Bivalves in the face of ocean acidification

Doctoral thesis

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Cover illustration by Nanna Hartmann: blue mussel (*Mytilus edulis*).

To my dad

PREFACE

The objective of this thesis was to study responses and adaptation potential of bivalve molluscs to global environmental changes, more specifically ocean acidification. Results were gathered throughout research conducted between 2014 and 2018. The majority of the research was conducted at the Department of Biological and Environmental Sciences of the University of Gothenburg (Sweden) under the supervision of Assistant Professor Sam Dupont (University of Gothenburg) and co-supervision of Dr. Pierre De Wit (University of Gothenburg). Part of the work was conducted at the Department of Fisheries and Wildlife and Coastal Oregon Marine Experiment Station (Oregon State University, Newport, Oregon, USA) within Professor Chris Langdon's group. The PhD project was part of the EU Marie Curie Initial Training Network CACHE (Calcium in a Changing Environment, EU FP7-PEOPLE-2013-ITN, Grant: 605051). The overall aim of CACHE was to understand calcium regulation and shell production in commercially important bivalve species.

ABSTRACT

Anthropogenic CO_2 emissions are leading to a gradual decrease in ocean pH and changes in seawater carbonate chemistry, a process known as ocean acidification (OA). Such changes in oceanic environmental conditions will have negative consequences for marine life and organisms producing calcium carbonate ($CaCO_3$) structures are amongst the most vulnerable due to the additional costs associated with calcification and maintenance of calcified structures under more acidic conditions. As calcifying animals of particular commercial and ecological relevance, bivalve molluscs have frequently been the object of OA research. In this thesis, responses to changes in seawater acidity in commercially important bivalve species were investigated with the aim of understanding their adaptation potential to OA. As the main focus was on blue mussels, the first part of the thesis provided an introduction to blue mussel-species complex in Europe which is characterized by the three species *Mytilus edulis*, *M. galloprovincialis* and *M. trossulus*. An analysis of potential consequences of interspecies hybridization for the aquaculture industry, especially in the context of changing environmental conditions, was provided. Possible positive and negative effects of hybridization were identified, the complexity of the blue mussel-species complex was highlighted and the implications of hybridization for adaptation were discussed. In the following section of the thesis, responses of *Mytilus edulis* larvae from a Swedish west coast population to elevated seawater acidity were investigated. By exposing larvae to a wide range of seawater acidity, the physiological tolerance threshold for normal shell development was identified and corresponded to pH_T (pH on the total scale) ~ 7.8 which approximates the lower extremes of the local pH range naturally experienced by the larvae. This suggests that these mussels are well adapted to their local environment characterized by considerable fluctuations in seawater pH. Additionally, this result allowed selecting an appropriate pH level (pH_T ~ 7.5, beyond the present range of natural variability), representing a realistic OA scenario for the investigated population and driving enough biological response to further investigate adaptation potential. This was achieved by measuring genetic variance and heritability of larval fitness-related traits (i.e. size and malformation of shell) through a cross-breeding experimental design and quantitative genetic techniques. Results showed high trait heritability under elevated seawater acidity, an indication of the potential of adapting to OA. Finally, in order to understand what functions and genes may be targeted by natural selection in the context of OA, genes involved in the initial phases of shell formation in Pacific oyster (*Crassostrea gigas*) larvae were identified. With a genome available, the Pacific oyster was an ideal candidate for this task. The identified genes were attributed to four categories (metabolic genes, transmembrane proteins, shell matrix proteins and protease inhibitors) and are candidates for genes under selection in the context of an acidifying ocean. Altogether the results of this thesis contribute to a better understanding of bivalve adaptation potential to global changes and provide critical information for future work (e.g. investigation of allele-specific associated tolerance to changes in environmental parameters).

Keywords: ocean acidification, CO_2, adaptation, larvae, *Mytilus edulis*, *Crassostrea gigas*

POPULÄRVETENSKAPLIG SAMMANFATTNING

Koldioxidutsläpp orsakar en minskning av havets pH och förändringar i havsvattenkemi, en process som kallas för havsförsurning. Detta kommer att ha negativa effekter speciellt för organismer som producerar kalciumkarbonat ($CaCO_3$). I denna avhandling har jag undersökt effekter av havsförsurning på olika musselarter och deras anpassningspotential. I första sektionen ligger fokus på blåmussel-artkomplexet i Europa och konsekvenserna av hybridisering mellan arter. Sedan undersökte jag effekterna av havsförsurning på blåmussellarver (*Mytilus edulis*) från den Svenska västkusten. Larver utsattes för ett brett pH spektrum för att identifiera den fysiologiska tolerans-tröskeln för normal skalutveckling. Denna tröskel var pH_T (pH på total skala) ~ 7.8 vilket motsvarar det lägsta pH larverna utsätts för i området och visar att musslorna är anpassade till sin lokala miljö. Detta var första steget i undersökningen av musslors anpassningspotential till havsförsurning och följdes av en studie där ärvbarhet av fitness-relaterade egenskaper hos mussellarver undersöktes med kvantitativa genetiska tekniker. Resultaten visade höga ärvbarhets-nivåer i lågt pH, en indikation på att musslorna har en potential att anpassa sig till havsförsurning. Slutligen identifierades gener som möjligtvis är involverade i de initiala faserna av skaldeponering hos larver av Japanska jätteostron (*Crassostrea gigas*). Japanska jätteostron har en tillgänglig genom-sekvens och var därför en idealisk kandidat för denna uppgift. Generna som identifierades (till exempel transmembranproteiner, skalmatrisproteiner och proteashämmare) kommer sannolikt att utsättas för selektion i samband med surare hav i framtiden. I allmänhet så bidrar resultaten från denna avhandling till en bättre förståelse av anpassningspotentialen till globala förändringar hos musslor och ger viktig information för framtida studier.

LIST OF PAPERS

This thesis is based on the following papers:

Paper 1: Michalek, K., Ventura, A. & Sanders, T., 2016. *Mytilus* hybridisation and impact on aquaculture: a minireview. *Marine genomics* 27, pp.3-7.

Paper 2: Ventura, A., Schulz, S. & Dupont, S., 2016. Maintained larval growth in mussel larvae exposed to acidified under-saturated seawater. *Scientific Reports* 6, 23728.

Paper 3: Ventura, A., Wegner, K. M., Lazareff, H. & Dupont, S., (manuscript). Assessing adaptation potential to ocean acidification in blue mussels, *Mytilus edulis*, from the Swedish west coast.

Paper 4: De Wit, P., Durland, E., Ventura, A. & Langdon, C. J., 2018. Gene expression correlated with delay in shell formation in larval Pacific oysters (*Crassostrea gigas*) exposed to experimental ocean acidification provides insights into shell formation mechanisms. *BMC Genomics* 19:160.

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BACKGROUND & AIMS

Global changes are affecting the natural environment and are mainly driven by human induced rise in the concentration of atmospheric CO_2. One of the consequences for the oceans is ocean acidification (OA), characterized by changes in seawater chemistry and a drop in pH. This can have a wide range of negative effects on marine life, from a reduction in growth rate to changes in behaviour. OA poses challenges for biocalcification (i.e. the biological production of calcium carbonate structures such as shells) representing a particularly big threat for calcifying organisms including bivalves, marine molluscs of particular ecological and commercial importance such as mussels and oysters. However, living organisms are capable of coping with environmental changes through adaptation, a process based on natural selection which improves population fitness in the new environment.

On this basis, the main goal of the present thesis is to understand whether commercially important bivalve species are capable of adapting to ocean acidification. Because the main focus is on Swedish blue mussels (*Mytilus edulis*), the stage is set by discussing the European blue mussel-species complex (i.e. *Mytilus edulis*, *M. galloprovincialis* and *M. trossulus*) and interspecies hybridization (i.e. the cross between different species). Particular attention is given to potential implications of hybridization for mussel farming, especially in the context of global changes, and the possible effects of hybridization on adaptation. The question of whether bivalves can adapt to OA is then tackled by first investigating responses of *M. edulis* larvae, the most sensitive mussel life stages, in order to determine their physiological tolerance threshold. This is followed by estimations of the bivalves' potential for adaptation by measuring the heritability (the amount of variance in an observable trait explained by genetic variance) of specific fitness-related traits. Furthermore, by analysing the expression patterns of genes likely associated with initial shell formation in larvae of the Pacific oyster (*Crassostrea gigas*), it was possible to identify which genes may be targeted by natural selection in the context of OA. In this case, *C. gigas* was chosen over *M. edulis* as it is a model species with an available genome making it an ideal species for this type of mechanistic investigations.

More specifically, the sections and aims of the thesis are:

I. <u>The blue mussel-species complex and hybridization</u>: the aim is to investigate blue mussel distribution patterns in Europe and whether hybridization can have

implications for mussel farming. Moreover, the potential for global changes to affect hybridization patterns, further exacerbating potential impacts on the aquaculture industry and implications of hybridization for adaptation, are discussed (**section I**).

II. Local environment and tolerance threshold: the aim is to find the physiological tolerance threshold to OA of blue mussel (*Mytilus edulis*) larvae from the Swedish west coast. This would reveal the limits of OA tolerance of the local population opening the question of OA adaptation potential (**section II**).

III. Heritability and adaptation potential: the aim is to measure genetic variance and heritability for *M. edulis* larval traits (i.e. larval shell length and malformations) affected by higher seawater acidity and associated with fitness. This would indicate potential for adaptation to OA (**section III**).

IV. Targets of selection: the aim is to identify genes linked to initial shell formation in Pacific oyster (*C. gigas*) larvae and which may be under selection in the context of OA. This is tackled by measuring differences in gene expression between larvae depositing their initial shell and non-shelled larvae of the same age but reared under more acidic conditions, a process which slows down shell deposition. Differentially expressed genes would pinpoint mechanisms and functions of initial larval calcification and indicate what genes/functions may be "targeted" by natural selection in the context of OA (**section IV**).

INTRODUCTION

Ocean acidification

Oceans absorb approximately one third of the atmospheric CO_2 (Sabine 2004) which is associated with a decrease in oceanic pH, a phenomenon known as ocean acidification (OA). Since pre-industrial times, a decrease in global surface ocean pH (currently ~ 8.1) by ~ 0.1 pH units has been calculated (Caldeira & Wickett 2003) and a further decrease corresponding to approximately 0.3-0.5 pH units by the end of the century is projected (Caldeira & Wickett 2003). As a result, oceans could experience the greatest acidification event of the last ~ 300 million years of our planet's history (Caldeira & Wickett 2003). In addition to a decrease in seawater pH, OA is characterized by complex changes in seawater carbonate chemistry (Fig. 1). When atmospheric CO_2 dissolves into the oceans it reacts with water molecules forming carbonic acid (H_2CO_3) which dissociates leading to the formation of carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-) ions, the increase of hydrogen ion (H^+) concentration and ultimately causing a decrease in seawater pH (see Riebesell et al. 2010). As more CO_2 dissolves and its partial pressure (pCO_2) rises, the chemical equilibrium is affected by the production of more H^+ and shifts towards formation of HCO_3^- at the expense of CO_3^{2-} concentration (see Riebesell et al. 2010). Formation/dissolution of calcium carbonate ($CaCO_3$) is driven by its saturation state (Ω) described by the following equation:

$$\Omega = \frac{[Ca^{2+}][CO_3^{2-}]}{K'_{sp}}$$

where $[Ca^{2+}]$ and $[CO_3^{2-}]$ are concentrations of calcium and carbonate ions whilst K'_{sp} is known as the stoichiometric solubility product and depends on factors such as salinity, pressure, temperature and mineral phase (see Riebesell et al. 2010). Calcium carbonate in the ocean exists under different isoforms such as calcite and aragonite and their stability is governed by their saturation states (Ω_{cal} and Ω_{arag}, respectively) with calcite being considerably less soluble than aragonite in seawater (Morse et al. 1980; Mucci 1983). From a chemical perspective and in the absence of protective coatings, calcium carbonate undersaturation ($\Omega < 1$) leads to dissolution of $CaCO_3$ biological structures (see Doney et al. 2009).

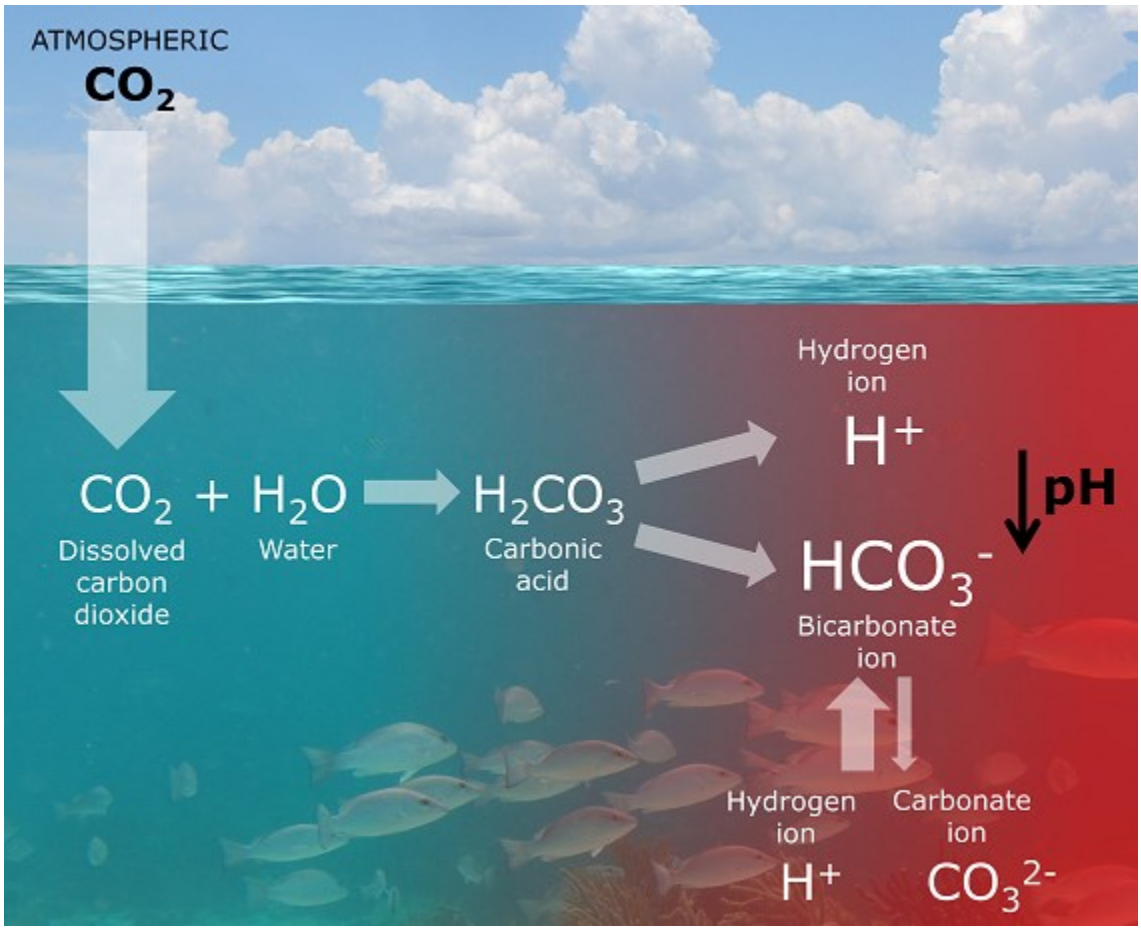

Fig. 1 Chemical reactions linked to CO_2 dissolution into the ocean. The different sizes of arrows in the bottom right corner indicate the shift of the chemical equilibrium as more CO_2 dissolves into seawater. Ultimately the process leads to an increase in HCO_3^- concentration at the expense of CO_3^{2-} concentration and a rise in H^+ concentration which causes a drop in pH (indicated by the red tint on the right side of the figure).

Biological impacts of OA

OA represents a serious threat to marine life (see Doney et al. 2009) and can affect organisms through different modes of action. Rising CO_2 concentration affects organisms' acid-base balance (Pörtner et al. 2004) which for example can lead to a drop in extracellular pH and metabolic rate as observed in the mussel *Mytilus galloprovicialis* exposed to seawater acidification (pH 7.3) (Michaelidis et al. 2005). Elevated seawater acidity may affect metabolic processes such as oxygen consumption and ammonia excretion (e.g. Liu & He 2012) and ultimately have impacts on higher order processes and functions such as behaviour (e.g. Simpson et al. 2011) and feeding. For example, under elevated *p*CO2, feeding of larvae of the gastropod *Concholepas concholepas* decreased and changes in feeding behaviour were also observed (Vargas et al. 2013). In *M. galloprovincialis* larvae, elevated pCO_2 had a negative impact on the feeding rates (Gray et al. 2017).

However, the magnitude of biological responses to OA varies across taxonomic groups (Kroeker et al. 2010; Wittmann & Pörtner 2013). For some organisms, seawater acidification may even have positive effects as shown for larval stages and juveniles of the sea star *Crossaster papposus* which, when reared under acidified conditions (pH ~ 7.7), had higher growth rates with no negative effects on survival and skeletogenesis (Dupont et al. 2010). Similar results were observed for colonies of the bryozoan *Electra pilosa* which showed higher growth rates under elevated pCO_2 (~ 1200 μatm) as compared to ambient conditions (~ 460 μatm) (Saderne & Wahl 2013). Larvae of the orange clownfish *Amphiprion percula* also had higher growth rates under elevated CO_2 concentrations (Munday et al. 2009).

OA is generally deemed to have particularly negative impacts on organisms forming calcium carbonate structures, such as bivalve molluscs. Originally it was believed that as OA leads to a decrease in CO_3^{2-}, it will limit the availability of material needed to build calcium carbonate structures ($Ca^{2+} + CO_3^{2-} = CaCO_3$). Roleda et al. (2012) challenged this idea, building on the knowledge that the substrate used by marine organisms for calcification is actually HCO_3^- (as opposed to CO_3^{2-}) or metabolic CO_2 converted to HCO_3^- by carbonic anhydrase. They also stressed that many marine calcifying organisms are capable of controlling the environment where deposition of calcium carbonate occurs. Hence, comprehending responses of marine calcifiers to OA requires an understanding of physiological mechanisms associated to biocalcification, a more complex processes than simple calcification, often occurring in isolated compartments (see Pörtner 2008) and consisting of highly controlled processes in many taxa (e.g. Addadi et al. 2006). It has been suggested that in molluscs the initial mineral phase forms in vesicles inside specialized cells that then transport the vesicles to the calcification site (Addadi et al. 2006). Furthermore there is evidence showing that bivalve larvae are capable of actively regulating Ω_{arag} by increasing pH and CO_3^{2-} at the calcification site during initial shell formation to favour the biocalcification process (Ramesh et al. 2017). The major cause behind negative OA effects on biocalcification may therefore be dissolution (Roleda et al. 2012), or more specifically increased energetic costs associated with maintaining optimal conditions at calcification sites to prevent dissolution of existing structures or to rebuild them. Shell dissolution caused by acidic seawater has been observed for example in pteropods in which the shells' outer organic layer (periostracum) did not appear to provide protection against dissolution (Bednaršek et al. 2012). Moreover for certain organisms, such as bivalve larvae, the calcification process may be negatively affected by factors such as energy budget restriction (Waldbusser et al. 2013).

Finally, different life-stages can respond differently to OA. Padilla-Gamiño et al. (2016) showed that early life stages of the coralline alga *Corallina vancouveriensis* are more resilient to OA than adults. Kroeker et al. (2010) conducted a meta-analysis to evaluate responses of several marine organisms to elevated CO_2 finding for example that larval stages of molluscs appear to be particularly vulnerable. Similar conclusions had previously been reached by Kurihara (2008) who stated that tolerance to acidification varies among life stages and cumulative effects across several life stages could lead to species extinctions.

OA and bivalves: physiological responses

The phylum Mollusca includes many species deemed as being particularly vulnerable to OA and bivalve molluscs have received a great deal of attention by the OA research community. These calcifying organisms represent an important component of both benthic and planktonic ecosystems, are first level consumers and contribute to the carbon cycle (Barros et al. 2013). Additionally they include some of the EU's commercially most important farmed shellfish, such as mussels and oysters (EUMOFA 2016) which are investigated in the present thesis.

A considerable body of literature covers the effects of OA on bivalves. The focus of many studies has been on adult and juvenile (including newly settled) bivalve calcification. Waldbusser et al. (2010) found that calcification rates in the hard clam (*Mercenaria* spp.) exposed to pH values of 8.02, 7.64 and 7.41 decreased with decreasing pH. Gazeau et al. (2007) reported a linear decline in calcification rates with increasing pCO_2 for the blue mussel (*Mytilus edulis*), yet Ries et al. (2009) showed no effect of elevated pCO_2 (~ 600, ~ 900 and ~ 2800 ppm) on calcification rate in the same species. Thomsen et al. (2013) demonstrated how freshly settled *M. edulis* from the Kiel fjord (Germany) tolerate elevated pCO_2 when food supply is abundant and observed impacts on calcification only at extremely high pCO_2 levels (3350 μatm). Their results highlight how other variables (e.g. food concentration) may strongly influence responses to changes in seawater chemistry. Elevated seawater acidity has also been shown to affect bivalves' capabilities of controlling biomineralisation, cause disorientation of calcite crystals and ultimately lead to reduced structural integrity of shells (Fitzer et al. 2014). On top of calcification processes, higher seawater acidity affects other functions and processes in adult and juvenile bivalve molluscs. Schalkhausser et al. (2013) observed a negative effect on king scallops' (*Pecten maximus*) clapping performance under elevated CO_2 concentrations, which also reduced the animals' thermal tolerance. O'Donnell et al. (2013) found that despite no evident effects on shell or tissue growth, byssus threads,

used by mussels as an anchoring tool, were weaker when produced under elevated pCO_2. Furthermore, OA may have implications for bivalve populations by affecting species interactions. For example, exposure to high CO_2 (1000 μatm) increased predation pressure on the Olympia oyster (*Ostrea lurida*) by the invasive Atlantic oyster drill (*Urosalpinx cinerea*) possibly as a result of smaller oyster size (Sanford et al. 2014).

Although the examples above demonstrate that seawater acidification can impact adult and juvenile bivalves, the most sensitive life stages of these organisms are undoubtedly larvae. Elevated CO_2 concentrations can lead to higher larval mortality rates. For example, Talmage & Gobler (2010) showed that larvae of two bivalve species (*Mercenaria mercenaria* and *Argopecten irradians*) had lower survival rates under future projected acidification scenarios (~ 1500 ppm CO_2). Not surprisingly, detrimental effects on calcification are among the most frequently described impacts of seawater acidification on bivalve mollusc larvae (e.g. Miller et al. 2009; Timmins-Schiffman et al. 2013; Waldbusser et al. 2015). Waldbusser et al. (2015) found that aragonite saturation state, more than any other parameter of carbonate chemistry (e.g. pH and pCO_2), explains decreased calcification in the early phase of bivalve development. This may be because during the initial shell formation phase, larvae have a limited capability to isolate the calcifying fluid from seawater, a restricted energy budget (i.e. energy derived from the mother) and are faced with a strong kinetic demand for precipitating $CaCO_3$ (Waldbusser et al. 2013). Ramesh et al. (2017) found that blue mussel larvae are capable of elevating Ω_{arag} at the calcification site, which favours shell precipitation and reduces dissolution. This process is however possible only up to a moderate level of seawater acidification. Higher seawater acidity often results in slower larval development, leading to a reduction in larval sizes. Bechmann et al. (2011) analysed the early development of *M. edulis* under increased seawater acidity (pH 7.6) finding that under these conditions larvae had a reduced growth rate compared to ambient conditions. Larval development in the Antarctic bivalve *Laternula elliptica* was found to be slower under decreased seawater pH (Bylenga et al. 2015). A developmental delay resulting in smaller larvae due to elevated CO_2 concentration (1000 μatm) has also been described for larvae of the Pacific oyster *C. gigas* (Timmins-Schiffman et al. 2013). Furthermore elevated CO_2 has been reported to cause malformations in bivalve shells (e.g. Kurihara et al. 2007; Parker et al. 2009). Another reported consequence of seawater acidification is a negative impact on larval feeding (e.g. Gray et al. 2017).

OA and bivalves: ecological and economic consequences

Bivalves often play important ecological roles within their ecosystems. Mussel beds, for instance, promote and maintain biodiversity (Koivisto & Westerbom 2010). They provide a habitat for invertebrate species (Seed 1996) and increase species richness by providing organisms unable to settle onto soft sediment with a substrate for attachment (e.g. Albrecht & Reise 1994; Buschbaum 2000) and by creating a habitat (i.e. sand trapped between mussel shells) for infaunal species in rocky environments (e.g. Tokeshi & Romero 1995). A study conducted on the west coast of Sweden provides evidence for mussels' physical structural properties being the main factor affecting the mussel-associated biodiversity (Norling & Kautsky 2007). Furthermore mussels' biological activity can also have a positive impact on macrofaunal abundance likely due to mussel biodeposition (i.e. transfer of nutrients from the water column to the sea bed) resulting in greater food abundance and reflected by higher carbon and nitrogen concentration in the sediment (Norling & Kautsky 2007). Similar results were reported for the eastern Swedish coast where even small mussel patches increase species richness and abundance (Norling & Kautsky 2008). By threatening the health and viability of bivalve populations, rise of seawater CO_2 concentration may reduce their positive ecological functions. Sunday et al. (2016) investigated the potential consequences of decrease in percentage coverage of *Mytilus* mussels due to declining pH along the US Pacific North-West. Based on projections, they concluded that a steep decline in species richness may be observed due to a drop in habitat structural complexity.

In addition to their ecological value, many bivalve species are of high commercial value. In 2014 the EU aquaculture production value for mussels increased to 438M€ compared to the previous year and the value for oysters was estimated at 445M€ (EUMOFA 2016). Impacts of seawater acidification on the profitable bivalve aquaculture industry have already been observed on the west coast of the United States, an area frequently affected by upwelling of deep acidified seawater (Feely et al. 2008). Waters beyond the euphotic zone tend to be richer in CO_2 as a result of respiration and upwelling of acidified waters is a naturally occurring phenomenon which is exacerbated by increasing ocean CO_2 uptake (Feely et al. 2008). Bivalve farming has been an important activity from both cultural and economic perspectives since the late nineteenth century in the Pacific North-west where several species including the mussels *M. trossulus* and *M. galloprovincialis* and the Pacific oyster *C. gigas* are farmed (Barton et al. 2015). Oyster hatcheries were successfully established in this area in the 1970s but during 2007 an unprecedented larval mortality event followed by a second one in 2008,

concurrent with a large upwelling event, led Whiskey Creek Shellfish Hatchery managers to investigate links between seawater chemistry and shellfish mortality (Barton et al. 2015). A later study conducted at Whiskey Creek Shellfish Hatchery confirmed a negative correlation between seawater aragonite undersaturation with reduced larval growth and larval production (Barton et al. 2012), suggesting potential strong negative implications of higher seawater acidity on shellfish farming.

Acclimatization and adaptation

The extent to which populations of marine organisms might be impacted by global changes, including changing seawater chemistry, are strongly dependent on their acclimatization and adaptation potentials. The former depends on phenotypic plasticity which is defined as the ability of a single genotype (i.e. specific set of genes carried by an individual) of exhibiting different phenotypes (i.e. organisms' observable traits determined by genotype and environmental factors) in response to environmental variation (Fordyce 2006). Plasticity may benefit a single life stage or also later life stages (reversible and developmental plasticity, respectively) or it may even improve offspring's performance in a stressful environment (transgenerational plasticity) (Sunday et al. 2014). The latter may occur through different processes (Sunday et al. 2014) such as epigenetics, which involves mechanisms like e.g. DNA-methylation but not changes in DNA sequence (Jablonka & Lamb 1998). It has been argued that plasticity can allow persistence through environmental changes providing additional time for adaptation to occur (Chevin et al. 2010). However, plasticity could also theoretically delay adaptation by shifting the average phenotype closer to the fitness peak (Sunday et al. 2014). As opposed to acclimatization, adaptation occurs through natural selection acting on genetic variance (Sunday et al. 2014) and involves changes in a population's genetic makeup. This process is of primary importance in preventing species from extinction in the context of rapidly changing environmental conditions.

The interest of OA researchers for acclimatization and adaptation has grown considerably in the last years. However, little is still known and there is a need for a better understanding of the modulating role of these processes in order to thoroughly understand the risk vulnerable populations are facing (Kelly & Hofmann 2013). Among a few examples showing evidence of acclimation (equivalent to acclimatization but occurring in a laboratory environment) potential and selectable OA tolerance is a study by Parker et al. (2012) who found that exposure to elevated pCO_2 of adult Sydney rock oysters (*Saccostrea glomerata*) during

reproductive conditioning alleviated the detrimental effects of acidified seawater on their larvae and suggested that marine organisms may be capable of acclimating. Yet negative carry over effects have also been described in oysters with juveniles grown under elevated CO_2 concentrations during the larval phase showing reduced growth rates (Hettinger et al. 2012). Another example is a study by Thor & Dupont (2015) who found that transgenerational effects reduced the magnitude of negative effects on fecundity in copepods (*Pseudocalanus acuspes*). The authors concluded that not accounting for such transgenerational responses may lead to erroneous overall conclusions regarding the future of marine populations in the context of OA.

Hybridization

By generating new genotypes, interspecific hybridization has the potential of influencing organisms' performance. Hybridization can lead to a process known as heterosis which consists in the enhanced performance of hybrid offspring in relation to a specific biological trait. For example, Liang et al. (2014) showed that the hybrid young offspring of the cultured abalone *Haliotis discus hannai* and *Haliotis gigantea* had higher heat stress and disease tolerance than their parents. Villegas (1990) found that hybrids of two tilapia species (*Oreochromis mossambicus* and *O. niloticus*) exhibited greater salinity tolerance than one of the parental species (*O. niloticus*). Positive effects of hybridization have also been observed in mussels (see Gardner 1994). However, hybridization does not always improve performance. Some of the potential negative consequences of hybridization are sterility (e.g. Buck 1960), reduced reproductive success (e.g. Muhlfeld et al. 2009) and slower growth (e.g. Beaumont et al. 2004).

In the context of evolutionary adaptation, selection against hybrids could occur as the new genotypes arising from hybridization have never been subjected to selection and may on average perform more poorly than parental genotypes (Burke & Arnold 2001). Yet, introgressive hybridization, the infiltration of genetic material of one species into the genetic material of another due to hybridization (Anderson 1953), may lead to greater genetic variability which in turn can favour adaptation (Lewontin & Birch 1966). Seehausen (2004) proposed that assuming hybridization increases response to selection rates and considering it is common following invasions of new environments, it may favour adaptive diversification in the invading populations.

Study organisms

The two organisms investigated in the present thesis are the blue mussel, *Mytilus edulis*, and the Pacific oyster, *Crassostrea gigas*.

The blue mussel, *Mytilus edulis*, (Linnaeus, 1758) (Fig. 2a), the main species of interest within the present thesis (**papers 1**, **2** and **3**), is abundant along rocky coastlines, can be found from the high intertidal to the subtidal zone in both sheltered and wave-exposed environments and tolerates conditions ranging from fully marine to estuarine (see Gosling 2015). The species is eurythermal and capable of surviving within a wide temperature range (Read & Cumming 1967; Williams 1970). In Europe, the species' distribution extends north to south from the Svalbard archipelago (Berge et al. 2005) to the west coast of France (see Gosling 2015), whilst in the western Atlantic the range stretches south to North Carolina (Jones et al. 2010). Additionally, *M. edulis* occurs in both Iceland (Varvio et al. 1988) and Greenland (Wanamaker et al. 2007). Mussels within the *Mytilus* genus have separate sexes and are broadcast spawners (see Gosling 2015). In the Northern Hemisphere, gonadal development starts during the last two months of the year and is completed in February. The first spawning event occurs in spring with a potential second event during early autumn (see Gosling 2015). Fertilized embryos develop into planktonic trochophore larvae and then secrete an aragonite D-shaped shell. At this stage the larvae are veligers and their first shell is known as prodissoconch I which later develops into a prodissoconch II. Larvae typically remain in the plankton for 4 to 8 weeks and become competent to settle once the pediveliger stage is reached, typically at a size of ~ 260 μm (Bayne 1965). *M. edulis* is one of the main species of interest to global mussel fisheries, yet the greatest proportion of global mussel landings is attributable to aquaculture (FAO 2017a; FAO 2017b). Depending on the production area, mussel farming is practiced using a variety of different techniques. In France one of the methods for culturing *M. edulis* consists in the "bouchot" technique, in which mussel-filled stockings are wrapped around wooden poles placed in the intertidal zone (see Gosling 2015). Other techniques include on-bottom, raft and longline culturing (see Gosling 2015), the latter being the most common technique in Swedish waters (Smaal 2002). In addition to *M. edulis*, two other species within the *Mytilus* genus are found in Europe: *M. galloprovincialis* and *M. trossulus*. Hybridization among the three species is common (Gosling 1992).

The Pacific oyster, *Crassostrea gigas*, (Thunberg, 1793) (Fig. 2b), the study organism in **paper 4**, is a suspension-feeding lamellibranch within the Pelecypoda class. A change of genus name from *Crassostrea* to *Magallana* has been proposed (Salvi et al. 2014; Salvi &

Mariottini 2017) and the latter is now the official genus name in The World Register of Marine Species (WoRMS; http://www.marinespecies.org/). However, due to the controversy surrounding this issue (Bayne et al. 2017), *Crassostrea* will be used throughout this thesis. The Pacific oyster has spread globally from its native geographical range which extends north to south from Primorskiy Kray and the Russian island of Sakhalin to the island of Kyushu (Japan) and the Chinese eastern coast (Arakawa 1990). The species was introduced to British Columbia between 1912 and 1913 (Quayle 1988) and subsequently to France in 1966 (Le Borgne et al. 1973). The optimal salinity range of this euryhaline species is 20-25 PSU but it can survive salinities exceeding 35 PSU (Helm 2005). The Pacific oyster, a protandric hermaphrodite, spawns at temperatures higher than 20°C (Helm 2005). Larval developmental stages are similar to those of *M. edulis* and the planktonic veliger larvae remain in the water column for approximately 3 weeks before settling onto hard substrates (Helm 2005). A considerable contribution to the global production of farmed oysters comes from *C. gigas* mainly cultured in Korea, Japan, France, Taiwan and the United States (see Gosling 2015). In France, the largest *C. gigas* producer in Europe, despite reliance on wild spat, production from hatcheries is increasing in relevance and in 2011 25% of oyster seed originated from hatcheries. Successful hatcheries were also established during the 1970s on the northwest coast of the United States were the production of *C. gigas* contributes over 80% to the annual farmed shellfish production in terms of live weight (Barton et al. 2015).

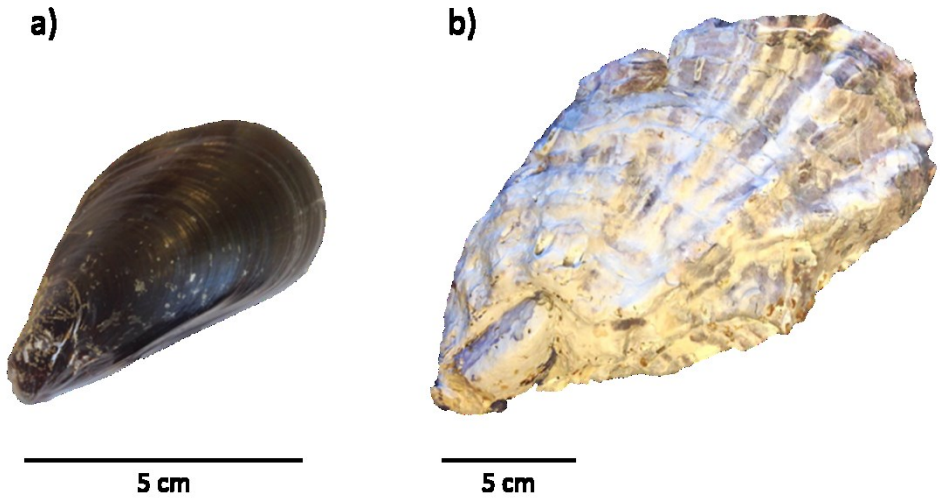

Fig. 2 Study organisms within the present thesis: a) *Mytilus edulis*; b) *Crassostrea gigas*.

I. THE BLUE MUSSEL-SPECIES COMPLEX AND HYBRIDIZATION

In this introductory section of the thesis blue mussel species distribution and hybridization patterns in Europe are discussed. The focus is on impacts of hybridization on mussel farming, especially in the context of global changes, and implications of hybridization for adaptation.

Blue mussels in Europe

M. edulis is one of the species within the blue mussel species-complex. In the Northern Hemisphere, it hybridizes with *M. galloprovincialis* and *M. trossulus* where their distributions overlap (Gosling 1992). All three species are found in In Europe. Additionally, *M. edulis* × *M. galloprovincialis* and *M. edulis* × *M. trossulus* hybrids have been reported (Fig. 3).

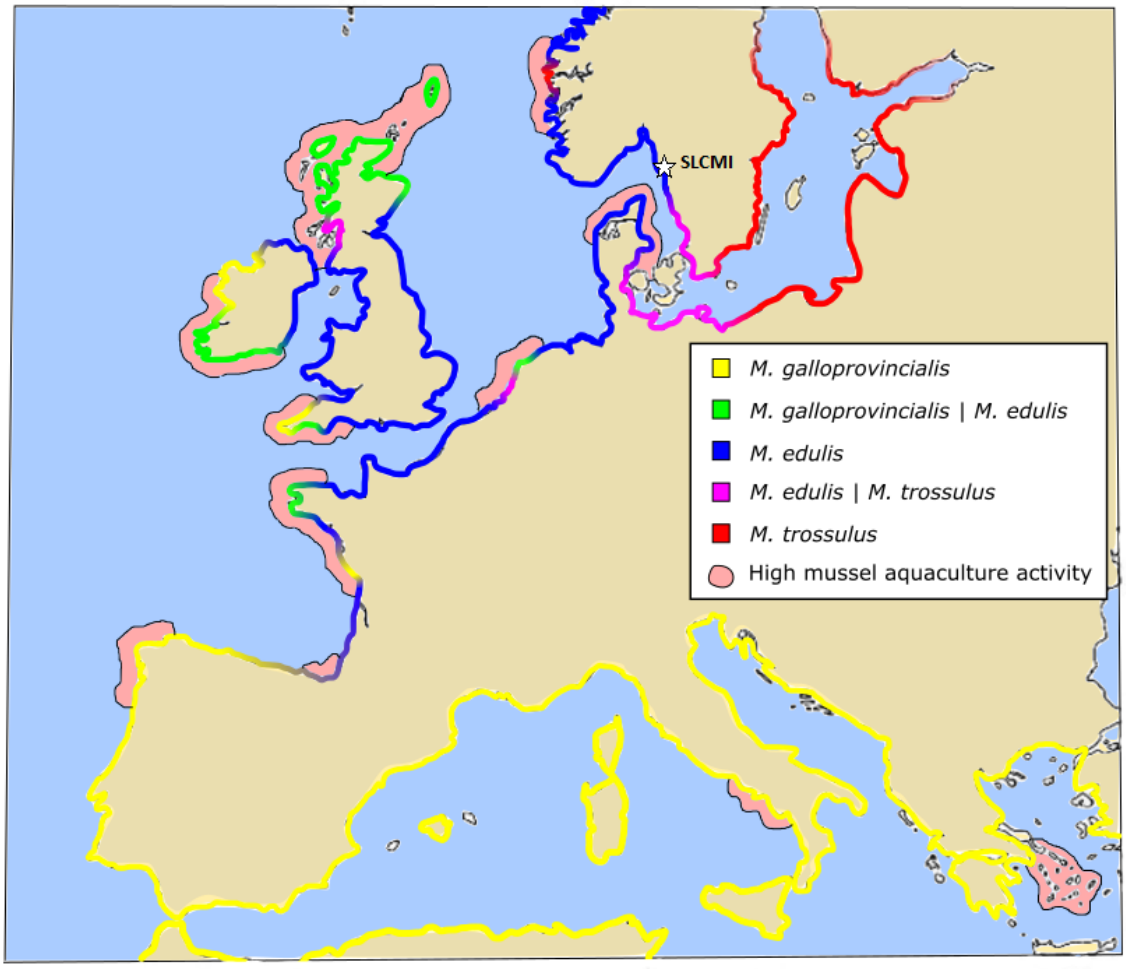

Fig. 3 Simplified representation of blue mussel genotypes' distribution and major mussel farming areas in Europe. Green and pink = hybrids (see text for details). SLCMI = Sven Lovén Centre for Marine Infrastructure, the research station where experiments included in **paper 2** and **3** were conducted. Modified from Michalek et al. (2016) (**paper 1**).

As previously mentioned, *M. edulis* occurs from Svalbard in the north (Berge et al. 2005) to the west coast of France in the south (see Gosling 2015). *M. galloprovincialis* has a more southerly distribution, and is found in the Mediterranean and Black seas (Śmietanka et al. 2004), with a range extending to the west coast of France and the British Isles (Koehn 1991). *M. galloprovincialis* hybridizes with *M. edulis* along the Atlantic coast of Europe (Beaumont et al. 2004 and references therein) and the hybrid zone here is best described as a mosaic of populations (i.e. pure *M. edulis*, *M. edulis* mixed with *M. galloprovincialis* or hybrids of the two species) (Daguin et al. 2001). *M. trossulus* is originally native to the northern Pacific coast of America (Seed 1992). Occurrences of this species have been reported along the coasts of Norway and the Barents and White Sea coasts of Kola Peninsula (Russia) (Väinölä & Strelkov 2011). Blue mussels in the central Baltic Sea have traditionally been considered as *M. trossulus* (e.g. Bulnheim & Gosling 1988), however later studies suggested the Baltic mussel population is best described as a hybrid swarm (Riginos & Cunningham 2005). Taking into account previous investigations (e.g. Larsson et al. 2017), Stuckas et al. (2017) proposed that the genetic transition of Baltic *Mytilus* species occurs between Öresund and west of Rügen Island (Germany). The authors sampled 25 populations mainly from the southern Baltic coast and covering an area extending from the Baltic proper (Askö, Sweden) to the northwest coast of Sweden (Tjärnö, Sweden). Using pure *M. edulis* and *M. trossulus* populations as references and nuclear marker analyses, they found that the Askö population (most northern population sampled in the Baltic proper) was one of the most *trossulus*-like whilst one of the most *edulis*-like corresponded to the Tjärnö population (most northern population sampled along the west coast of Sweden), located ~ 70 km north (in a straight line along the coast) of where mussels used for experiments included in the present thesis were collected (see Fig. 3). It should however be mentioned that despite it being categorized as *M. edulis*-like, the Tjärnö population was still found to be genetically divergent from a pure "reference" North Sea *M. edulis* population (Stuckas et al. 2017).

Mussel hybridization implications

In the review **paper 1** the consequences that hybridization among blue mussel species may have for the mussel aquaculture industry were investigated. The focus was on European waters and the complexity of the investigated topic was highlighted as impacts are strongly dependent on cultured species, environmental conditions and mussel farming techniques. As mentioned above, hybridization between different species can lead to improved performance. An example is represented by the hybrid young offspring of the cultured abalone *Haliotis*

discus hannai and *Haliotis gigantea* which show higher heat stress and disease tolerance (Liang et al. 2014). Within the blue mussel species complex, no decrease in fitness (e.g. parasite resistance, growth rate, fecundity) was observed for *M. edulis* × *M. galloprovincialis* hybrids compared to parental types in the hybrid zone of North Western Europe (see Gardner 1994). However, Beaumont et al. (2004) found that *M. edulis* × *M. galloprovincialis* hybrid larvae grew slower than larvae of the pure species, which the authors suggested could represent a disadvantage due to potentially higher exposure to planktonic predation. Moreover, there may be negative consequences associated with hybridization linked to undesirable features of hybrid adults. In an aquaculture context, *M. trossulus* is a less valued species due to its grey meat colour, lower meat yield and shell thinness (Penney et al. 2007; Penney et al. 2008). In 2006, the occurrence of *M. trossulus* was recorded for the first time in Loch Etive (Scotland) (Beaumont et al. 2008). Farmed fragile-shelled individuals were identified as either pure *M. trossulus* or *M. edulis* × *M. trossulus* hybrids, the presence of which negatively affects mussel aquaculture as they are more easily damaged during handling operations (Beaumont et al. 2008). Additionally, the occurrence of other species or their hybrids may constrain export of mussels due to regulations controlling introduction of non-native species (Beaumont et al. 2008). Ultimately, thin-shelled mussels had a strong negative impact on the mussel farming business in Loch Etive (Beaumont et al. 2008).

Global environmental changes add a layer of complexity to our understanding of the impacts of mussel hybridization because environmental changes can act as drivers for changes in species distributions, potentially affecting hybridization patterns. An example is offered by changes in distribution of almost two-thirds of fish species in the North Sea during the last 25 years as a result of rising temperatures (Perry et al. 2005). Along the eastern coast of the United States, the southern limit of the range of *M. edulis* has shifted ~ 350 km to the north throughout the last 50 years, potentially due to high summer temperatures (Jones et al. 2010). In addition to temperature, global changes will affect other environmental variables such as salinity in certain environments like the Baltic Sea where salinity is expected to drop (Neumann 2010). Moreover, salinity itself is coupled to other parameters associated to biological processes. Intracellular ion- and osmoregulation contributes significantly to the cellular energy budget and most likely limits distribution of marine species in low saline estuaries. Furthermore, calcification in bivalve larvae is strongly linked to calcium concentration ($[Ca^{2+}]$) (Thomsen, Ramesh et al., 2017) and total dissolved inorganic carbon (CT) (Thomsen et al. 2015). The former linearly declines with decreasing salinity (Broecker & Peng 1982) which is also true for the latter as it correlates with total alkalinity (TA), which

in turn positively correlates with salinity in oceanic waters (Millero et al. 1998). *M. trossulus* larvae have been shown to be more tolerant than *M. edulis* larvae to hyposaline conditions, which may be a factor governing distributions of these species, with *M. trossulus* being the only one found in certain low salinity environments (Qiu et al. 2002). As previously mentioned, in the Baltic Sea, a brackish water environment with unfavourable conditions for calcification for the reasons discussed above, blue mussels exist as a hybrid swarm (Riginos & Cunningham 2005). It has been shown that calcification in larvae of the more *M. trossulus*-like mussels from the central Baltic (Stuckas et al. 2017), compared to larvae of the more *M. edulis*-like individuals from the western Baltic (Stuckas et al. 2017), is less affected by low $[Ca^{2+}]$ and $[Ca^{2+}][HCO_3^-]/[H^+]$ (where $[HCO_3^-]$ and $[H^+]$ are concentrations of bicarbonate and hydrogen ions, respectively) possibly as a plastic response or a result of genetic adaptation of these low-salinity tolerant mussels to the more calcification-adverse environment (Thomsen, Ramesh et al., 2017). Despite the fact that the central Baltic mussels showed no higher tolerance to low aragonite concentration (Thomsen, Ramesh et al., 2017), it remains to be thoroughly investigated whether there is an association between low salinity, low $[Ca^{2+}]$ and low $[Ca^{2+}][HCO_3^-]/[H^+]$ tolerance and greater OA tolerance in more *M. trossulus*-like mussels. Should this be the case, it could be speculated that changes in not only salinity but also seawater acidity might affect blue mussel species distributions which could have consequences for the aquaculture industry due to potentially greater occurrence of less desirable species (e.g. *M. trossulus*) and hybrids.

Furthermore, hybridization may also influence evolutionary adaptation but there are contrasting hypothesis surrounding this topic. On one hand, it has been suggested that hybridization-driven introgression may be the source of greater genetic variability on which selection can act and then could ultimately promote adaptation. Some evidence is provided by Lewontin and Birch (1966). The authors' experiments supported their hypothesis that introgression of genes of the fly *Dacus neohumeralis* into *D. tryoni* could lead to selection of better extreme environment-adapted genotypes by increasing genetic variability. On the other hand, as new genotypes which have never been exposed to selection, hybrid genotypes may actually be less well adapted than the parental ones (see Burke and Arnold 2001).

In the context of mussel adaptation to global changes, it is likely that consequences of hybridization will be closely linked to specific local environmental conditions. Nevertheless, hybridization will affect the genetic characteristics of a population and should not be ignored in the context of adaptation potential investigations. Hence, the mussel population which will be investigated in the rest of this thesis was selected based on practical reasons (e.g. proximity

to field station where experiments were carried out) but also due to its genetic composition (i.e. limited introgression of *M. trossulus* genes), allowing a certain degree of confidence in characterizing it as a relatively "pure" *M. edulis* population from a hybridization perspective (see paragraph "Blue mussels in Europe" for details). This limits the addition of a layer of complexity to adaptation potential investigations, introduced by the possible implications of hybridization for adaptation.

Summary

The blue mussel-species complex in European waters is characterized by three species which hybridize in areas of co-occurrence. There are currently knowledge gaps preventing a thorough understanding of the impacts of hybridization on mussel farming and here it is clarified that impacts may be both positive and negative. Moreover, global changes, including changes in seawater chemistry, are likely to play a role in shaping species distributions, indirectly influencing the impacts of hybridization on mussel aquaculture. Finally, hybridization can have consequences for evolutionary adaptation, either promoting it by enhancing genetic variability or hindering it by generating new genotypes which have never been subjected to selection. Hence, hybridization should be accounted for in investigations of adaptation potential.

II. LOCAL ENVIRONMENT AND TOLERANCE THRESHOLD

In order to understand the susceptibility to OA of a mussel population from the Swedish west coast and gather information for further investigation of adaptation potential, the hypothesis that the tolerance threshold would match the lower limits of the present range of seawater acidity is tested. Acidity levels beyond the environmental variability represent realistic OA scenarios and can be further utilized for adaptation potential investigations.

Local environment and OA scenarios

Uncovering which traits are affected by elevated seawater CO_2 concentration is a key factor for understanding how organisms and populations may respond to OA (Kelly & Hofmann 2013). This requires testing relevant scenarios for the investigated population. A considerable portion of ocean acidification experimental research has, throughout the years, focused on understanding the biological impacts using projected near-future scenarios (Caldeira & Wickett 2005) for the open ocean (Cornwall & Hurd 2016) (i.e. pH ~ 8.1 vs. 7.7). However, many marine organisms targeted by OA studies, including bivalve molluscs, inhabit coastal environments such as fjords, estuaries and the intertidal. Seawater chemistry in coastal ecosystems is more complex and less stable compared to the open ocean due to inputs from land (see Aufdenkampe et al. 2011) and the presence of coastal ecosystem engineers (e.g. corals, bivalves and macroalgae) affecting biogeochemical processes (Gutiérrez et al. 2011). As a result, coastal environments can be characterized by large fluctuations in several abiotic parameters including carbonate chemistry parameters (Dorey et al. 2013; Waldbusser & Salisbury 2014). As a consequence, the open ocean scenarios fall within the current range of natural variability of seawater carbonate chemistry experienced by coastal organisms. Furthermore, being within the natural variability range, these scenarios cannot technically be considered OA scenarios. This is of critical importance as true stress occurs when an organism is exposed to conditions that are outside of its present environmental niche (Van Straalen 2003). Different biological responses can occur when an organism is exposed to stressing conditions as compared to variation of their natural environment. For example, Thor & Dupont (2015) hypothesized that plasticity was the main response when copepods were exposed to low pH within the range of their natural variability while selection occurred when they were exposed to realistic OA scenarios deviating from the present pH variability. This stresses the importance of accounting for the characteristics of a population's niche when

investigating the consequences of environmental changes (Hendriks et al. 2015; Dorey et al. 2013).

Environmental variability and physiological thresholds

The breadth of a population's tolerance range to an environmental parameter often reflects the magnitude of its natural range of variability. Assuming adaptation to local conditions, it can be hypothesized that the extremes of this range are likely to represent the population's tolerance threshold. This has been documented in the context of OA. For example, Jager et al. (2016) found that the tipping point (i.e. tolerance threshold) for larvae of the green sea urchin (*Strongylocentrotus droebachiensis*) corresponded to the local extreme of current natural variability in seawater acidity (pH = 7.5). Vargas et al. (2017) conducted a meta-analysis of studies investigating responses to elevated pCO_2 of several marine organisms inhabiting different coastal environments along the Chilean coast, a highly heterogeneous geographical area in terms of oceanographic conditions. The authors found that the magnitude of the effect of elevated pCO_2 on organisms belonging to the same species but inhabiting different environments in terms of seawater acidity levels, was often different, ranging from negative to positive. For example, copepods (*Acartia tonsa*) from high-pCO_2 estuarine environments showed more tolerance to elevated pCO_2 levels than conspecifics from oceanic environments. Furthermore, physiological traits of those organisms naturally experiencing elevated mean pCO_2 levels and high pCO_2 variability were less dramatically affected by seawater acidification in comparison to organisms inhabiting more stable environments with lower mean pCO_2 conditions. Taking into account the local chemistry, they showed that the intensity and direction of the biological impact could be predicted by how much the tested treatments deviated from the extreme of the natural variability.

Physiological thresholds in Swedish blue mussel larvae

One of the main objectives of **paper 2** was to investigate the physiological tolerance threshold to seawater acidity in a blue mussel (*M. edulis*) population from the west coast of Sweden. The study was conducted in June at the Sven Lovén Centre for Marine Infrastructure (Fiskebäckskil, Sweden). The research station is located at the mouth of the Gullmar fjord. We hypothesized that as they inhabit an area characterized by wide fluctuations in seawater acidity (Dorey et al. 2013) (Fig. 4), larvae of mussels from this area would be able to tolerate the pH/pCO_2 regime they are currently experiencing and their physiological threshold would

approximate the lower limit of their experienced natural variability.

Focus was on larvae, one of the most OA sensitive bivalve life stages, which, during laboratory experiments, were exposed to a range of seawater acidity representing what they are experiencing in the field today (Dorey et al. 2013). However, in order to find their physiological threshold for normal shell development the boundaries were also pushed and larval performance at even higher acidities was tested. As previously mentioned bivalve larval shells are sensitive to changes in seawater acidity and higher acidity has been found to cause malformations in larval shells. These malformations may affect the larvae's swimming capability (Beiras & His 1995) which in turn could compromise their chances of survival (Kurihara et al. 2008).

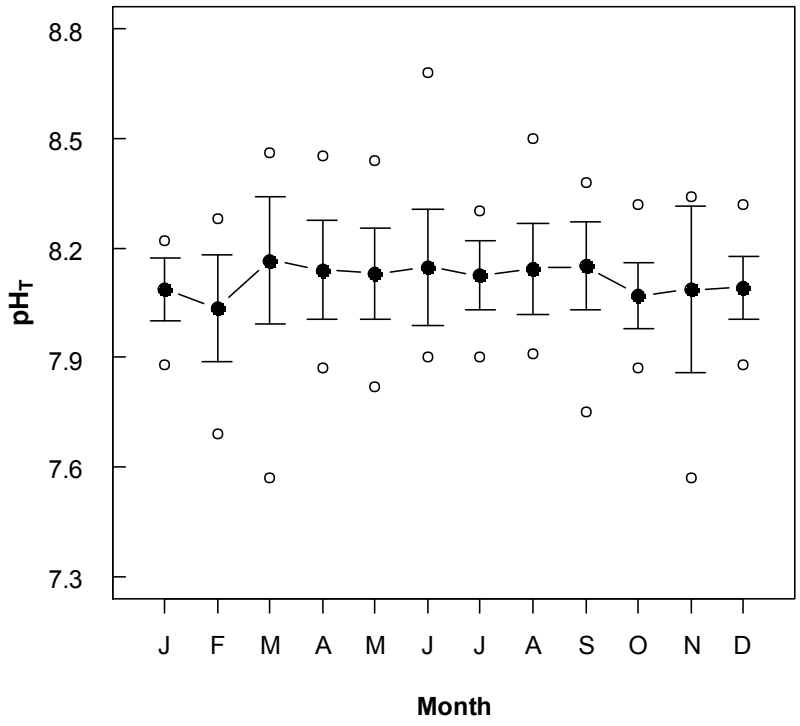

Fig. 4 Variation in pH_T (pH on the total scale) in the Gullmar fjord (Sweden) based on monthly data between 1921 and 1987. Black dots represent average values (±SD); empty circles show maximum and minimum values. Modified from Dorey et al. (2013).

An increase in the percentage of malformed larvae with decreasing pH was observed and the tipping point for normal development was identified at $pH_T = 7.76$. As predicted and as described for other species (e.g. Jager et al. 2016), this corresponds to the lower limits of the present pH range experienced by these larvae (late spring seawater pH values of $pH_T \sim 7.8$-7.9) (see Fig. 4). Despite a large decrease in the percentage of normally developed larvae

beyond the tipping point, a considerable number of normally developed larvae were observed at a lower pH_T (i.e. ~ 7.6). This represents a realistic OA scenario for the larvae and can be interpreted as an indication of potential for adaptation. One intriguing result was the presence of very few normally developed larvae (~ 1%) in cultures within the lowest pH treatment (pH_T ~ 7.1). This could be due to a negligible degree of cross-contamination during sampling and does not affect main conclusions but does call for caution in the context of examining extreme acidity levels at which larvae are capable of developing non-malformed D-shaped shells.

Plasticity in physiological mechanisms, such as active elevation of Ω_{arag} at the site of calcification (Ramesh et al. 2017), might be the reason larvae were capable of producing and maintaining normal shells at moderate acidity levels up to their physiological threshold. Additional support for this is provided by results on larval calcification and shell dissolution in **paper 2**. It was found that, compared to ambient conditions, shells of normally developed larvae previously grown under ambient pH and then exposed to extremely low pH values (pH ~ 7) in a short term physiological assay dissolved significantly more; however, calcification rates for these larvae were also significantly higher, indicating a compensatory mechanism leading to comparable net calcification rates between ambient and low pH conditions. It should be noted that, despite potentially limited genetic variance in the tested larval pool due to a single male/female cross, non-malformed larvae observed under considerably acidic conditions (e.g. pH_T ~7.6) may be the result of more tolerant genotypes present in the larval pool. Finally, it is worth mentioning potential variability in egg energy content could be playing a role and may to some extent also explain observations of both malformed and normally developed larvae at the same time points within the same larval cultures.

Summary

Larvae of a population of mussels from the Swedish west coast were capable of developing normal shells up to a pH level approximating the lower limits of their experienced natural range (pH_T = 7.76). Moreover, presence of normal larvae at lower pH corresponding to realistic OA scenarios (i.e. pH_T ~ 7.6) suggests a potential for adaptation to near future environmental conditions. Finally, results of **paper 2** give an indication that more dramatic levels of acidification would not only represent locally relevant OA scenarios but also elicit strong responses on traits such as shell development, which represents useful information for investigations of adaptation potential (see following section).

III. HERITABILITY AND ADAPTATION POTENTIAL

The next step in understanding how bivalves may cope with OA is to investigate if they have the potential to adapt to changes in near-future seawater chemistry. This is achieved by testing the hypothesis that considerable additive genetic variance in fitness-related larval traits affected by seawater acidification exists.

The study of evolutionary processes

The analysis of evolutionary adaptation to environmental changes is often performed through one of the following approaches: selection experiments or measurements of standing genetic variance in traits affected by environmental changes (see Sunday et al. 2014). An example of the first approach is a study by Pespeni et al. (2013) who provided evidence of potential for rapid adaptation to OA in the sea urchin *Strongylocentrotus purpuratus*. By rearing larvae under ambient and elevated CO_2 concentrations for seven days, the authors described CO_2-driven changes in allele (i.e. variant forms of genes) frequencies but no strong effects on sea urchin larval morphology, suggesting selection for genotypes associated with better performance under elevated CO_2 concentration. Evolution experiments may also be conducted over several generations (see Sunday et al. 2014). Lohbeck et al. (2012) investigated whether *Emiliania huxleyi*, a key calcifying phytoplankton species, is capable of adapting to OA. The authors performed multigenerational selection experiments starting from both a single (single-clone experiment) and multiple genotypes (multi-clone experiment) to test for evolutionary adaptation driven by new mutations or standing genetic variation, respectively. Both experiments were run for ~ 500 generations under ambient and elevated CO_2 concentrations (400, 1100 and 2200 pCO_2) and results provided evidence of partially restored calcification rates due to evolutionary adaptation. A study on *Gephyrocapsa oceanica* by Jin et al. (2013) represents another example of multigenerational selection experiment (~ 670 generations) providing evidence of evolutionary adaptation to OA in coccolithophores. The feasibility of multigenerational selection experiments is constrained by the study organisms' generation times which may be several years. In this case, the second approach for studying evolutionary adaptation mentioned above (i.e. measurement of standing genetic variance in relevant traits) may be more feasible. Conducting breeding experiments combined with quantitative genetic analyses which focus on potential genetic variation in phenotypic traits that may be under selection in the context of environmental changes, may in fact provide insight into adaptation

potential (Shaw & Etterson 2012). This approach has been employed by several authors to investigate the adaptation to OA in marine invertebrates. Kelly et al. (2013) investigated adaptation to OA in the purple sea urchin (*Strongylocentrotus purpuratus*) by using a modified North Carolina II breeding design (i.e. a breeding design involving all possible matings between two sets of individuals; Lynch & Walsh 1998) and analysing genetic variance for larval size through a mixed-effects model known as the "animal model" (Kruuk 2004). Results indicated considerable additive genetic variance and heritability for the investigated trait under acidified conditions (~ 1200 μatm). This suggests that size of larvae has the potential to evolve. Larval size has been the target of other OA adaptation potential studies (e.g. Sunday et al. 2011) as it is considered a trait with important implications for population fitness. Vulnerability of invertebrate larvae to predation is often size-dependent (Rumrill 1990) and greater larval size may be an advantageous trait reducing predation pressure (but see review by Pechenik 1999). Additionally there is evidence for larger larval shell length (*M. edulis* and *C. gigas* larvae) being positively associated with faster swimming (Troost et al. 2008) and it could be speculated that larger larvae may have a competitive advantage both in relation to escaping from predation and feeding. Hence larger larval body size may certainly be considered a fitness advantage in the face of OA.

Blue mussel adaptation potential to OA

In **paper 3** it was investigated if and to what extent the same *M. edulis* population investigated in **paper 2** has the potential for evolutionary adaptation to OA. Following a North Carolina II breeding design, two adult males were mated with two females generating four families which corresponded to one experimental block. The entire study consisted of four experimental blocks for a total of eight male and eight female parents (sires and dams, respectively) and sixteen families. Larval families were exposed in triplicate to an ambient (pH_T ~ 8.1) and a low pH (pH_T ~ 7.5). The latter was selected based on results of **paper 2** suggesting that on top of being a relevant acidification level representing a realistic OA scenario for the investigated population, it would elicit strong measurable responses in larvae. The experiment continued until most larvae reached the D-shaped veliger stage (72 hours post-fertilization). Larvae were then photographed and measured in order to estimate additive genetic variance, phenotypic variance and heritability for larval shell size and larval shell malformation under ambient and elevated CO_2 employing a quantitative genetic approach

based on the implementation of an "animal model" using the MCMCglmm package in R (Hadfield 2010).

Shell size of larvae were significantly lower under elevated acidity (~ 15% lower mean size) and proportion of malformed larvae was significantly higher (~ 86% higher mean). There was considerable additive genetic variance under elevated seawater acidity for both traits and heritabilities for size were 0.489 (confidence intervals (CI) = 0.401 – 0.630) and 0.104 (CI = 0.058 – 0.236) under low and ambient pH, respectively. Heritability for malformation was low (0.017, CI = 0.009 – 0.0615) at ambient seawater acidity but significantly higher (0.857, CI = 0.609 – 0.879) under acidified conditions.

The considerably high heritabilites for both shell size and malformation under elevated seawater acidity, which were also higher compared to ambient conditions, represent a good indication that the investigated population has the potential to adapt to OA. Furthermore, heritability measures for size are in agreement with results in other mussel populations (Thomsen, Stapp et al. 2017; Toro & Paredes 1996). The potential for coping with elevated CO_2 concentrations through evolutionary processes has previously been investigated in other bivalve populations. Sunday et al. (2011) used quantitative genetic tools to investigate the adaptation potential to OA in the mussel *Mytilus trossulus*. Parker et al. (2011) showed how a faster-growing pathogen-resistant selectively bred line of the Sydney rock oyster (*Saccostrea glomerata*) appears to be more tolerant to elevated pCO_2 compared to the wild populations, suggesting a positive genetic correlation between these two traits and the existence of selectable genetic variation for resilience to elevated pCO_2 in bivalve molluscs. By comparing responses of blue mussel (*M. edulis*) populations from two different locations, one of which (Kiel fjord, Germany) is periodically subjected to upwelling events leading to regular rises in CO_2 concentration during the mussels' reproductive period, Thomsen, Stapp et al. (2017) were able of demonstrating an evolutionary response to OA in a wild mussel population. This was indicated by greater survival and calcification capability under elevated pCO_2 in mussels from the Kiel fjord. The authors also conducted a multigenerational experiment involving both "tolerant" and "sensitive" families from the Kiel fjord mussel population and showed that larger larval size of "tolerant" families had a heritable component and was passed on between the F_1 and F_2 generations (first and second filial generations, respectively). However, larger size did not directly translate into increased survival in the F_2 generation. This suggests that caution must be exercised when formulating conclusions regarding adaptation potential to OA based solely on single generation experiments and heritability of few traits. Nevertheless, our experiments provide crucial information for the investigation of adaptation potential to

OA in bivalves, as they provide valuable information on of the genetic variance of fitness-related traits, which could likely be a source of resilience in the face of OA.

Summary

Results of **paper 3** on genetic variance and heritability of fitness related traits (i.e. larval shell size and malformation) under elevated seawater acidity reflect a good adaptation potential to OA in the investigated mussel population. Despite the limitations associated to the single generation experimental design and investigation of a few traits, the study represents a valid and needed contribution for understanding how mussel populations may cope with OA.

IV. TARGETS OF SELECTION

Identifying genes involved in initial larval shell production may indicate potential targets of selection under OA scenarios. To identify these genes, it is tested whether bivalve larvae secreting their first shell show a different pattern of gene expression compared to larvae of the same age, but developing their shell later as a consequence of more acidic rearing conditions. It is then suggested that differentially expressed genes are mostly associated with shell deposition. The Pacific oyster, Crassostrea gigas*, was selected over* M. edulis *in this study as it is a model species with an available genome representing an ideal candidate for these investigations.*

Bivalve larval calcification

Knowledge of shell formation mechanisms in bivalve larvae is currently patchy. Yet, the process of larval calcification is of great relevance as it may represent the bottleneck determining the susceptibility of bivalves to OA. The larval shell is secreted during early development and larvae of the Pacific oyster (*C. gigas*) produce theirs as early as 14-18 hours post-fertilization (Waldbusser et al. 2016). Larval shell is mainly composed of aragonite which is secreted between the larval epithelium and the periostracum (i.e. organic coating of the shell) (Marin et al. 2007) produced by the shell gland at the trochophore developmental stage (Kniprath 1981). It is during the transition phases in larval development that many major changes at the molecular level occur. An example is represented by the major changes in gene expression during transition from the trochophore to the veliger stage in the pearl oyster *Pinctada fucata* (Li et al. 2016). Protein expression also provides useful insight regarding dynamics involved in larval shell formation. In comparing protein expression in trochophore and D-shaped veliger larvae of *C. gigas*, Huan et al. (2012) identified 50 differentially expressed proteins involved in processes including cell proliferation and protein modification. Despite the characterization of the *C. gigas* genome (Zhang et al. 2012) and existing information regarding genes potentially involved in oyster larval shell formation, a more detailed understanding of the initial calcification process at a molecular level would benefit from a more fine-tuned investigation of gene expression patterns focusing especially on the crucial phases during which shells are deposited.

Molecular dynamics of shell formation in Pacific oyster larvae

One of the effects of low aragonite conditions, a consequence of seawater acidification (Waldbusser et al. 2015), is a delay in larval shell formation (Kurihara et al. 2007; Talmage & Gobler 2009). In **paper 4** the molecular processes associated with initial shell formation in larvae of the Pacific oyster (*C. gigas*) were investigated. The main reason for choosing this specific species was the greater amount of genetic information available for *C. gigas* (genome published by Zhang et al. 2012) compared to *M. edulis* which allowed more accurate analyses of molecular functions and processes. Seawater acidification was employed as a tool for generating a delay in shell formation in *C. gigas* larvae. This allowed comparing gene expression patterns at the same time for different developmental stages (with or without shells) which in turn allowed the identification of genes and functions potentially involved in the initial phases of bivalve shell deposition. It should be mentioned that on top of delaying shell formation, seawater acidification may also elicit other physiological responses in larvae (not necessarily related to calcification processes) which may have affected gene expression patterns. Two replicate experiments were conducted at the Department of Fisheries and Wildlife and Coastal Oregon Marine Experiment Station (Oregon State University, Newport, Oregon, USA). Larvae were sampled every two hours between 6 and 18 hours post-fertilization for gene expression analyses using RNA-Seq. There were differences in larval calcification rates between acidified and non-acidified conditions (Fig. 5) and 55 differentially expressed transcripts shared by the two experiments were identified. Thirty-seven of these had at least one type of annotation and could be attributed to one of these categories: metabolic genes, transmembrane proteins, shell matrix proteins and protease inhibitors.

The description of differentially expressed genes for transmembrane proteins and shell matrix proteins presented in **paper 4** provides a likely picture of the initial phases of calcification, being characterized by the production of shell matrix proteins which are then transported across membranes to the calcification site. Several of the differentially expressed transcripts that were identified belonged to the category of protease inhibitors, indicating that protection of the larval shell protein matrix is key during initial shell formation. Damage to the shell protein matrix due to hydrolysis by endopeptidases (Berezney 1979) may be associated with an incorrect deposition of the aragonite crystals and the considerable number of malformed larvae observed under low aragonite conditions (see Waldbusser et al. 2015).

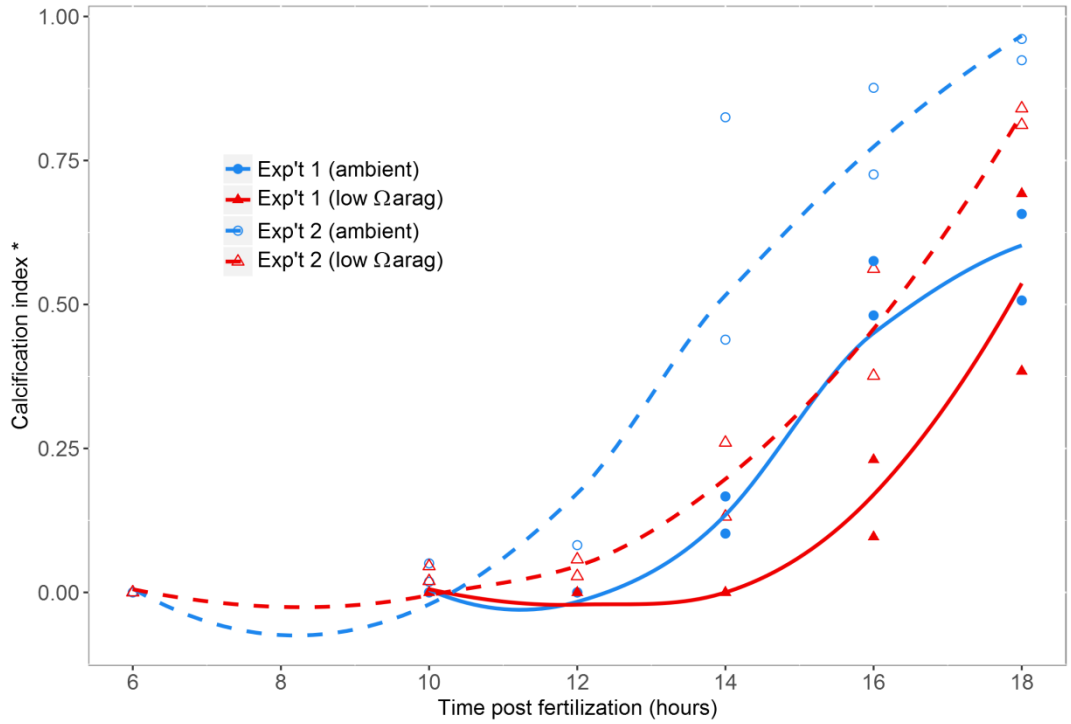

Fig. 5 Larval calcification expressed as calcification index (CI) ($CI = (FC + (PC * 0.5))/TL$, where FC, PC and TL are numbers of fully calcified, partially calcified and total larvae from each sample) between 6 and 18 hours post fertilization. Non-acidified and acidified conditions are shown in blue and red respectively whilst solid lines/symbols and dashed lines and empty symbols represent the first and second replicate experiments, respectively. From De Wit et al. (2017) (**paper 4**). http://creativecommons.org/licenses/by/4.0/.

Despite their possible involvement in other physiological processes, the genes identified in **paper 4** appear to be associated to functions likely linked to the initial larval calcification. These genes may be important in the context of responses of oyster larvae to projected seawater acidification. For instance, in wild populations these genes may be the "targets" for natural selection and sufficient genetic variation at these loci may provide scope for resilience. Standing genetic variation has been described as a key factor for populations' ability to cope with environmental changes (Pespeni et al. 2013). A study on evolutionary changes in purple sea urchin (*Strongylocentrotus purpuratus*) larvae exposed to simulated ocean acidification (Pespeni et al. 2013) showed that higher seawater acidity lead to considerable changes in allele frequencies despite that no dramatic phenotypic changes were observable. The major differences were in genes associated with membrane composition and ion transport, suggesting greater survival chances for larvae capable of better regulating internal pH under elevated seawater acidity. One of the most relevant drivers of OA impacts on calcifying organisms is in fact acid-base regulation as calcification produces protons that must be pumped out of the calcification space, a process which may be negatively affected by an extracellular accumulation of protons associated to a decrease in pH (Hofmann &

Todgham 2010). The results by Pespeni et al. (2013) indicate that rapid adaptation to OA is possible if there is sufficient intra-population variability for genes associated with resilience to elevated OA. Due to the known dramatic impacts of increasing seawater acidity on calcification, this stresses the importance of a better knowledge of which genes are involved in the calcification process in the context of understanding population responses to OA. Although it is not possible to directly extrapolate results of **paper 4** to other bivalves, there is evidence suggesting similarities across species. Thomsen et al. (2015) conducted a meta-analysis to compare calcification performance at different calculated seawater carbonate ion concentrations in larvae of several bivalves including mussels (e.g. *M. edulis*, *M. trossulus* and *M. galloprovincialis*), oysters (e.g. *C. gigas*), scallops (e.g. *Pecten maximus*) and clams (e.g. *Macoma balthica*). They found similar responses among different species and populations, a result suggesting that mechanisms determining calcification sensitivity to seawater carbonate chemistry in larvae are likely similar among several bivalve taxa. From these results it can be speculated that the genes and functions identified in **paper 4** and suggested as being involved in Pacific oysters' initial shell formation, may be similar in other species such as *M. edulis*.

Summary

The bivalve larval stage is considered one of the most sensitive and important of life stages in the context of OA impacts. Calcification is known to be strongly affected by seawater acidification which stresses the importance of understanding what processes are involved in larval shell formation when investigating effects of OA. Finally, intra-population genetic variation for responses to seawater acidification can allow for resilience to OA through rapid evolutionary responses. In **paper 4**, some candidate genes involved in the calcification process were identified. Several of these are coding for transmembrane proteins, shell matrix proteins and protease inhibitors. This indicates that these genes may be involved in larval calcification of Pacific oysters and potentially other bivalve species. As oceans become more acidic these genes represent potential targets for natural selection.

OVERALL CONCLUSIONS & FUTURE DIRECTIONS

The main conclusions that can be drawn from the present thesis in relation to the specific aims outlined above are:

I. In Europe, the three species within the blue mussel-species complex hybridize where their ranges overlap which, as stated in **paper 1**, can have both positive and negative implications for mussel farming. These impacts can be further influenced by environmental changes which will affect species distributions and hybridization. The latter may influence populations' tolerance to environmental parameters and their adaptation potential.

II. Blue mussel larvae from the investigated population on the Swedish west coast can develop normal shells up to seawater acidity levels approximating the lower limits of their natural experienced variability, as indicated by result of **paper 2**. This shows that the larvae tolerate the wide fluctuations in seawater acidity they naturally experience. Furthermore, some larvae had a normal development at pH below the tipping point suggesting scope for adaptation to OA.

III. Genetic variance and heritability for the fitness related traits (i.e. larval shell size and shell malformation) investigated in **paper 3** for the same mussel population mentioned above, was considerable with heritability being higher under elevated seawater acidity compared to ambient conditions. This suggests the population has the potential of adapting to OA.

IV. The genes identified in **paper 4** as being involved in initial shell formation of *C. gigas* larvae, can be included in four categories: metabolic genes, transmembrane proteins, shell matrix proteins and protease inhibitors. This stresses the key importance of the shell protein matrix and its integrity for successful shell deposition. Additionally, as calcification is strongly affected by seawater acidification it highlights potential targets for selection in the face of OA and suggests that sufficient within-population variability for these genes may play a role in adaptation to OA.

This thesis contributes to a better understanding of the how bivalve molluscs may respond to global changes including what their adaptation potential to OA may be. Furthermore, it

proposes interesting avenues for future research. For example, the potential for specific alleles of providing greater tolerance to changes in certain environmental parameters (e.g. salinity and/or acidity) was mentioned in the initial section of the thesis. This could be tested by exposing a mixed *M. edulis-trossulus* population, including individuals with different levels of introgression, to salinity and/or acidification stress and conducting a selection experiment to investigate selection of specific tolerant genotypes. In 2017, I started to investigate this question together with my colleagues Elliot Scanes, Kirti Ramesh, Isabelle Casties, Loreen Knöbel, Heiko Stuckas, Jennifer Schulze, Trystan Sanders, Sam Dupont and Frank Melzner, at GEOMAR (Helmholtz Centre for Ocean Research, Kiel, Germany). Results of the selection experiment conducted in spring are currently being analysed. Additionally, the experiment in **paper 4** could be repeated on *M. edulis* to confirm similarities in calcification mechanisms between oyster and mussel larvae. Finally, by standardising larvae for size, impacts of OA on expression of genes involved in calcification could be disentangled from other physiological processes. This would provide further evidence that the genes identified in **paper 4** are indeed involved in initial larval calcification.

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