 to August 2018), the experimental condition (+3 °C above annual sea temperature and ~ 900 µatm CO_2) negatively impacted reproduction, females did not start the laying process. Therefore, a follow-on experiment was conducted on adults to understand the physiological (i.e. metabolic, growth and ingestion rate) and molecular response (i.e. *hsp*70) to high temperature and pCO_2. The expression of the 70 kDa heat shock protein (*hsp*70) was chosen in this study because of its prominent response to temperature and acidosis stress in the cell (Cummings et al., 2011). Thus, it can be used as a proxy to measure the stress under climate change conditions on *O. erinaceus*.

5.2 Material and methods

5.2.1 Animal collection and maintenance

Ocenebra erinaceus adults (from both sexes) with sizes ranging between 30 and 40 mm were collected by hand from the low intertidal in the Solent, UK (50° 51' N, 001° 21' W) to understand the effects of high temperature and pCO_2 on: (a) maternal exposure and the potential carry-over effects on offspring and (b) physiological and molecular responses. Gastropods were collected in different periods: for maternal effects in October 2017 and for physiology and molecular responses between October and November 2018. In both periods, specimens were transferred to the National Oceanography Centre, Southampton, UK (NOCS) facilities in thermally insulated boxes. Specimens were maintained for thirty days prior to experimentation under aquarium conditions of continuously re-circulated seawater that originated from Empress Doc (at 14-15 °C, salinity 33-34). Individuals were fed *ad libitum* with mussels and water changes were conducted twice per week. Then, individuals were transferred to one of the two experimental conditions: control (14 °C – 400 µatm CO_2) and climate change conditions (17 °C – 900 µatm CO_2). The climate change conditions were designed according to the worst-case scenario RCP8.5 (IPPC 2013).

For the maternal effects experiment, 35 gastropods (17-18 specimens per tank) were exposed to control and climate change conditions, respectively, for an experimental period of ten months. The beginning of the experimental period started when females finished their reproductive cycle and started a new one in October. The ratio between females and males was 40-60% in each tank. At the end of the experimental period, of 35 individuals, only six control females laid egg masses (n = six egg masses); however, females exposed to climate change conditions (high T°/pCO_2) did not lay capsules. Thus, it was not possible to assess the potential effects of maternal exposure in offspring performance. The implication of this finding is considered further in the Discussion (see section 5.4).

Therefore, follow on experiment was designed to understand to what extent high temperatures and pCO_2 impact the physiological (i.e. growth, weight, ingestion and routine metabolic rate) and molecular response (*hsp*70 expression) of adults of *O. erinaceus*. Forty gastropods (i.e. twenty individuals per tank) were exposed to control and climate change conditions, respectively for a period of 135 days. The effects of temperature and pCO_2 were measured at regular intervals of 0, 15, 50, 95 and 135 days in control and climate change gastropods to observe the effects to short (15 days) and long-term exposure (135 days).

5.2.2 CO_2 microcosm system

The 'CO_2 system' for both experiments was designed following the methodology described by Hauton et al., (2009) and Riebesell et al., (2011). Two experimental systems were set up: two control tanks (14 °C and pH 8.0 ~ 400 ppm of CO_2) and two CO_2 injection tanks system (+3 °C and pH 7.7 ~ 900 µatm CO_2; Hauton et al., 2009). Each tank, whether a control or climate change condition, consisted in a 'mixing tank' (50 L) and separate 'incubation tank' (20 L) that were maintained constantly -using water bath- at 14 or 17 °C, respectively. Seawater was mixed between the mixing and incubation tanks with a peristaltic pump and was continually aerated. Twice per week, systems were turned off and 100% seawater of the mixing tank was changed. In CO_2 tanks, pH was adjusted to the desired setpoint using AquaMedic™ pH computers with calibrated electrodes. After every water change electrodes were recalibrated using pH 7 and 4 buffers (Mettler Toledo). When the measured pH fell below to the set point (7.5), the pH computer opened a gas solenoid valve to deliver 100% CO_2 directly in the mixing tank.

Temperature and salinity was measured every two-days with EC170 meter and pH was measured three times per week using a three-decimal-place SevenMulti pH meter. Water samples were taken and fixed using mercuric chloride every fortnight in control and CO_2 system mixing tanks, according to Riesbell et al., (2001). Total alkalinity (A_T) and dissolved inorganic carbon (DIC) was determined to characterize the carbonate chemistry in each tank, using VINDTA 3C (Versatile instrument for the determination of total inorganic carbon and titration alkalinity) following standard protocol at the carbonate facility at NOCS. pCO_2 was calculated from measured pH, A_T, temperature and salinity using CO2calc.

5.2.3 Physiological parameters

Growth (shell and wet weight) and routine metabolic rates were measured in the same specimens at regular intervals (0, 15, 50, 95 and 135 days) in five individuals exposed to control and ten individuals exposed to climate change conditions. Gastropods were individually tagged with coloured numbered tags (Queen bees marking kit – Abelo, UK), glued on the shell. Shell growth (in mm per day) was estimated with a calliper and weight (in grams per day) with a balance SI-234 DENVER. Shell and weight growth was estimated with the following equation: $GR = (GR_{t(1)} - GR_{t(0)})/T$, where GR is the growth rate estimated in mm or grams per day, $GR_{t(1)}$ is the final shell length or weight measure, $GR_{t(0)}$ is the initial shell length or weight measure and T is the number of days between the initial and final measure. Routine metabolic rate (RMR) was determined in hermetically sealed chambers filled with 365 ml of filtered seawater (0.5 µm and salinity 33-34), saturated at 100% oxygen and at the same temperature and CO_2 concentration of experimental

conditions. A pre-calibrated oxygen sensor FIBOX, PreSens fiber optic equipment calibrated with sodium sulphite, according to manufacturer's protocols was attached to the inner wall of the respiratory chambers. In each chamber, the oxygen concentration was measured at the start of the measurement and after four hours. As controls, oxygen concentration was determined in three chambers with the same physical characteristics but without animals. RMR was expressed as mg O_2 h^{-1} $individual^{-1}$.

Ingestion rate was determined according to following Navarro et al. (2002), with minor modifications. Ingestion rate was estimated every week in control and climate change tanks. Each tank, containing twenty snails, was fed with 5 mussels *of Mytilus edulis* every week. At the end of every week, only empty valves of the prey were collected and replaced by new live mussels. The shell length of empty valves was measured to estimate the food ingested by *O. erinaceus* snails. A regression curve was calculated to estimate the dry mussel weight (dry meat (g) = 0.00006 * mussel length (mm) $^{3.4412}$; n = 23 mussels with sizes ranging between 20 and 55 mm). To estimate the individual food intake, the total dry mussel meat ingested per week was divided by the number of snails in each aquarium.

Molecular analysis of the *hsp*70 expression was conducted in snails from control and climate change experimental conditions at regular intervals (i.e. 0, 15, 135 days). At 95-days exposure, the expression of *hsp*70 was only measured in snails exposed to climate change conditions. Please refer to 5.2.4 section for methodology description.

5.2.4 Molecular analysis: degenerate polymerase chain reaction

(a) Isolation of specific gene sequences

Degenerate primers were first designed for *Ocenebra erinaceus* for the genes of interest (GOI): the 70kDa heat shock protein (*hsp70* gene) and three endogenous reference genes (ERG) for quantitative real-time PCR: glyceraldehyde-3-phosphate dehydrogenase (*gapdh* gene), β-actin (*β-act* gene) and β-tubulin (*β-tub* gene). To find the target genes, the database of the National Centre for Biotechnology Information NCBI (www.ncbi.nlm.nih.gov) was used (Table 5.1). Primers were designed based on conserved regions that were identified from protein sequences alignments of the CLUSTAL Multiple Sequence Alignment tool, using the default settings (EMBL-EBI, European Bioinformatics institute https://www.ebi.ac.uk/Tools/msa/clustal). Each alignment was examined and any mismatches were corrected manually. A minimum of three primers (forward and reverse) were designed per gene (Table 5.2) and synthesised by Eurofin MWG operon, Ebesberg, Germany.

Degenerate primers were used in a conventional PCR reaction to isolate fragments of each gene. PCR reactions were performed according to the manufacturer's protocol using 0.25 µl of GoTAq DNA polymerase (5u/µl; Promega Corporation), 2 µM of each of sense and antisense degenerate primers and 1 µl of template cDNA (whole-tissue snails) in a final volume of 25 µl with the following PCR cycle conditions: 1 cycle of 95°C for 2 min, 35 cycles of [95°C 1 min, 'AT'°C 1 min, 72°C 30 s], followed by 72°C for 8 min (i.e. 'AT' annealing temperature of each pair of degenerate primers, Table 5.2). PCR products obtained from degenerate PCR were gel-purified and potential positive gene fragments were cut from agarose gel (1% agarose). The DNA from the gel was extracted using QIAquick® Gel extraction kit (Qiagen Ltd. UK) following the manufacturer's protocol.

Table 5.1 Sequences used for alignments to design degenerate primers. Listed are the sequences, species name and accession numbers from the NCBI database. 'GOI' means gene of interest and 'ERG' means endogenous reference genes, 'SL' means aminoacidic sequence length.

Protein name	Species names and accession numbers
HSP70 (GOI)	*Pomacea canaliculata* (KF356182) 906 SL ; *Haliotis gigante* (MF375339) 804 SL; *Cellana toreuma* (JX169849) 861 SL; *Biomphalaria glabrata* (AF025477) 1085 SL; *Haliotis fulgens* (MH220529) 827 SL; *Pomacea caniculata* (KM405321) 871 SL; *Mytilus galloprovincialis* (AY861684) 793 sl; *Crassostrea gigas* (AB122063) 743 SL; *Rattus norvegicus* (L16764) 810 SL
β-Actin (ERG)	*Cepaea nemoralis* (MH035489) 472 SL*; Haliotis diversicolor* (EU244396) 499 bp; *Hypriopsis cumingii* (HM045420) 479 SL, *Loligo pealei* (AY701849) 718 SL; *Crassostrea brasiliana* (KY707329) 377 SL; *Azumapecten farreri* (KJ081194) 613 SL; *Gecarcinus lateralis* (L76943) 463 SL*; Penaeus monodon* (MG775230) 434 SL*; Macaca mulatta* (NM_001033084) 523 SL*; Pan troglodytes* (AB188274) 610 SL
GAPDH (ERG)	*Homo sapiens* (AF261085) 434 SL; *Cryptocercus punctulatus* (JQ686947) 439 SL; *Bufo gargarizans* (KX698086) 381 SL*; Scophthalmus maximus* (KU057925) 435 SL
β-Tubulin (ERG)	*Aplysia californica* (AY25666) 701 SL*; Patella vulgate* (X79469) 484 SL*; Crassostrea gigas* (AB196534) 526 SL*; Loligo pealei* (AY701850) 622 SL*; Crassostrea brasiliana* (KY707330) 446 SL*; Saccostera kegaki* (AB374929) 531 SL*; Artemia franciscana* (EU179855) 509 SL*; Penaus monodon* (EU083324) 263 SL*; Homo sapiens* (AF141349)530 SL

Table 5.2 Forward (F) and reverse (R)degenerate primers for heat shock protein (*hsp*70 gene), glyceraldehyde-3-phosphate dehydrogenase (*gadph gene*), β-actin (*β-act* gene) and β-tubulin (*β-tub* gene). Primer sequence, length and annealing temperature (AT) are indicated for each one. The successful working primers used for sequencing are in **bold**. 'bp' means primer length in nucleotide basepairs Degenerate bases code: N (A,C,G,T); V (G,C,A); B (G,T,C); H (A,T,C); D (G,A,T); K (G,T); S (G,C); W (A,T); M (A,C); Y (C,T); R (A,G).

Gene/Primer name	Primer Sequence 5 to 3'	bp	AT (°C)
*hsp*70-**F1**	**GAYATGAARCAYTGGCCNT**	**19**	**54.5**
*hsp*70-R1	TCNCCYTCRWANACYTGDAT	20	54.9
*hsp*70-F2	GGNATHGAYYTNGGNCANAC	20	58.0
*hsp*70-**R2**	**TANGCNCANGCYTCRTCNG**	**19**	**58.8**
*hsp*70-	GTNRTNCANGTNCCNGCNTA	20	58.3
*hsp*70-	TANGCNCANGCYTCRTCNG	19	58.8
β-Act-F1	AAYGARYTNMGNGTNGCNCC	20	59.4
β-Act-R1	GGNCCNSWYTCRTCRTAYTC	20	59.4
β-Act-**F2**	**ATGGTNGGNATGGGNCARAA**	**20**	**57.3**
β-Act-**R2**	**TCYTTYTGCATNCKRTCNGC**	**20**	**57.3**
β-Act-F3	CCNGCNATGTAYGTNGCNAT	20	58.3
β-Act-R3	ATYTCNCKYTCNGCNGTNGT	20	5.3
gapdh-F1	AAYGGNTTYGGNMGNATHGG	20	58.0
gapdh-R1	ACNGTYTTYTGNGTNGCNGT	20	57.3
gapdh-F2	TAYGTNGTNGARWSNCANGG	20	57.3
gapdh-R2	CCRTCNACNGTYTTYTGNGT	20	57.3
gapdh-**F3**	**CNCCCNATGTTYGTNRTNGG**	**20**	**59.4**
gapdh-**R3**	**ATNCCNGCNYYNGCRTCRAA**	**20**	**59.4**
β -Tub-**F1**	**ATHGGNGCNAARTTYTGGGA**	**20**	**55.9**
β -Tub-**R1**	**CANACNGCNGTYTTNACRTT**	**20**	**55.3**
β -Tub-F2	ATHWSNGAYGARCAYGGNATHGA	23	59.2
β -Tub-R2	GGNGGDATRTCRTCRCANAC	20	59.0
β -Tub-F3	CCNGAYAAYTTYGTNTTYGG	20	55.3
β -Tub-R3	ARCATYTGYTCRTCNACYTC	20	55.3

(b) Cloning and sequencing

Extracted PCR products were cloned using the pGEM®-T Easy Vector cloning kit (Promega Ltd.). PCR products were ligated overnight at 4 °C into 1 µl of pGEM®-T easy vector (50ng). The overnight ligation reaction was added into JM109 High Efficiency Competent *Escherichia coli* cells. Successful transformant *E. coli* was grown overnight at 37 °C on Luria Bertrani (LB) agar plates containing 100 µg ml^{-1} ampicillin. Successful colonies containing the pGEM®-T Easy vector were identified by colour; colonies containing the insert developed a white colour (i.e. absence of β-galactosidase activity); whilst blue colour indicates β-galactosidase activity. A PCR was conducted on five colonies per gene with M13 primers to confirm the presence of a DNA/gene insert of the correct size. Then, three colonies were picked for each gene and cultured overnight in 15 ml centrifuge tubes with 5 ml LB broth and 100 µg ml^{-1}. Plasmid DNA was isolated from bacterial colonies using the Qiaprep Spin Miniprep kit (QIAGEN) and sequenced using vector-specific (M13) primers by SourceBioscience LifeScience, Nottingham. Returned sequences were excised from the vector sequence with BioEdit software (Hall, 1999). The nucleotide sequences were used to deduce amino acid sequences for each gene fragment and these were compared against the ENA database search engine (https://www.ebi.ac.uk/ena/data/sequence/search) using a BLAST sequence similarity search.

(c) Total RNA and cDNA

To study the effects of high temperature and pCO_2 on the *hsp*70 expression, eight to ten snails per interval period (0, 15, 50, 95 and 135 exposure) from control and climate change experimental conditions were snap frozen in liquid nitrogen (N_2). *hsp*70 expression was quantified from gill tissues because it has been reported that mollusc gills are thermally labile (Hofmann & Somero, 1996). Total RNA was extracted from gills in 1 ml of TRI reagent (Sigma Aldrich) following the manufacturer's instructions. RNA purity was confirmed by analysing the A_{260}/A_{280} ratio using a ND-1000 spectrophotometer (NanoDrop, thermo Scientific). Ratio values higher than 1.8 were further processed to DNA. The RNA integrity and quantity was measured also by an automated capillary electrophoresis system (Experion Bio-Rad laboratories) with StdSens chips and reagents. The integrity was analysed with the ratio between two large ribosomal RNA molecules (28S/18S rRNA). RNA samples with values of RQI >7.5 were considered acceptable for further analysis.

Genomic DNA contamination of total RNA samples was removed before cDNA synthesis; 4 µg total RNA was treated with Promega RQ1 Rnase-Free DNase (Promega Corporation) following the manufacturer's protocol. DNASE-treated total RNA was reverse transcribed in a 22 µl reaction using Superscript III reverse transcriptase (Invitrogen, UK) and oligo$(dT)_{20}$ primers.

(d) Quantitative real-time PCR analysis (qPCR) for gene expression

qPCR primers were design based on the nucleotide sequences per each gene obtained from cloning section. Each primer pair was designed using PrimerQuest Tool (Integrated DNA Technologies) according to the recommended criteria. Using the OligoAnalyser tool, qPCR primers were checked for hairpins, self- and cross-dimers. Primers were synthesized by Eurofins MWG Operon, Germany (Table 5.3). All real time qPCR reactions were performed in a LightCycler 96 (Roche, Switzerland); each 22 µl reaction contained: 12.5 µl of Precision Plus 2x qPCR Master mix with SYBR green (PrimerDesign, UK), 2 µl of each primer and 2 µl of template cDNA with the following PCR conditions: 1 cycle of 95°C for 5 min, 40 cycles of [90°C 10 s, 60°C 1 min], followed by 72°C for 45 sec.

Each gene was run in duplicate in 96-plates and NTCs ("no template controls") were included for every primer pair. Optimized primer concentrations per each gene are described in table 5.3. After each run a melt curve analysis was performed to determine if a single product (i.e. the specific gene) was amplified (Please refer to section Appendix A, Fig. 6.2). Standard curves of 10-fold serial dilutions were conducted to estimate the amplification efficiency and the linearity (r^2), according to the MIQE guidelines (Bustin et al., 2009, Appendix A, Fig. 6.3) with efficiency ranging between 90 – 105% and linearity greater than r^2=0.98 (Table 5.3). Gene expression was estimated with the relative quantification method*, gadph* and *β-Act* were used as references genes after assessing their stability as endogenous genes with qBase+ software (Biogazelle, UK). geNorm analysis (software qBase+) was used to calibrate and normalize the relative quantities (CNRQs) of the gene of interest expression (*hsp*70).

Table 5.3 Summary of real-time PCR primers and validated optimal reactions conditions. (F) Forward; (R) Reverse; (AC) Assay concentration; (AS) Amplicon size; (RF) Reaction efficiency; (Tm) melting temperatures ; (R^2) coefficient of determination.

Gene assay	Sequence	Tm (°C)	AC (nM)	AS (nt)	RF	Regression relation	R^2
*hsp*70	F: GCTTCTGGATCTTAGGGATAC R: GCCAGTGGAGAAGTCTATG	59	50 300	101	1.7	Cq = (-4.315 x log dilution) + 21.04	0.99
β-act	F: CTTCGTTCCTGGGTATGG R: GTACAGGTCTTTACGGATGT	59	50 300	92	1.8	Cq = (-3.6895 x log dilution) + 23.23	0.97
gapdh	F: GTGACGCTCAACAAAGAAG R: TCTGTGTAGCCCATGAATC	58	50 300	95	1.9	Cq = (-3.4070 x log dilution) + 22.55	0.93

5.2.5 Statistical analysis

Two-way repeated measures ANOVA were used to compare the impact of high temperature and pCO_2 on growth (shell length and wet weight) and routine metabolic rates at regular intervals (0, 15, 50, 95 and 135 days) followed by Holm-Sidak method *post hoc* analysis. Two-way ANOVA was used to analyse the ingestion rate and relative expression of *hsp*70 between control and stressed gastropods followed by Tukey *post hoc* analysis. Normality and homogeneity of variance were confirmed before respective analysis and statistical significance was identified at $p < 0.05$ (Please refer to Table 6.1, section Appendix A for raw data).

5.3 Results

The maternal effects study was ended after 10-months, when none of the stressed females (i.e. under climate change conditions) laid egg masses. However, six control females started laying capsules in April 2018; the female ranged in sizes from 30 to 40 mm and the egg masses sizes ranged from 20 to 30 capsules. Therefore, a follow on experiment was conducted on adults (male/female) to understand the physiological (i.e. metabolic, growth and ingestion rate) and molecular response (i.e. *hsp*70) to climate change conditions (+3 °C and ~ 900 µatm CO_2).

5.3.1 Seawater parameters

The seawater conditions for adult physiology experiment are summarised in Table 5.4. The mean (±SD) pCO_2 values were 499 ± 60 and 1013 ± 157 µatm for nominal control (14°C, ~400 µatm) and climate change conditions (17°C, ~900 µatm), respectively. The carbonate content decreased with increased pCO_2, however, in both experimental conditions, seawater was not undersaturated with respect to aragonite or calcite.

Table 5.4 Environmental seawater parameters for control and climate change conditions in specimens from **(a)** maternal effects and **(b)** physiology experiments. All parameters were measure each fortnight. (DIC) means dissolved inorganic carbon. 'Ωca' means the saturation state of calcite minerals, 'Ωar' means the saturation state of aragonite minerals. Values are given as mean ± standard deviation

Parameters	**(a) Maternal effects**		**(b) Physiology**	
	Control	High T°/ pCO_2	Control	High T°/ pCO_2
pH	8.114 ± 0.04	7.646 ± 0.2	8.022 ± 0.2	7.678 ± 0.1
Temperature (°C)	14.6 ± 0.3	17.3 ± 0.2	14.6 ± 0.4	17.2 ± 0.2
Salinity	32.85 ± 0.1	33.65 ± 0.21	33.2 ± 0.8	33.4 ± 0.5
pCO_2 (µatm)	475 ± 9	1072 ± 120	499 ± 60	1013 ± 157
Total Alkalinity (µmol $Kg^{-1)}$	2104 ± 164	2232 ± 290	2314 ± 71	2221 ± 52
DIC (µmol Kg^{-1})	1936 ± 150	2136 ± 238	2140 ± 79	2146 ± 71
$[CO_3^2]$ (µmol kg^{-1})	114 ± 17	65 ± 30	128 ± 14	76 ± 11
Ωca	1.7 ± 0.3	1.55 ± 0.7	3.1 ± 0.3	1.8 ± 0.2
Ωar	2.7 ± 0.4	1.23 ± 0.7	2.0 ± 0.2	1.2 ± 0.2

5.3.2 Physiological parameters in follow on experiment

No significant effects were found on shell length growth in gastropods exposed for 135 days to high T°/ pCO_2 ($p > 0.05$; two-way ANOVA repeated measures; Table 5.5; Table 6.1). Control and stressed gastropods grew at the same rate with values ranging between 0.003 and 0.008 mm on average per day (Fig. 5.1). Wet weight growth was significantly impacted by the exposure to high T°/ pCO_2 ($p < 0.05$; two-way ANOVA repeated measures; Table 5.5). A *post hoc* analysis (Holm-Sidak method) showed that stressed gastropods lost weight at the beginning of the experimental exposure (i.e. the first 15-days); however, thereafter gastropods in the modified conditions started to recover after 50 days of exposure, with similar weight increases to the control snails (Fig. 5.2). RMR was not significantly affected by the increase in temperature and pCO_2 during the experimental period ($p > 0.05$; two-way repeated measures ANOVA; Table 5.5). However, at the beginning of the exposure (i.e. 0-50 days), stressed gastropods showed a slight increase in their RMR (Fig. 5.3). After fifty days, stressed gastropods showed full acclimation with similar RMR to the control gastropods.

High T°/ pCO_2 did not affect the ingestion rate of stressed gastropods ($p > 0.05$; Two-way ANOVA; Table 5.6), however, the experimental period affected the food intake in control and stressed gastropods ($p < 0.05$). Tukey *post hoc* analysis showed that during the first fifty days, stressed and controls gastropods consumed 6.5 ± 4 mg of dry meat per day (i.e. the equivalent for wet mussel meat); however, after 50 days of exposure, stressed and control gastropods increased consumption to 10-15 mg of dry meat per day, respectively. There was a slight increase in food intake in stressed gastropods after fifty days; however, the differences were not significant.

High T°/ pCO_2 did not significantly affect the *hsp*70 expression during the experimental period ($p > 0.05$; Two-way ANOVA; Table 5.6; Fig. 5.5). However, the expression between control and stressed gastropods at the beginning of the experiment (0-15 days) was significantly different to the expression at 95 and 135 days ($p < 0.05$); stressed gastropods showed a slight decrease respect to control gastropods in the *hsp*70 expression (Fig. 5.3).

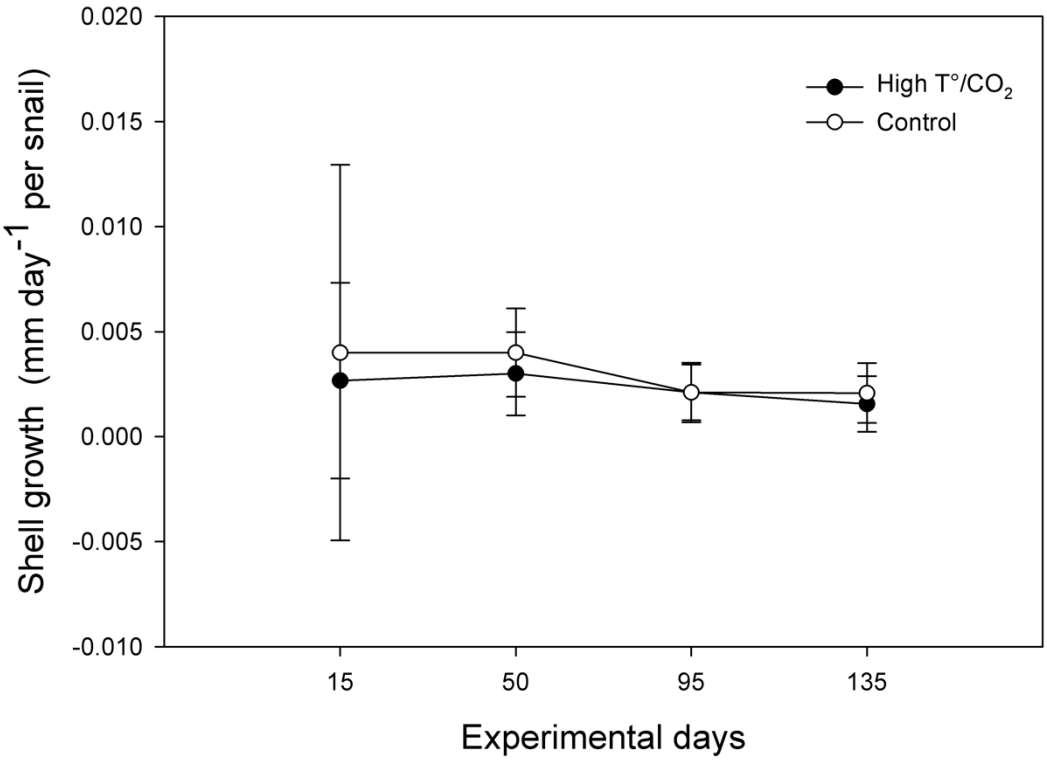

Figure 5.1 Shell growth (mm per day) in *Ocenebra erinaceus* gastropods exposed to control (14 °C - 400 ppm CO_2) and climate change conditions (17 °C - 900 µatm CO_2) for each of the four intervals of the experimental period. Values are given mean and standard deviation, n = 5 control, n= 10 stressed snails (same gastropods measured each time).

Table 5.5 Summary of two-way repeated measures ANOVA for growth (shell and wet weight) and routine metabolic rate in snails exposed to control and high T°/ pCO_2 within and between intervals (0, 15, 50, 95, 135 days). Factors: 'Group' (control or climate change conditions) and 'Exp. Days' (experimental days). Source of variation (source), degrees of freedom (df), variance ratios (F).

Variable	Source	df	F	p
Shell length rate	Group	1	0.212	0.65
	Exp. Day	3	1.116	0.35
	Interaction	3	0.130	0.94
Weight rate	Group	1	5.94	**0.03**
	Exp. Day	3	1.074	0.371
	Interaction	3	3.554	**0.02**
OCR	Group	1	0.382	0.547
	Exp. Day	4	1.385	0.252
	Interaction	4	1.229	0.310

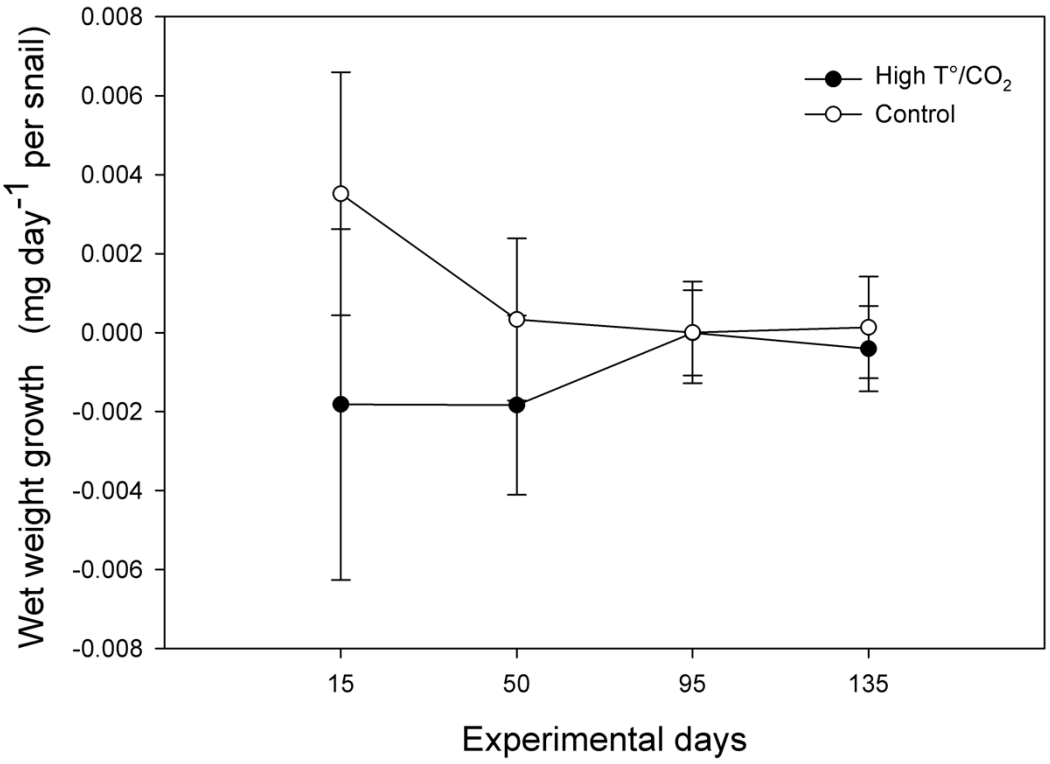

Figure 5.2 Wet weight growth estimated in days in *Ocenebra erinaceus* gastropods exposed to control (14 °C - 400 µatm CO_2) and climate change conditions (17 °C - 900 µatm CO_2) for each of the 4 intervals of the experimental period. Values are given mean and standard deviation, n = 5 control, n= 10 stressed gastropods (same gastropods measured each time).

Table 5.6 Summary of two-way ANOVA's for statistical comparison of ingestion rate and *hsp*70 gene expression of gastropods exposed to high T°/ pCO_2 within and between experimental period (0, 15, 50, 95, 135 days). Source of variation (source), degrees of freedom (df), variance ratios (F)

Variable	**Source**	**df**	**F**	**p**
Ingestion rate	Group	1	2.611	0.112
	Exp. Day	3	6.141	**0.001**
	Interaction	3	0.437	0.727
HSP70 expression	Group	2	0.111	0.741
	Exp. Day	1	8.400	**0.001**
	Interaction	2	0.243	0.786

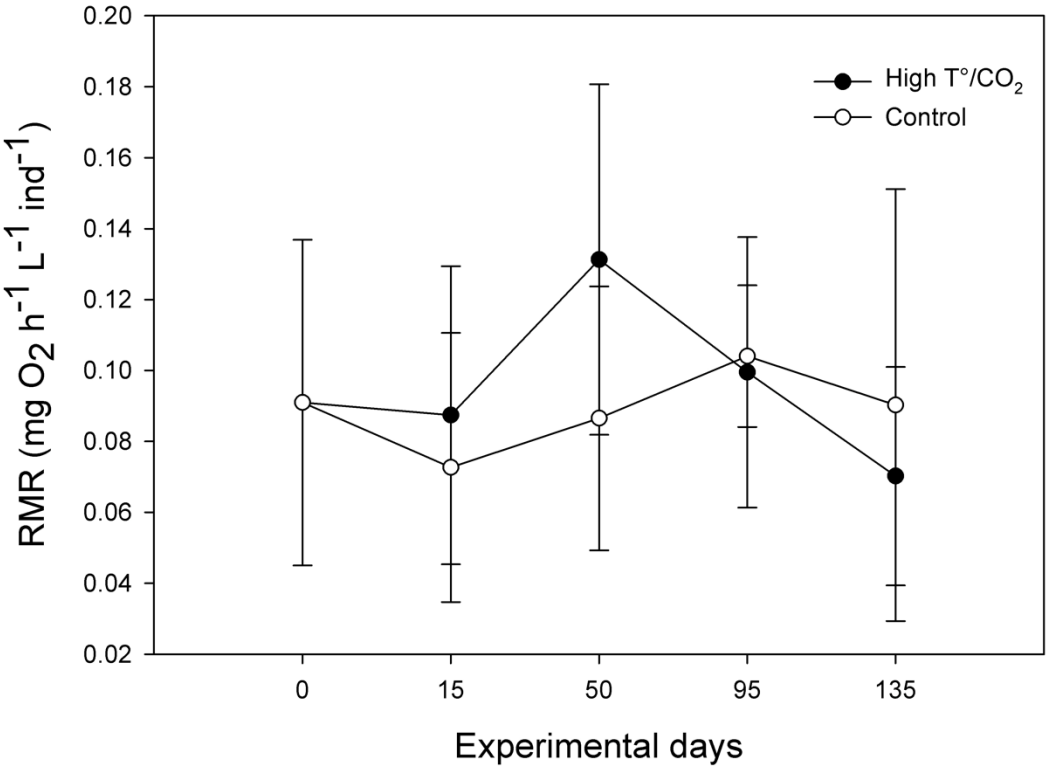

Figure 5.3 Routine metabolic rate (mg O_2 h^{-1} L^{-1}) in *Ocenebra erinaceus* gastropods exposed to control (14 °C - 400 µatm CO_2) and climate change conditions (17 °C - 900 µatm CO_2) for each of the 5 intervals of the experimental period. Values are given mean and standard deviation, n = 5 control and n = 10 stressed gastropods (same gastropods measured each time).

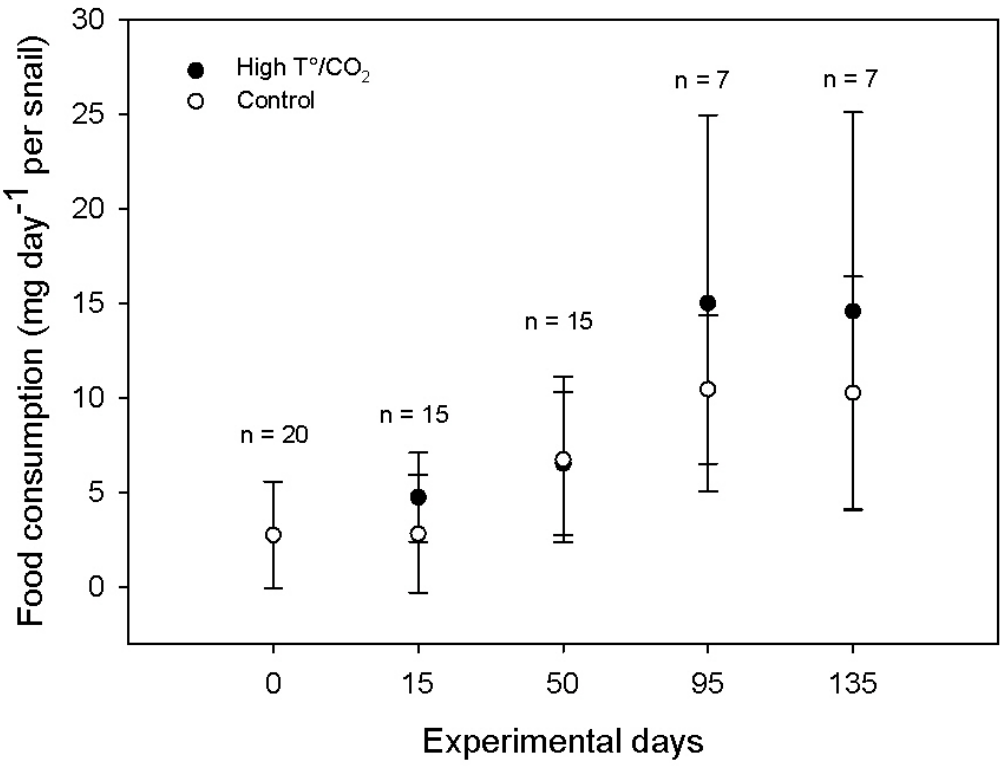

Figure 5.4 Ingestion rate (mg of dry meat mussel) in *Ocenebra erinaceus* gastropods exposed to control (14 °C - 400 µatm CO_2) and climate change conditions (17 °C - 900 µatm CO_2) for each of the 5 intervals of the experimental period. Values are given mean and standard deviation.

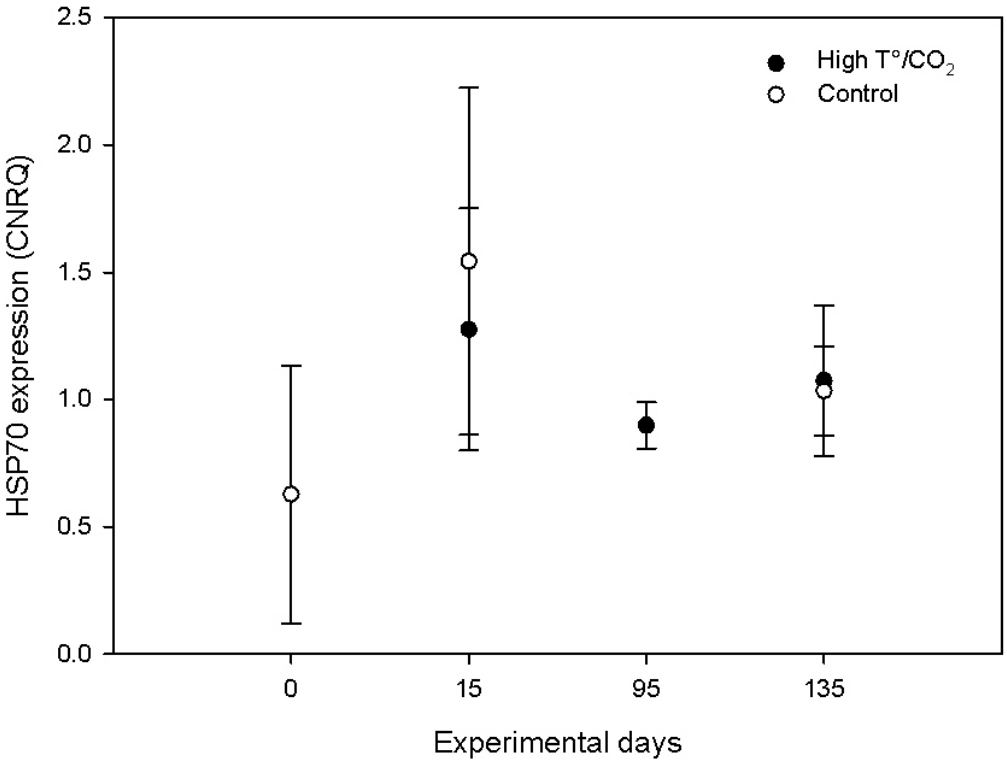

Figure 5.5 Normalized expression values of the *hsp*70 gene in gill tissues from gastropods exposed to control (14 °C - 400 µatm CO_2) and climate change conditions (17 °C - 900 µatm CO_2) for each of the 4 intervals of the experimental period At 95 days of exposure, the expression was only measured in gastropods exposed to high T/pCO2. *hsp*70 expression is normalized to multiple reference genes (*gapdh* and *β-act*), giving calibrated, normalized, relative quantities (CNRQ) with standard deviation, n = 8 snails control and n = 8 stressed gastropods per experimental days.

5.4 Discussion

The physiological impact of high temperature and pCO_2 on marine organisms are species-specific (Foo et al., 2012; Kroeker et al., 2013) and largely depends on the physiological plasticity that organisms have to acclimate to rapid environmental changes (e.g. climate change). The principal aim of this study was to determine whether females exposed to climate change experimental conditions (+3 °C above normal and 900 µatm CO_2) would improve the physiological response of offspring. However, after 10 months, females under high T°/pCO_2 did not lay eggs. Therefore, as maternal effects were not possible to establish, the physiological and molecular response was studied in adult gastropods over 135 days to understand the impact of high T°/pCO_2 exposure. The result showed that *O. erinaceus* adults from the Solent population were able to physiologically acclimate after 95 days of exposure if food resources are not limited. After long-term exposure, however negative impacts on reproduction were observed, which shows that this population could be at risk of extinction with future climate change.

The first fifty days of exposure to high T°/pCO_2 affected gastropods physiology, with a slight increase in routine metabolic rate and a decrease in growth rate (i.e. wet weight). Increases in metabolic rates have been reported in several marine organisms exposed to high pCO_2 with limited extracellular pH regulatory capacity, such as molluscs and echinoderms (Kroeker et al., 2013). For example, Uthicke et al., (2014) found, after ten weeks of exposure to high T°/pCO_2 (i.e. +3 °C and 963 µatm CO_2), an increase in metabolic demands and a decrease in growth rates in the Pacific sea urchin *Echinometra* sp. Similarly, adults of the temperate intertidal gastropod *Littorina littorea* exposed for 30 days to elevated temperature and pCO_2 concentration (+ 5 °C, 1000 µatm CO_2) showed lower shell growth rates, reduction on shell thickness and increase in ATP production (Melatunan et al., 2013). Therefore, the slight increase in metabolic rate observed during the first fifty days could be the result of the maintenance of internal homeostasis. As the increase in metabolism is energetically demanding, it is concluded that tissue catabolism provided the necessary energy for homeostasis.

After 95 days of exposure, *O. erinaceus* adults showed full acclimation to the experimental conditions. Gastropods exposed to high T°/pCO_2 recovered, growing at similar rates (i.e. shell and wet weight) and exhibiting similar metabolic rates compared to control gastropods. The ingestion rate increased in both experimental groups; however, a slight increase was observed in snails exposed to high T°/pCO_2. Thus, snails can acclimate at the expense of an increase in food intake. Indeed, a meta-analysis study conducted on the role of food supply in marine calcifiers (i.e. corals, molluscs and echinoderms) under acidified and warming conditions found that the negative effects of climate change on growth and calcification rates decreased when there are sufficient food

resources (Ramajo et al., 2016). For example, calcification rates in juveniles of the blue mussel *Mytilus edulis* were not affected by high pCO_2 (i.e. from 1000 to 3000 µatm CO_2) when food supply was abundant (Thomsen et al., 2013). Indeed, food deprivation in the marine copepods *Calanus* sp. exposed to climate change conditions (+2 °C and 1000 µatm CO_2) exacerbated the impact of temperature and pCO_2 on their physiology (Mayor et al., 2015). Therefore, our study showed that *O. erinaceus* likely increased feeding rates to compensate for the increase in respiration rates. Thus, so long as food resources remain abundant, *O. erinaceus* adult could acclimate to future climate conditions. However, if food is no longer abundant (e.g. climate change effects in the abundance of other calcifying organisms like mussels or oysters), gastropods are unlikely to compensate and will be at risk in future acidified and warm scenarios.

Most of the coastal and estuarine species are already experiencing natural variation in seawater chemistry with temperature, dissolved oxygen and pH values that are projected at the end of this century (Duarte et al., 2013; Hofmann et al., 2011). *Ocenebra erinaceus* from the Solent population inhabit a flooded river-valley estuarine system experiencing natural short-term fluctuations in pH and temperature (e.g. from river discharges; Shi, 2000). The full acclimation observed in stressed gastropods proved that *O. erinaceus* adults have developed physiological strategies to cope with fluctuating environments. The molecular level, *hsp*70 gene expression in gill tissue was not significantly different between control and snails exposed to high T°/pCO_2. *Hsp*70 expression has been widely used to study the acute effects of temperature and acidification on organisms because of their protective and restorative function against damage or denaturation (Whiteley and Mackenzie, 2016). For example, high temperatures increased the *hsp*70 expression after 5 days of exposure in gills of the blue mussel *Mytilus edulis* (Tedengren et al., 2000) or high pCO_2 (~ 1500 µatm CO_2) increased the expression after 72 hours in juveniles of the neogastropod *Concholepas concholepas* (Lardies et al., 2014). Few studies have attempted to study the combined effects of temperature and pCO_2 on *hsp*70 expression. For example, Liu et al., (2012) conducted an acute exposure for 96 hours to elevated temperature (+ 3 C) and elevated pCO2 (~1400 µatm CO_2) in the pearl *Oyster Pinctada fucata*. They found that warming and acidified seawater conditions activated the *hsp*70 expression. After 24 hours, the impacts of these two stressors were synergistic; pCO_2 exposure aggravated the sensitivity to temperature in *P. fucata*. After 96 hours, the expression decreased as a result of the decrease in the energy budget to meet the energy requirements for *hsp*70 gene expression (Liu et al., 2012).

Two hypotheses can be suggested to explain why there were no effects on *hsp*70 expression:

(1) the temperature and alkalinity stress (high T°/pCO_2) on gill cells of *O. erinaceus* was insufficient to activate the *hsp*70 shock response. *Ocenebra erinaceus* adults were very tolerant to high T°/pCO_2

in all the physiological variables studied in this chapter; therefore, to activate the heat shock response, it may be concluded that a more extreme stressful environment must be imposed. Alternately,

(2) gill cells of gastropods exposed to high T°/pCO_2 could have activated the heat shock response (i.e. *hsp*70) during the first hours after the shock stress; however, the expression in this study was measured after 15-days of exposure, thus, it was undetectable. Mollusc stress proteins response can persist for days or weeks after the first initial exposure. For example, high *hsp*70 expression was observed after 2-weeks following heat shock in gills of the temperate pacific oyster *Crassostrea gigas* or after 5 days in gills in *M. edulis* (Bradley et al., 1998; Clegg et al., 1998). However, in long-term exposure like in this study, it is possible that the buffer capacity was sufficient to cope with high temperature and pCO_2, and there was no need to induce the expression of heat shock proteins.

It has been suggested that HSP70 is a suitable stress biomarker because it is ubiquitous, highly conserved in almost all organisms and sensitive to stress (Tomanek and Somero 1999). However, there are some limitations to consider, for example: the time after initial stress exposure, duration of the stress, ontogenetic stage, number of environmental stressors that are producing stress, what form of HSP70 is expressed, amongst others (Morris et al., 2013). Therefore, the expression of HSP70 can be induced by a wide range of factors, which make them not suitable for estimating environmental stress in organisms.

After long-term female exposure (i.e. 10-months) to modified conditions, reproduction did not start; females did not lay eggs under climate change experimental conditions (+3 °C above normal and ~ 900 µatm CO_2). A possible explanation for this result could be that energetic demands were so high that females exposed to high T°/pCO_2 only invested energy in survival instead of reproduction. For example, histological examination of female gonads of the Pacific sea urchin *Echinometra* sp. exposed to similar experimental conditions used in this study, identified that females were unable to generate a new cohorts of gametes, as eggs were degenerate and/or reabsorbed (Uthicke et al., 2014). Although, sea urchin females were exposed only for 10 weeks, and possibly the effects observed might be the result of experimental stress, the results could explain the observation made after 10 months in *O. erinaceus* females. *Ocenebra erinaceus* is species that only exhibits one reproductive peak per year (Martel et al., 2004); thus, the cessation of reproduction in stressed females could directly impact the maintenance of the Solent population in the future. However, in order to determine the impact of future ocean conditions on the species, more comparative studies among geographic populations are needed.

5.5 Conclusion

This study showed that adults of *Ocenebra erinaceus* from the Solent, UK population are not able to fully compensate to the effects of high temperature and pCO_2 (RCP8.5 climate change sceneries) if food resources are limited. After 95 days exposure, all the physiological variables measured in this study were no longer affected, which means that stress snails can physiologically acclimate. However, full acclimation of *O. erinaceus* came with high energetic demands resulting in high ingestion rates. After ten months of exposure, reproduction ceased under climate change conditions demonstrating that adults invested energy toward survival and not reproduction. The implications of this study suggest the Solent population of *O. erinaceus* could decline, or even become locally extinct under future climate change. However, this study was focused in one population and further studies are required to explore the true potential for this extreme outcome more widely in this species.

Chapter 6 Synthesis and future perspectives

Global temperatures are likely to increase by 3°C above pre-industrial levels at the end of this century (IPPC 2013); this temperature rise will impact the physiology, ecology and distribution patterns of terrestrial and marine species, especially for ectotherms species were nearly all-physiological and behavioural traits are sensitive to temperature (Castañeda et al., 2004; Jones et al., 2009; Somero, 2005). One of the main predicted effects of global warming is the shift of biogeographic ranges and causing local extinctions (Thomas et al., 2004). Scientific interest around intraspecific differences across a species' distribution have grown during the last decades, as researchers have realized that it is not possible to make robust predictions regarding species range shifts and local extinction without considering the diversity of physiological adaptations among populations to local environmental conditions (Kawecki and Ebert, 2004; Kuo and Sanford, 2009; Sanford and Kelly, 2011).

The effects of increased temperatures could be more pronounced for species that undergo direct development, with restricted connectivity and low gene flow (Sanford and Kelly, 2011). Reduced gene flow increases the potential for individuals to evolve local adaptations to native environmental conditions. *Ocenebra erinacea* is a temperate shallow water mixed developing species with a short pelagic phase, that enclose their embryos inside of hard structures (i.e. capsules) for extended periods of up to 3 months at 15°C (Smith et al., 2015). As embryos are not able to seek preferred environments in which to develop, they will need to depend on their physiological adaptations to survive under global warming scenarios. Therefore, it is important to fully understand the physiological adaptations of encapsulated embryos, how their thermal tolerances vary among populations and how they will respond to elevated temperatures. The aim of this thesis was to identify:

a) the thermal tolerance response of early stages of *O. erinaceus* from two populations with different thermal histories (Chapter 2);

b) the influences of geographic origin on aerobic metabolic response during early stages of *O. erinaceus* (Chapter 3);

c) the possible effects of the physical characteristic of the capsule wall on the embryos' aerobic response (Chapter 3);

d) whether the intraspecific differences observed between these two populations are due to local adaptation (Chapter 4); and

e) whether maternal effects will improve the offspring performance under climate change scenarios (RCP8.5) for a thermally sensitive population (Chapter 5).

To achieve these aims, the thermal tolerance response was studied in two populations: one from the middle of their geographic distribution, the Solent, Southampton, UK experiencing colder waters during winter and spring, and the other one from the southern end of the geographic distribution: Arcachon, France (Fig. 2.2). The results of this thesis provide valuable new understanding about physiological adaptations to local thermal conditions during the early ontogenetic stages and the potential impact of future ocean conditions in adult physiology.

6.1 Thermal tolerance response during early stages (Chapter 2,3,4)

Ocenebra erinaceus exhibits intraspecific differences during early development in terms of its thermal tolerance and physiological response (Fig. 6.1). The warm water population (i.e. low-latitude) showed a wide, eurythermal tolerance range between 12 and 18 °C, with optimal temperatures between 12 and 16 °C (Fig. 2.5b; 2.6b; 2.7b). Temperatures outside of the natural thermal range (i.e. 10 and 20 °C) caused sub-lethal effects, increasing the abnormalities during the intracapsular development. On the contrary, the cold water population (i.e. high-latitude) showed a narrow thermal tolerance range between 12 and 16 °C and optimal temperatures between 14 and 16 °C. Temperatures outside of the natural thermal range (i.e. 10 and 18 °C) caused lethal effects, with high mortalities up to 70% (Fig. 2.5a; 2.6a; 2.7a). Interestingly, both populations live in temperatures near to their upper thermal tolerance limits; thus, an increase of one or two degrees from the maximal temperatures that embryos normally experience in the field increased mortality or abnormalities during early development (Fig. 2.5; 2.6). Similar findings have been observed in tropical and intertidal species, where species evolved greater tolerance to high temperatures at the expenses of acclimatisation capacity (Stillman and Somero, 1996; Stillman, 2003). Thus, *O. erinaceus* embryos are living at the expense of their acclimatisation capacity, warming will adversely affect this species during early development.

The thermal tolerance response of these two populations can be explained by physiological adaptations of embryos to local thermal conditions. Warm water embryos were able to adjust their aerobic metabolism when they were exposed to high temperatures (Chapter 3); as temperatures increased, the respiration rate increased (Fig. 3.4). However, to tolerate high temperatures, embryos incurred high energetic demands to increase the aerobic response, leading to embryonic abnormalities at 18 and 20 °C (Table 3.2). On the other hand, cold water embryos showed limited metabolic acclimation to high temperatures; as temperature increased, the respiration rate remained constant (Fig. 3.4). As increases in metabolism are energetically demanding (Verberk et

al., 2016), cold-adapted embryos showed no metabolic compensation at elevated temperatures, which is correlated with the high mortalities observed at elevated temperatures (Table 3.2).

The contrasting thermal tolerance ranges observed in this study between the warm and cold water population of *O. erinaceus* contradict the climate variability hypothesis (Gaitan-Espitia et al., 2016; Yu et al., 2018), because broader tolerance ranges were observed in the warm water, low-latitude population. Therefore, this thesis supports the idea that thermal tolerance variation among populations might be a result of physiological adaptations to local thermal conditions (Gleason and Burton, 2013; Jansen et al., 2007; Kelly et al., 2012; Kuo and Sanford., 2009). It was shown that each population was locally adapted to their natal environmental conditions, exhibiting similar maternal investment (i.e. Fecundity, Fig. 4.3). However, adaptation to native conditions had cost, leading to trade-offs among reproductive traits such as capsule size and the number of eggs in response to high temperatures and low oxygen saturation (Fig. 4.6). One explanation could be that warmer environments appear to select for larger females depositing larger capsules to improve oxygen availability to a greater quantity of eggs per capsule. On the contrary, colder environments could be selecting for smaller female sizes laying smaller capsules.

According to the "local and foreign" criteria (Kawecki and Ebert 2004), this thesis showed that transplanted females of *O. erinaceus* (i.e. from warm to cold location) had lower fitness in the foreign environment than in their native habitat (Fig. 4.10). Transplanted females reduced their maternal investment (i.e. low fecundity, smaller capsules and eggs), which suggest that females were maladapted to live under the foreign environment (Chapter 4). In fact, offspring from transplanted females showed a poor performance under the new environment (i.e. high mortality, presence of soft capsules and small juvenile size; Fig. 4.8; 4.9). Therefore, these results support the idea that the intraspecific differences observed in thermal tolerance ranges of early stages in *O. erinaceus* are due to local adaptation to the regional thermal conditions.

Setting the development of encapsulated embryos under future warming scenarios in relation to other types of development (e.g. planktotrophic), encapsulation seems to be a maladaptive strategy. In encapsulated species, oxygen availability inside capsules decreases as embryonic development progresses due to oxygen embryonic demands (Fig. 3.2; Cancino et al., 2011). Segura et al. (2010) demonstrated that to increase the oxygen availability inside capsules, the capsule wall became thinner as development progresses to increased permeability. However, even though the permeability increased, it is insufficient to fulfil the embryonic oxygen demands at the end of the intracapsular development (Segura et al., 2010). In Chapter 3, the oxygen content within capsules decreased as development progressed; however, at high temperatures, metabolic demands increased causing oxygen depletion inside capsules limiting the embryonic development. When

embryos were removed from their capsule chambers, they consumed 100% more oxygen than encapsulated embryos (Chapter 3). Thus, encapsulated embryos are vulnerable to temperatures that extend beyond their normal thermal range due of the physical characteristic of the capsule wall, which makes this type of development at risk under warming scenarios.

Therefore, the impact of global warming in *O. erinaceus* will depend on the geographic origin and the physiological adaptations of embryos to local environmental conditions. In this study, the warm water population was physiologically more adapted to warmer conditions, which suggests that the differences appear to be genetically based. However, genetic analysis (e.g. population divergence estimate; Whitock, 2015) are necessary to elucidate if this intraspecific differences have a genetic base. In response to global warming, local extinctions could happen at northern locations of the geographic distribution of *O. erinaceus*, as embryos showed low metabolic adjustment to temperatures outside of the normal tolerance range. However, warm-adapted populations could expand their distribution range, shifting the distribution poleward and filling the niche space left empty by local extinction (Thomas et al., 2004).

6.2 Impacts of future ocean conditions on maternal effects and offspring performance (Chapter 5)

Although the main aim of Chapter 5 was to measure maternal effects and the potential impact on offspring performance; after 10-months, females from the Solent, UK, under Future Ocean conditions did not lay capsules. Thus, it was not possible to assess trans-generational effects. The second experiment was focused on understanding how this thermal sensitive population (i.e. Solent adult population) will respond to future ocean conditions. The physiological variables measured in this chapter were: growth, oxygen consumption and ingestion rate and *hsp*70 expression. The results showed that the physiological impact of high temperature and pCO_2 is species-specific and largely depends on the physiological plasticity that organisms have to acclimate to rapid environmental changes. Adults from the cold water population were able to fully compensate after 135 days for the effects of high temperature and pCO_2 (8.5 RP5 climate change sceneries) if food resources are not limited. During the first 15-days, snails increased their metabolic rates and decreased growth rates under future climate conditions. After 95 days of exposure, all physiological variables measured in this study were no longer affected, which means that stressed snails can physiologically acclimate. However, the full acclimation of *O. erinaceus* came with high energetic demands resulting in high ingestion rates. Potentially, so long as food resources remain abundant, snails can acclimate to future climate conditions. However, if food is no longer abundant (e.g. climate change effects in the abundance of other calcifying organisms like mussels or oysters), then future conditions are likely to become challenging for gastropods.

Despite the fact that adult have the potential to acclimate to future ocean conditions, not all-physiological process can acclimate. After ten months of exposure, reproduction ceased under future conditions, demonstrating that some physiological process such as metabolic response or growth rate can acclimate but other do not; which is reflected in the cessation of reproductive investment. In future scenarios, as it was observed during early stages (Chapter 2-3), the cold water population could decline or even become locally extinct. However, this study was focused on one population and further studies are required to explore the true potential for this extreme outcome more widely in this species.

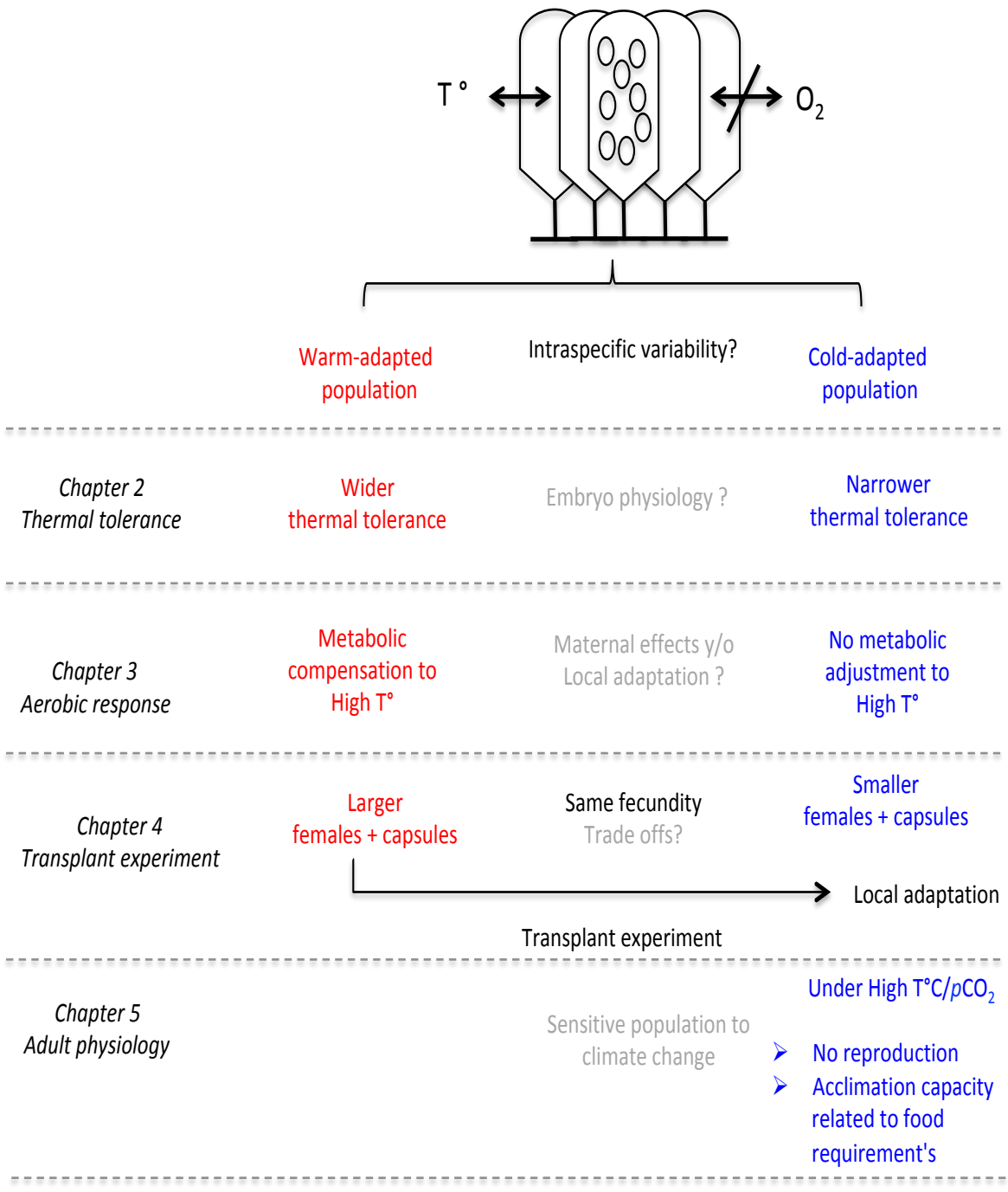

Figure 6.1 Schematic illustrations summarizing the results obtained in this thesis. Intraspecific differences were studied in two populations with different thermal history during the intracapsular development of *Ocenebra erinaceus*. Thermal tolerance response varied between populations as a result of physiological adaptations to local conditions. Populations were locally adapted to environmental conditions, leading to trade-offs in their reproductive traits. Finally, the thermo-sensitive adult population (i.e. cold-adapted) could be at risk in future ocean conditions if food resources are not sufficient to meet the energy requirements.

6.3 Current limitations

6.3.1 Thermal tolerance response

This thesis was focused on understanding the intraspecific differences during early stages between two populations with different thermal histories. The potential influence of maternal effects or non-genetic developmental plasticity could not be eliminated from the results. Female thermal history before spawning can induce phenotypic carry-over effects on the offspring and, therefore, can influence the thermal tolerance response of embryos (Donelson et al., 2011; Munday, 2014; Shama et al., 2014; Sheader, 1996). In this study, it is possible that the colder temperatures experienced by cold-adapted females influenced the thermal tolerance of embryos, resulting in poor acclimation to temperatures outside of their normal thermal range. In future studies it would be interesting considering maintaining individuals from warm and cold-adapted populations over more generations under common garden conditions, to reduce effects of maternal environmental history.

6.3.2 Local adaptation

In this study, local adaptation was suggested as a possible explanation for the intraspecific differences observed in the thermal tolerance response during early development (Chapter 4). However, from the experimental design, phenotypic plasticity and genetic differentiation cannot be distinguished. Although females were maintained under Solent climatic conditions for 9 months to reduce effects of environmental history, female origin could have, nonetheless, influenced the response of early stages. Therefore, genetic analyses (e.g. Genome-scan techniques; Whitlock, 2015) are necessary to elucidate whether there is a genetic differentiation between these two populations. Moreover, only the warm-adapted population was transferred to the climatic conditions of the cold-adapted populations. Thus, to understand the adaptive cost to live under each native location is necessary to do a fully reciprocal transplant experiment. However, this design has the risk to introduce nonlocal genotypes into study populations (Sanford and Kelly 2011). For this reason, it was only measured in Solent climatic conditions, since the NOCS has the facilities to maintain the snails under controlled conditions.

6.4 Future research

Climate change has already resulted in shifting the distribution ranges of many species (Parmesan and Yohe 2003, Thomas et al., 2004); however, many of the climate change predictions did not consider that populations within a single species are also adapting to environmental changes at different rates (Donelson et al., 2019, Kelly et al., 2012). This thesis marks an important step in our understanding about intraspecific differences across a species' distribution range and how thermal tolerance during the early stages can be influenced by physiological adaptation. However, to have a better understanding of how global warming will shift the distribution of marine species, especially those that are more at risk (i.e. encapsulated species), more studies focusing on intraspecific differences across species' distribution range are needed.

For example:

(1) it is necessary to identify whether the differences among populations are due to phenotypic plasticity or local adaptation. If the differences are plastic, each population has the potential to achieve the full range of tolerances; however, if differences are due to local adaptations, as observed in this study (Chapter 4), poleward populations may not able to achieve higher thermal tolerance without the gene flow from warm-adapted populations (Sanford and Kelly 2009);

(2) the thermal tolerance response varies according to the ontogenetic stages (Truebano et al., 2018), therefore more studies are needed that consider thermal sensitivities at different ontogenetic stages, e.g. embryo, juvenile and adults;

(3) trans-generational studies can help to understand whether females experiencing different thermal conditions can ameliorate the impact of global warming on offspring (Donelson et al., 2011). In this study, embryos live at temperatures very close to their respective upper thermal limits; however, if mothers experience warmer temperatures, they could improve the offspring phenotype and increase their performance. And finally,

(4) future studies should consider studying the physiological adaptations of populations from 'the trailing edge' (i.e. warmest area of species range), 'the core' and 'leading edge' (i.e. coolest area, Donelson et al., 2019) of species range. This will allow us to make more powerful predictions about thermal ranges shifts in marine organisms.

6.5 Final conclusions

Global warming is already altering the physiology and shifting the distribution of marine ectotherms. As marine ectotherms are unable to regulate body temperatures independently from the marine environment, global warming poses a great threat for marine ectotherms. It has been reported that global warming will affect the reproduction of many marine species; which, over long time scale, will affect the ecosystem functioning and human food resources. This is particularly evident in neogastropod species with encapsulation as a mode of development. Neogastropoda is one of the most abundant group of the Caenogastropoda clade, having an important role in supporting coastal fisheries. From the intertidal to the deep ocean, neogastropods encapsulate their embryos for a period or during the whole early development. In the intertidal, encapsulation can confer protection during the intracapsular development; however, increases in temperatures lead to oxygen depletion inside capsules producing high mortality and abnormalities. This thesis has shown that during early stages, *O. erinaceus* exhibit population-scale intraspecific differences in its optimal temperatures and thermal limits. These differences arise because embryos of *O. erinaceus* are physiologically adapted to their native environmental conditions. However, with the projected increase in seawater temperatures, local extinction will be more expected in populations located at northern locations, since embryos are not adapted to experience warmer temperatures. This may lead to the expansion of the distribution range of southern populations to fill the niche space left empty by northern populations. Therefore, this work has proven that temperate encapsulated shallow water neogastropods will be at risk of local extinctions in warming scenarios.

Appendix A

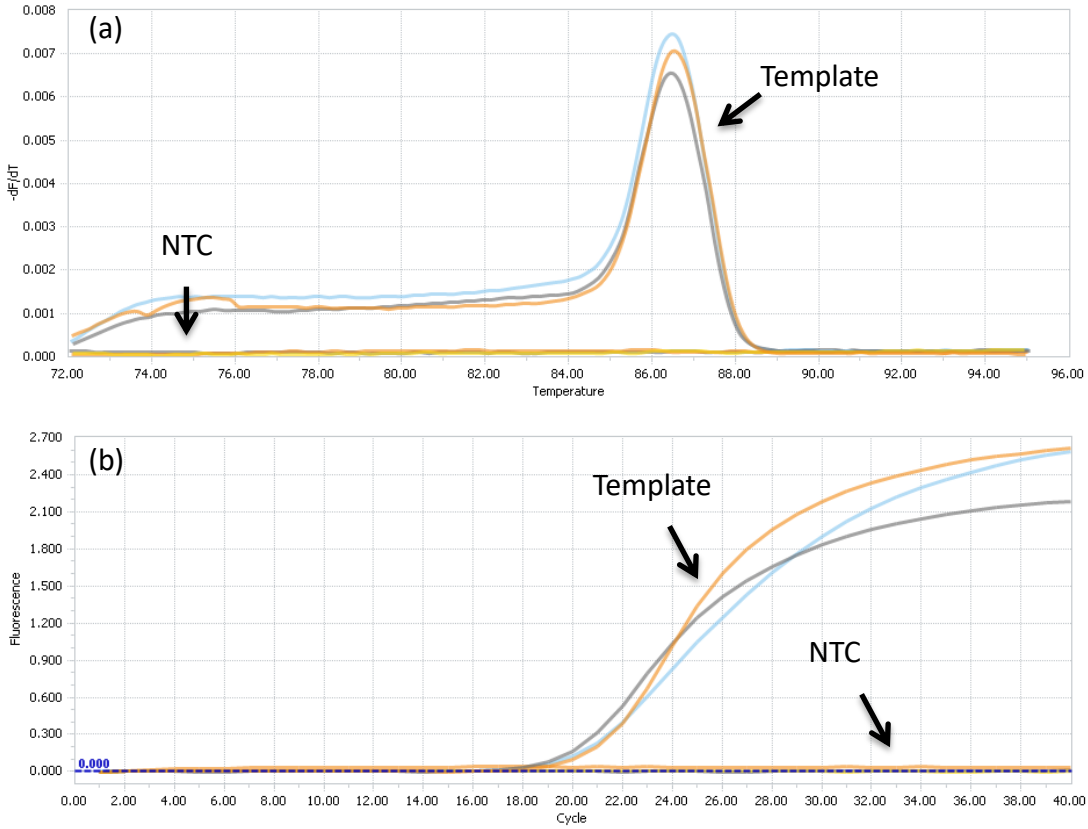

Figure 6.2 Example of melt curve **(a)** and amplification plot **(b)** for *hsp70* gene in *Ocenebra erinaceus*, showing a single target. 'NTC' is the no template control.

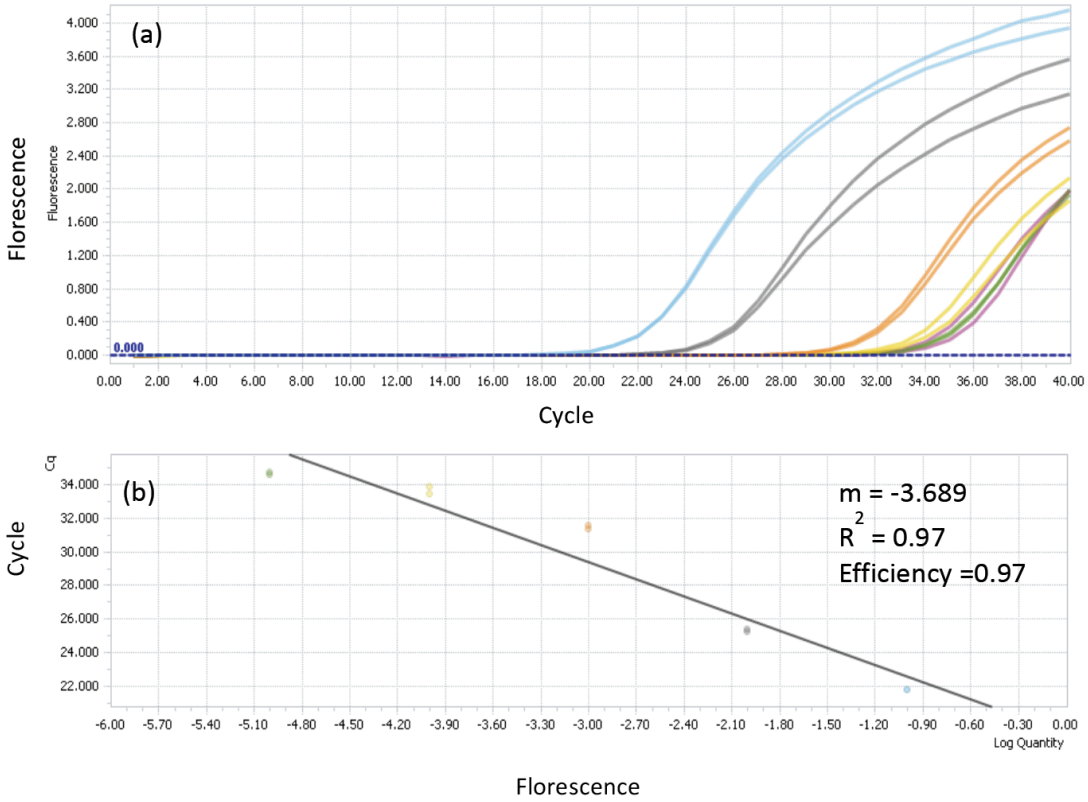

Figure 6.3 Example of **(a)** standard curve of 10-fold serial dilution in semi-logarithmic scale in gadph gene in Ocenebra erinaceus to estimate the Cq values. **(b)** Linear regression between Cq values in **(a)** to estimate amplification efficiency and linearity (R2).

Table 6.1 Shell and wet weight growth, routine metabolic rate, ingestion rate and HSP70 expression raw data in *Ocenebra erinaceus* gastropods exposed to control exposed to control (14 °C - 400 µatm CO_2) and climate change conditions (17 °C - 900 µatm CO_2) for each of the 4 intervals of the experimental period. Values are given mean and standard deviation.

Exp. Days	Shell growth (mm day^{-1})		Wet weight growth (mg day^{-1})	
	High T°/CO_2	Control	High T°/CO_2	Control
15	0.0027 ± 0.0047	0.0033 ± 0.0082	-0.0018 ± 0.004	0.0035 ± 0.003
50	0.0030 ± 0.0033	0.0033 ± 0.0082	-0.0018 ± 0.001	0.0003 ± 0.002
95	0.0021 ± 0.0020	0.0018 ± 0.0021	0.0000 ± 0.002	0.0000 ± 0.002
135	0.0016 ± 0.0013	0.0019 ± 0.0014	-0.0004 ± 0.001	0.0001 ± 0.001
Exp. Days	**Routine metabolic rate (mm O_2 h^{-1} L^{-1} ind^{-1})**		**Ingestion rate (mg day^{-1})**	
	High T°/CO_2	Control	High T°/CO_2	Control
0	0.0909 ± 0.0459	0.0909 ± 0.0459	2.7421 ± 2.8325	2.7421 ± 2.8325
15	0.0874 ± 0.0420	0.0727 ± 0.0379	4.7336 ± 2.3652	2.8052 ± 3.1186
50	0.1313 ± 0.0494	0.0865 ± 0.0372	6.5164 ± 3.7968	6.7237 ± 4.3806
95	0.0995 ± 0.0382	0.1040 ± 0.0200	15.0125 ± 9.9469	10.4456 ± 3.9356
135	0.0702 ± 0.0308	0.0902 ± 0.0609	14.5790 ± 10.527	10.2623 ± 6.1632
Exp. Days	**HSP70 expression (CNRQ)**			
	High T°/CO_2	Control		
15	0.6276 ± 0.5060	0.6276 ± 0.5060		
50	1.2761 ± 0.4765	1.5428 ± 0.6811		
95	0.8983 ± 0.0900			
135	1.0742 ± 0.2952	1.0338 ± 0.1742		

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