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Improving our understanding of evolutionary persistence in an increasingly high CO2 world: Insight from marine polychaetes at a low pH vent system

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XXVIII CICLO

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by

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Noelle M. Lucey

Abstract

The main aim of my thesis was to determine how marine metazoans might persist as ocean acidification (OA) conditions intensify. This was done using a combination of field surveys, field transplants and laboratory experiments with polychaetes from a localized low pH/high pCO_2 marine ecosystem (Ischia, Italy), formed from volcanically derived CO_2. This gas bubbles through the sea floor and drives the seawater pH down, resulting in a low pH ecosystem representative of global OA projections for, or before, the year 2100. My first objective was to identify OA-tolerant phenotypes by assessing the distributions, abundances and life history traits of marine polychaetes persisting in low pH environments (Chapter 2 and 5). To do this, I characterized the distribution of dominant calcifying polychaetes at sites associated with low pH vents and used a comparative species recruitment experiment to investigate life history responses underlying species' CO_2 tolerance/vulnerability. I first found two dominant, closely related species of polychaete: *Pileolaria militaris* Claparède, 1870 and *Simplaria* sp. (Serpulidae, Spirorbinae). I then found that higher reproductive output and faster settlement rates were important traits in determining species' abilities to persist in low pH environments (Chapter 2). Afterwhich, I overviewed the life history strategies of all dominant polychaetes in the same low pH vent, and performed breeding experiments on *Platynereis dumerilii* (Audouin & Milne Edwards, 1834), one of the few species in low pH areas known to have broadcasting, pelagic development. Through genetic barcoding approaches, I found that this presumed *P. dumerilli* from the low pH was incorrectly identified, and was actually the direct developing brooder sister species, *Platynereis massiliensis* (Moquin-Tandon, 1869). By reanalayzing the sister species distributions, I found that direct development and brooding were related to low pH persistence (Chapter 5). My second objective was to use reciprocal transplant experiments to compare the relative importance of local adaptation and/or plasticity as potential mechanisms responsible for the differential tolerances of populations of the polychaete species (*Simplaria* sp.) to low pH. Laboratory transplants indicated that a local adaptation response had occurred through genetic accommodation in the *Simplaria* sp. population from a low pH site. However, neither local adaptation nor plasticity appeared responsible for this species natural low pH persistence when transplanted in the field (Chapter 3 & 4). My final objective was to create a framework using the polychaete vent model to identify other types of marine metazoans that are likely to be able to adapt to, and survive, under the predicted environmental conditions (Chapter 5). By integrating and linking the life history strategies of the polychaete vent assemblage to those of other marine

invertebrates, I show that brooding may be a key trait of species likely to persist in future oceans pH. I conclude by summarizing how research regarding evolutionary responses may be advanced to add confidence to our projections of future marine life responses.

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Piero first introduced me to the topic of evolution in marine systems and his overall enthusiasm of its relevance played a large role in carrying me through this Ph.D. with bright eyes and curiosity. Simon Rundle was instrumental is guiding and focusing my research, as well as introducing me the fascinating world of plasticity. His enthusiasm, calmness and ability to settle my uncertainties meant so much to me. Likewise, Silvia Cocito's steadfast calm helped when all was going awry. I also want to thank Maria Cristina Gambi for teaching me the complexities of polychaete taxonomy with her detailed and ardent attention. Her love of marine biology and polychaetes really made it a great experience. More generally, I want to thank all my advisors for having the patience to work remotely at various periods throughout this doctorate. Despite the challenges of Skype and email with many people from different countries and backgrounds, everyone was responsive, collaborative and helpful.

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee. Work submitted for this research degree at the University of Pavia and Plymouth University has not formed part of any other degree either university or at another establishment.

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Chapter 1

1 Introduction

1.1 Summary

A pressing question for today's society is what will happen to marine life as the ocean chemistry conditions change in response to increasing carbon dioxide emissions. In this introduction I briefly describe the background to ocean acidification and the approaches used to predict the resultant responses of marine life. I then detail the study site and study organisms used in this thesis. I conclude by outlining the approaches to be used in this thesis to investigate how marine polychaetes are able to persist in a present-day marine ecosystem where volcanically-derived CO_2 emissions trickle through the seafloor and lower the pH (and increases the pCO_2) to levels that are representative of global ocean acidification projections for year 2100.

1.2 The ocean acidification process

Since the pre-industrial era, increased burning of fossil fuels has increased the carbon dioxide (CO_2) in the atmosphere by nearly 40%, with approximately half of this increase occurring in the last three decades (Feely et al., 2009). The world's oceans have passively absorbed about 50 % of all this atmospheric CO_2 (Sabine et al., 2004). As the CO_2 is absorbed into the seawater, it reacts through a series of chemical events leading to an excess of H^+ ions and lowering of pH, calcium carbonate ion concentrations and calcium carbonate mineral saturation states (Ω) worldwide. This process is called ocean acidification (OA).

The chemical reactions that result in OA begin when carbon dioxide gas from the atmosphere ($CO_{2\,(atm)}$) enters surface seawater and dissolves to form aqueous carbon dioxide ($CO_{2\,(aq.)}$). This is due to the atmospheric-ocean equilibrium exchange, which holds a proportional ratio of CO_2 concentrations ($[CO_2]$) in the atmosphere and surface water. The equilibrium is reached within several months. After the CO_2 is in the seawater, two main equilibrium reactions occur between the aqueous carbon dioxide ($CO_{2\,(aq.)}$), water (H_2O), carbonic acid (H_2CO_3), bicarbonate ions (HCO_3^-), and carbonate ions (CO_3^{2-}). The first reaction creates carbonate ions (CO_3^{2-}) and protons (H^+) lowering seawater pH (Equation 1).

Equation 1. $$CO_{2\,(atm)} \leftrightarrow CO_{2(aq.)} + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+$$

The second reaction consumes carbonate ions (CO_3^{2-}) but does not change pH (Equation 2):

Equation 2. $CO_{2\,(aq.)} + H_2O + CO_3^{2-} \leftrightarrow 2HCO_3^-$

Both reactions occur in modern oceans and result in lowering pH and decreasing carbonates. Furthermore, a third reaction involves the formation and dissolution of solid calcium carbonate minerals ($CaCO_{3(s)}$) (Equation 3):

Equation 3. $CaCO_{3(s)} \leftrightarrow Ca^{2+}_{(aq)} + CO_3^{2-}$

The '(s)' indicates a solid state, and '(aq)' the dissolved state of calcium carbonate minerals ($CaCO_3$).

At equilibrium, the calcium ion concentration ($[Ca^{2+}]$) times the carbonate ($[CO_3^{2-}]$) concentration is equal to a constant called the apparent solubility product ($[Ca^{2+}] \times [CO_3^{2-}] = K'_{sp}$). The saturation state of carbonate mineral phases (Ω_{sp}) is defined by $\Omega_{sp} = [Ca^{2+}]\,[CO_3^{2-}] / K'_{sp}$, with 'sp' representing the $CaCO_3$ 'species'. The apparent solubility product varies with temperature, salinity, pressure, and also the different calcium carbonate minerals, or 'species' (e.g., calcite, aragonite, and high-Mg calcite). The three primary biogenic carbonate-containing mineral phases in seawater are aragonite, calcite and magnesium (Mg) calcite. Aragonite and calcite are naturally occurring polymorphs of calcium carbonate with differing crystal lattice structures and hence different solubilities (aragonite is 1.5 times more soluble than calcite at 25 °C). Mg calcite is a variety of calcite that substitutes Ca^{2+} ions for Mg^{2+} ions in a disordered calcite lattice (Gattuso, 2011). Depending on the molar ratio of Mg^{2+} and Ca^{2+} ions, its solubility can be more or less than aragonite (Feely et al., 2009). The decrease in seawater carbonate ions from Eq. 2 reduces the saturation state of $CaCO_3$ in seawater. When $\Omega_{sp} = 1$, the solution is in equilibrium with that mineral phase; if $\Omega_{sp} < 1$, the solution is supersaturated with respect to that particular mineral phase; and when $\Omega_{sp} > 1$, it is undersaturated (Gattuso, 2011). Throughout this thesis, the term 'OA-conditions' and 'low pH conditions' are used to refer to the cumulative reactions of OA described in this section, unless otherwise stated.

1.3 Ocean acidification trends

Ocean acidification is clearly documented from ocean time-series measurements beginning in the 1990s (Dore et al., 2009; Feely et al., 2009). The trends through the following two decades illustrated the existence of a strong correlation between rising levels of CO_2 in the atmosphere, rising levels of CO_2 in the ocean and decreasing seawater pH (Dore et al., 2009). Multiple time-series records and survey measurements

from seawater collections in other oceanic systems over the last 20-30 years have further validated this correlation with pH (Brewer, 2013; Doney et al., 2009a). From this, predictive models have demonstrated that the current ocean surface water pH is already on average 0.1 pH unit lower than it was prior to the industrial revolution, this is approximately a 30% increase in acidity (IPCC, 2014; Caldeira and Wickett, 2003).

Based on the current emission trends, the Intergovernmental Panel of Climate Change (IPCC) has modeled future emission scenarios with biogeochemical projections to better understand how emitting atmospheric CO_2 at levels that are greater, lower, or unchanged from current figures will affect oceanic pH (IPCC, 2014). Unchanging current emission patterns, termed as "business-as-usual scenario," or the IPCC's Representative Concentration Pathway (RCP) 8.5, predict the pH will decrease an additional 0.3- 0.4 pH units sometime between the year 2081 and 2100, a 100-150% increase in acidity from present (IPCC, 2014; Orr et al., 2005). However, even if emissions are completely stopped during the next century, the effects of past CO_2 input will continue to load the natural oceanic carbonate system for the next 100 -1000 years. If all emissions were stopped today, pH would continue to increase because oceans would still need 100-300 years, at minimum, to stabilize, and reach a new equilibrium (IPCC, 2014).

The closest comparable event to that which is occurring now happened 55 million years ago during the Paleocene-Eocene Thermal Maximum (PETM) (Zachos et al., 2005). A rapid decrease in oceanic pH was identified with the event, yet, this decrease was ten times slower than that of the current rate of OA (Hönisch et al., 2012). Furthermore, between the last 800,000 years and the preindustrial era, the concentration of CO_2 in the atmosphere and in the surface seawater has been in equilibrium, at an average stable ocean pH around 8.2 units (Lüthi et al., 2008). As such, it is believed that the contemporary seawater chemistry is changing at a rate faster than ever before (Hönisch et al., 2012).

1.4 Current ability to predict the effects of OA on marine organisms

As research further advances our knowledge of the OA processes and associated trends, there has been a growing concern that marine organisms will experience severe altered health and function, leading to reduced marine biodiversity (Raven et al., 2005; Widdicombe and Spicer, 2008). This concern has prompted a large research effort attempting to predict what will happen to marine life (Gaylord et al., 2014; Harley, 2011; IPCC, 2014; Kroeker et al., 2013a). This research has predominantly used pCO_2 perturbation experiments where single life history stages of various marine species are exposed for short periods to decreased pH/elevated pCO_2 (perturbation experiments, where pCO_2 is the partial pressure of CO_2 gas in the seawater).

These short-term experiments have revealed a range of impacts that vary within the life-stages, species, intra-species, and populations (reviewed in: Dupont et al., 2010a; Fabry et al., 2008; Gazeau et al., 2007; Kleypas et al., 2006). Positive effects appear to be primarily associated with non-calcifying algae and marine plants that can benefit from the increased CO_2 (Garrard et al., 2014; Koch et al., 2013; Reusch, 2014). Negative effects are primarily associated with calcifying algae and marine metazoans (Gazeau et al., 2007; Kamenos et al., 2013; Kroeker et al., 2013a). Marine metazoans with calcium carbonate skeletons, such as molluscs, crustaceans and echinoderms, are supposed to be particularly susceptible to OA (Kroeker et al., 2013a; Wood et al., 2008). However, recent experimental manipulation studies have also revealed no effects, little effects, and/or mixed effects in extant marine metazoans, including calcifiers, exposed to future OA conditions (reviewed in Browman (2016), and references within. While this is by no means an exhaustive account of the current knowledge on the potential impacts of marine species, it demonstrates that no cohesive conclusion or inference from these short-term experiments is clear.

One important characteristic of these studies is that they are based on time-scales that do not represent realistic exposure periods (Bell and Collins, 2008; Doney et al., 2009a; Gattuso, 2011). They may potentially misrepresent the complexity of OA impacts on marine life by neglecting to simulate real-world scenarios where OA effects occur in ecosystems that are modified over time scales difficult to simulate in laboratory settings (Visser, 2008; Widdicombe and Spicer, 2008; also see Browman, 2016). However, some real-world scenarios are illustrating how marine metazoans are already being challenged by OA. Because the magnitude of OA varies with depth (Caldeira and Wickett, 2003), latitude (Orr et al., 2005), and habitat, there are a number of areas around the world that appear to be most vulnerable to OA (Gruber, 2011; Hauri et al., 2009). The most well known areas are upwelling gradients such as those found on the west coast of the United States and Chile (Feely et al., 2008; Hauri et al., 2009). OA is causing exceedingly high pH variability at these sites, raising the baseline, and marine life is reacting (Waldbusser and Salisbury, 2014). Pteropod (small planktonic molluscs) assemblages have migrated away from such sites (Bednarsek and Ohman, 2015; Bednarsek et al., 2012). The change in distribution patterns associated with these migrations have only occurred in the last few years, with no significant changes recorded in 2009 distributions (Ohman et al., 2009). In addition to pteropod distributions, oyster larvae in similar upwelling areas have been severely compromised by OA (Barton et al., 2012). Commercial aquaculture facilities off the coast of Oregon and Washington, USA have documented a direct correlation between larval deformation and mortality and OA-induced seawater conditions (Barton et al., 2015).

Again, these sensitivities have only been realized in the last few years as natural variability in seawater chemistry is exceeded by the effect of OA. These cases are

predicted to increase and result in subsequent loss of species diversity (Doney et al., 2012), ascendance of weedy taxa, shifts in patterns of demographic connectivity (Gaylord et al., 2014), modified consumer-resource relationships/ food web interactions (Bednarsek and Ohman, 2015), and increased economic calamities (Cooley et al., 2012; Mathis et al., 2014; Turley et al., 2010). Additionally, they confirm predictions that water chemistry is quickly changing due to OA and support the view that some marine organisms/taxa are, and will, continue to be adversely affected.

1.5 Processes of evolutionary persistence

In order to survive the mounting OA conditions, populations of marine organisms must be able to maintain their capacity to function under the new environmental settings and/or successfully adapt (Bell and Collins, 2008). This continued or prolonged existence can be defined here as evolutionary persistence. Such persistence to OA may occur if the organisms either already possess phenotypes tolerant to OA conditions (Melzner et al., 2009), or can persist *via* phenotypic plasticity and/or genetic adaptation (Bell and Gonzalez, 2009; Kelly et al., 2013). The subsections below overview these processes, and focus on how they can – and are – beginning to be applied to ocean acidification research on marine metazoans.

Box 1. Key terms in adaptation and plasticity research

Adaptation: refers to both the current state of being adapted to a given environmental condition (i.e. to possess an **adaptive trait** with a current functional role in the life of an organism that is maintained and evolved by means of natural selection), as well as the dynamic evolutionary process that leads to the adaptation of a trait in a organism. Adaptations enhance the fitness of individuals by natural selection in natural populations (Falconer and Mackay, 1996).

Local adaptation: the fine-tuning of a population to their local environment *via* natural selection, which results in resident genotypes that have a higher fitness in their native habitat than genotypes from more distant populations (Bell 2008, Sanford and Kelly 2011).

Darwinian fitness: a measure of the capacity of a variant type to invade and displace the resident genotype in competition for the available resources (Demetrius and Ziehe, 2007; Garland and Rose, 2009). Evolution selects for highest fitness as a function of lifetime fecundity and survival (Roff, 1997).

Reaction norm: the expected phenotype of a given genotype as a function of the environment (Chevin et al., 2010).

Genotype: at the population level, it is the average difference among genotypes, across environments; at the individual level it is the actual set of genes affecting the phenotype and shaping all aspects of the norm of reaction (i.e. both its plasticity and 'height' in an environment-phenotype space) (Pigliucci, 2005).

Phenotype: the composite of an organism's observable characteristics or traits, including its morphology, development, biochemical or physiological properties, phenology, behavior, and products of behavior; an expression of both genetic and environmental factors (Chevin et al., 2010).

Phenotypic plasticity: at the population level, it is considered the average differences found among environments, across genotypes; at the individual level, it is an attribute of the individual reaction norm, indicating that the genotype (through interactions with the environment) generates different phenotypes depending on the external conditions. The only case of zero plasticity is when the reaction norm is flat and parallel to the environmental axis (DeWitt and Scheiner, 2004; Pigliucci, 2005; West-Eberhard, 2003).

Adaptive plasticity: phenotypic changes that move the phenotype closer to the fitness optimum for genetic selection (Ghalambor et al., 2007).

Non-adaptive plasticity: the environmentally induced phenotype in the new environment is further away from the new adaptive peak compared to the ancestral phenotype (Ghalambor et al., 2007).

Developmental plasticity: occurs when exposure to a novel environment at a specific life stage affects its performance in that environment at a different phase of life, either intra-or trans- generationally (West-Eberhard, 2003).

Genetic accommodation is a modern term used to describe the process of heritable changes that occur in response to a novel induction. It is a mechanism of evolution wherein a novel phenotype, generated either through a mutation or environmental change, is refined into an adaptive phenotype through quantitative genetic changes, and can result in either increased or decreased environmental sensitivity of a plastic phenotype (Crispo, 2007).

Genetic assimilation: a type of genetic accommodation where the new mean fitness phenotype is favorably selected by the process of directional selection, and is genetically determined and canalized (a loss of plasticity or a flat reaction norm) (Ghalambor et al., 2007).

1.5.1 Tolerant phenotypes

Various aspects of an organism's ecology, life history, and physiology are responsible for its tolerance in new environments, and these are important components to the probability of their persistence (Bradshaw, 1984; Lee, 1999; McLain et al., 1999). However, little research focus is currently placed on identifying those marine metazoans that might be more tolerant to the 'new oceanic environment' resulting from the changes in ocean chemistry (OA). Identifying and comparing vulnerable and tolerant counterparts (i.e. congeneric species) and their associated phenotypes, is an important first step in understanding what processes might lead to ecological persistence in a future ocean (Calosi et al., 2013; Lewis et al., 2013; Maas et al., 2011; Melzner et al., 2009; Reznick and Ghalambor, 2001).

The identification of OA tolerant metazoans has been aided by the recent use of natural systems with levels of pH/ pCO_2 comparable to levels of future projections (Ricevuto et al., 2014; Somero, 2012). These systems are found throughout the world in coastal upwelling areas (Feely et al., 2008), fjords (Thomsen et al., 2010), acidified estuaries (Chaparro et al., 2011), coastal aquaculture sites (Parker et al., 2010) and volcanic CO_2 vents/seeps (Fabricius et al., 2014). Volcanic vent systems that exclusively emit CO_2 have been specifically identified as unique proxies for OA studies as they are not driven by potentially confounding oceanic processes such as low oxygen or high nutrients (Hall-Spencer et al. 2008). Additionally, they are found in oceans around the world (e.g. Mexico (Crook et al., 2016), Papua New Guinea (Fabricius et al., 2011), and Greece and Italy (Hall-Spencer et al., 2008; Johnson et al., 2013).

A myriad of marine metazoan species that inhabit such systems have been identified, and they all have a 'standing' tolerance to the low pH conditions produced by their specific site. These have been primarily been classified from abundance and distribution data. For instance, certain species of copepod, amphipod, and non-calcifying polychaetes have been identified as comparatively tolerant species at some volcanic vents; see: (Cigliano et al., 2010; Fabricius et al., 2011; Kroeker et al., 2011). In a low pH fjord, mussels and gastropods were found to dominate (Thomsen et al., 2010). These findings have both dissuaded across-taxa generalizations and encouraged more detailed research into specific phenotypes affiliated with the tolerant organisms/taxa.

The phenotypes of tolerant metazoans may best be investigated with comparative studies that look at closely related congeners from these natural systems, and make inferences on how differences in their ecological, life history, or physiological traits may be driving their differential tolerances (Somero, 2010). For example, Dupont et al. (2010b) found that invertebrates with lecithotrophic larvae were found with a higher level of OA tolerance compared to those with planktrophic larvae within a low pH fjord system. The patterns derived from such systems can also test theories of phenotypes that promote

persistence. For instance, the theory that marine ectothermic metazoans with extensive extracellular fluid volumes may be less vulnerable as their cells are already exposed to much higher pCO_2 values than those of unicellular organisms and gametes, for which the ocean is the extracellular space (Melzner et al., 2009), can be strengthened/tested by looking at which organism classes are found in natural systems. By identifying these tolerant organisms, scientists can then do more focused research on their biology to look for potential mechanisms or traits underpinning their tolerance. This can then direct research towards understanding the extent to which these traits, phenotypes are 'standing'- driven by genotype, or 'flexible' –driven by phenotypic plasticity, and if either, or both, can change or adapt in species' populations.

1.5.2 Adaptation and phenotypic plasticity: mechanisms of OA persistence?

Adaptation and plasticity theory

Adaptation is considered to be the most important way in which marine life will persist in future oceans (Kelly and Hofmann, 2012; Sunday et al., 2013). Genetic adaptation can occur *via* natural selection acting upon existing phenotypic/genotypic variation and upon genetic mutations (Bell and Collins, 2008). Phenotypic plasticity has also been proposed as a potentially important *mechanism* by which natural populations might adapt rapidly to novel environments (Ghalambor et al., 2015; Merilä, 2015; Réale et al., 2003). However, very little research has explored the adaptive potential of marine organisms in response to OA, and even fewer studies have looked at the importance of phenotypic plasticity as a mechanism in adaptive evolution (Munday et al., 2013; Sunday et al., 2013).

Plasticity has often been thought to constrain or slow down the rate of evolution (de Jong, 2005; Huey et al., 2003). Conversely, the perspective in this thesis assumes that phenotypic variation, even when environmentally induced and not under strict genetic control, will play an important role in creating the conditions that may result in an adaptive genetic response (Ghalambor et al., 2007; West-Eberhard, 2003). In this perspective, populations subjected to new environmental conditions may be initially established *via* phenotypic plasticity, and then through evolutionary processes (i.e. genetic accommodation; Box 1) become genetically established (Ehrenreich and Pfennig, 2015; Pigliucci, 2005; West-Eberhard, 2003). This is presumed to be a mechanism that rapidly facilitates the process of evolution, and holds high relevance in terms of OA change (Hallgrímsson and Hall, 2005; Scoville and Pfrender, 2010).

Phenotypic plasticity is defined as the ability of a single genotype to express different phenotypes (i.e. its morphology, life history, or behavior) in response to different environmental conditions (Box 1; West-Eberhard (2003)). Plasticity can occur at various time-scales within the lifetime of the organism, or when the environment

experienced by the parents influences the performance of offspring in the same environment through nutritional, somatic, cytoplasmic, or epigenetic transfer between generations (West-Eberhard, 2003). Phenotypic plasticity can also be described as moving in an adaptive or non-adaptive direction in regard to the organisms' fitness (Ghalambor et al., 2007). It may change between categories (i.e. from non-adaptive plasticity to adaptive), or from one developmental life stage to another (i.e. high plasticity as juveniles but not as adults) (see Box 1 for definitions and references). Here, I consider how any plasticity that is either adaptive or non-adaptive with respect to fitness, may promote adaptive evolution.

The predominant hypothesis for how phenotypic plasticity will promote evolutionary adaptation is that marine organisms more able to exhibit plastic responses will be more likely to adjust to the changes of OA (Chevin et al., 2010). In this way, plasticity may act as an initial mechanism by which marine organisms could persist in a novel oceanic environment (Merilä and Hendry, 2014). Here, plasticity is considered to be adaptive, where the phenotypic changes aid in survival under the changed conditions, and bring the phenotype closer to the optimum for genetic selection (Ghalambor et al., 2007). An alternative hypothesis for how phenotypic plasticity will promote evolutionary adaptation is *via* non-adaptive plasticity. This is where plastic changes do not contribute to increased fitness (Box 1), representing a fundamentally different kind of environmentally induced plasticity that may be more realistic in terms of the 'stressful' pressures of OA (Ghalambor et al., 2007).

Non-adaptive plasticity has primarily been associated with limiting population persistence (Chevin et al., 2010), however non-adaptive plasticity is perceived to be the most likely type of plasticity to occur in response to stressful environments (Ghalambor et al., 2007). The available evidence on OA effects demonstrates that for most marine organisms (excluding plants), OA causes stress and is comparable to other stressful new environments (Hallgrímsson and Hall, 2005). Ghalambor et al. (2007) define stress as being outside the range of conditions typically experienced by population, and the two main challenges for a newly established population in a new stressful environments as (1) maintaining homeostasis and proper development, and (2) responding to strong directional selection. Populations can overcome the first challenge by buffering themselves against the stress so that proper development and function can still occur; i.e. phenotypic buffering, where the level of stress is moderate (Reusch, 2014; Scharloo, 1991). This can result in a change in phenotypic variance, and disappearance of the phenotypes unable to cope with the stress (Pfennig et al., 2010). As a result, non-adaptive plasticity may facilitate adaptive genetic changes by increasing the strength of natural selection on the phenotypes that are able to persist under the novel conditions (Ghalambor et al., 2015; Merilä, 2015).

The extent to which plasticity (either adaptive or non-adaptive) acts as a mechanism of genetic adaptation in nature, however, are still wrapped in contention (de Jong, 2005; Ghalambor et al., 2015; Pigliucci, 2005; West-Eberhard, 2003). Unraveling this contention is particularly relevant, in terms of the novelty of the rapidly changing and restructuring OA is causing in global oceanic environments (Palumbi, 2001).

Experimental approaches

In marine systems, two main approaches have been identified to determine the potential of adaptive evolution *versus* plasticity. The first approach is based on quantitative genetic breeding designs that are used to identify, and separate, additive genetic variance in traits such as CO_2 tolerance from phenotypic variance (Foo et al., 2012; Kelly et al., 2013; Sunday et al., 2011). Breeding designs only describe the *potential* for adaptive evolution by assuming variance for traits analyzed will result in evolution *versus* studying the end result of past evolutionary adaptation (Reusch, 2014). To date, breeding designs have not focused on traits that are necessarily fitness related (e.g. growth rates, not survival), and they primarily consider phenotypic variance to be a complicating factor (Kelly et al., 2013; Sunday et al., 2011).

In the second approach, synchronic comparisons (through space) of populations are made in natural systems, and these case studies report phenotypic differences that are consistent with local adaptation and/or plasticity among subpopulations from contrasting habitats (Garland and Rose, 2009; Reznick and Ghalambor, 2001; Sanford and Kelly, 2011). Synchronic comparisons can test species with longer life spans as they have had time to adapt in the natural systems that would not be possible in an experimental setting (Bell and Collins, 2008; Sanford and Kelly, 2011). These studies may also be most realistic, as they represent past adaptations that have occurred through a complex web of evolutionary pathways impossible to account for with breeding experiments or experimental evolution (Bell and Collins, 2008).

Synchronic comparisons can be made with reciprocal transplant experiments. This approach is considered to be best way to assess if changes are adaptive by isolating phenotypic plasticity from adaptation results (Merilä and Hendry, 2014; Nuismer and Gandon, 2008). They determine levels of adaptation and plasticity among populations living in low pH areas, and whether low pH persistence of the population is enabled by forms of adaptation and/or plasticity (Ayrinhac et al., 2004; Etterson and Shaw, 2001). In this approach, individuals are taken from different field habitats and held in their respective (and their own) 'habitat' conditions for multiple generations. Following this grow-out period, their progenies are relocated to the *in situ* source and test habitats, after which their fitness (e.g. survival) is quantified (Falconer and Mackay, 1996; Kawecki and Ebert, 2004). The performance of local genotypes can then be explored using reaction norms and analysis of variance to test for the relative importance of local adaptation

(significant differences between trait means between populations), plasticity (significant effects of environment) or genotype * environment interactions (Nuismer and Gandon, 2008). The resulting reaction norms from such experiments can be used to infer if genotypes differ in their plasticity (West-Eberhard, 2003). Reaction norms bridge the gap between phenotypic plasticity and quantitative genetic studies of natural selection by connecting quantitative phenotypic plasticity and genotype (Dam, 2013; Murren et al., 2014; Pfennig et al., 2010). By examining fitness-influencing (phenotypic) traits through reaction norms, important distinctions between adaptive (genotype) and plastic (plasticity) drivers can help to elucidate the role of plasticity in promoting genetic adaptation.

Evidence from synchronic comparisons of adaptation and plasticity

Synchronic comparisons based in low pH/high pCO_2 systems described above have only recently been utilized. From the few existing studies, only one case of local adaptation in direct response to low pH (OA conditions) has been identified. Pespeni et al. (2013a, 2013b) have found the first evidence of local adaptation to OA conditions in the widely distributed green sea urchin – yet this was based on molecular evidence, and no phenotypic differences were observed. Their experiment was established in the same upwelling area where pteropods and oysters are currently being threatened on the west coast of the USA (Section 1.4; Bednarsek and Ohman, 2015). Current work to identify the functionality of genes associated with this adaptation is in progress, and the results will likely establish a needed bridge for research identifying local adaptations using molecular markers (Sanford and Kelly, 2011).

Reciprocal transplants based at low pH /high CO_2 volcanic vent systems using field collected and transplanted specimens provide additional examples of such synchronic comparisons. The limpet, *Patella caerulea*, from a low pH vent site calcified more under all conditions, suggesting some adaptively increased calcification rates (Rodolfo-Metalpa et al., 2011). Other studies have identified plasticity as the primary mechanism for observed phenotypic changes. For instance, the polychaete *Amphiglena mediterranea* from a low pH vent site in Ischia, Italy, increased its metabolism under low pH conditions, an effect which was attributed to phenotypic plasticity (Calosi et al., 2013). Likewise, the gastropod *Hexaplex trunculus* from another low pH vent site (Vulcano, Italy) also demonstrated increased metabolic activity in low pH conditions, which the authors also attributed to plasticity (Harvey et al., 2016).

These synchronic comparisons demonstrate how measurements of *potential* adaptive change and plasticity can be assessed using a variety of measurements of different phenotypic traits (i.e. acute metabolic change, growth). Unfortunately these studies have provided little evidence that the assessed phenotypic traits are representative of actual fitness (i.e. Foo et al., 2012; Sunday et al., 2011). None of the described

synchronic studies so far have looked at long-term fitness-related traits related to reproduction and survival. Linking lifetime fecundity and survival to the reciprocal transplant approach described here by incorporating effects through full life cycles will be an important way to add validity to these studies' evolutionary interpretations (Irschick, 2003; Reznick and Ghalambor, 2001).

1.6 Study Site

The above sections emphasize how little is known about the evolutionary persistence of marine metazoans with respect to OA. They also highlight the approaches that can be used to identify standing OA 'tolerant' phenotypes, phenotypic plasticity and/or genetic adaptation in response to OA. This thesis explores these approaches using natural systems. The natural system that this thesis uses to explore OA persistence is located on the north-eastern coast of Ischia Island (Naples, Italy), around the Castello Aragonese islet on the Mediterranean Sea (Figure 1.1, 40° 43' 83" N, 13° 57' 08" E). Here, an underwater volcanic vent system is responsible for CO_2 gas bubbling up through the seafloor into shallow waters (de Alteriis et al., 2010; Tedesco, 1996). This volcanically derived CO_2 gas mixes with seawater decreasing the pH of the venting area from an ambient value of ~8.17 to as low as 6.57 (Hall-Spencer et al. 2008). Additionally, the business-as-usual IPCC pH projections for 2100 (pH 6.5-7.8, see Kroeker et al. 2011; IPCC, 2014) are well represented by this low pH ecosystem (IPCC, 2014).

The vents are located along both sides of a peninsula called the Castello Aragonese islet: on the north side, which is relatively exposed to the dominant northwestern winds; and on the south side, which is a bay-protected area. Due it its geography, the area is designated as two gradients, the north and south gradients, both approximately 300 m in length. The venting area in the south is approximately 3000 m^2 and gases are emitted at a rate of 1.4 x 10^6 L d^{-1}. In the north, the area is only 2000 m^2 and the venting rate is slightly decreased at 0.7 x 10^6 L d^{-1} (Hall-Spencer et al., 2008). Specific quantities of the emitted gases are comprised of the following: 90-95 % CO_2, 3-6 % N_2, 0.6-0.8 % O_2, 0.2 - 0.08 % CH_4, and 0.08-0.1 % Ar. No sulfur is present (Cigliano et al., 2010). Neither seasonal, tidal nor diurnal variation in gas flows have been recorded, however pH does not stay static due to variable bubbling intensity.

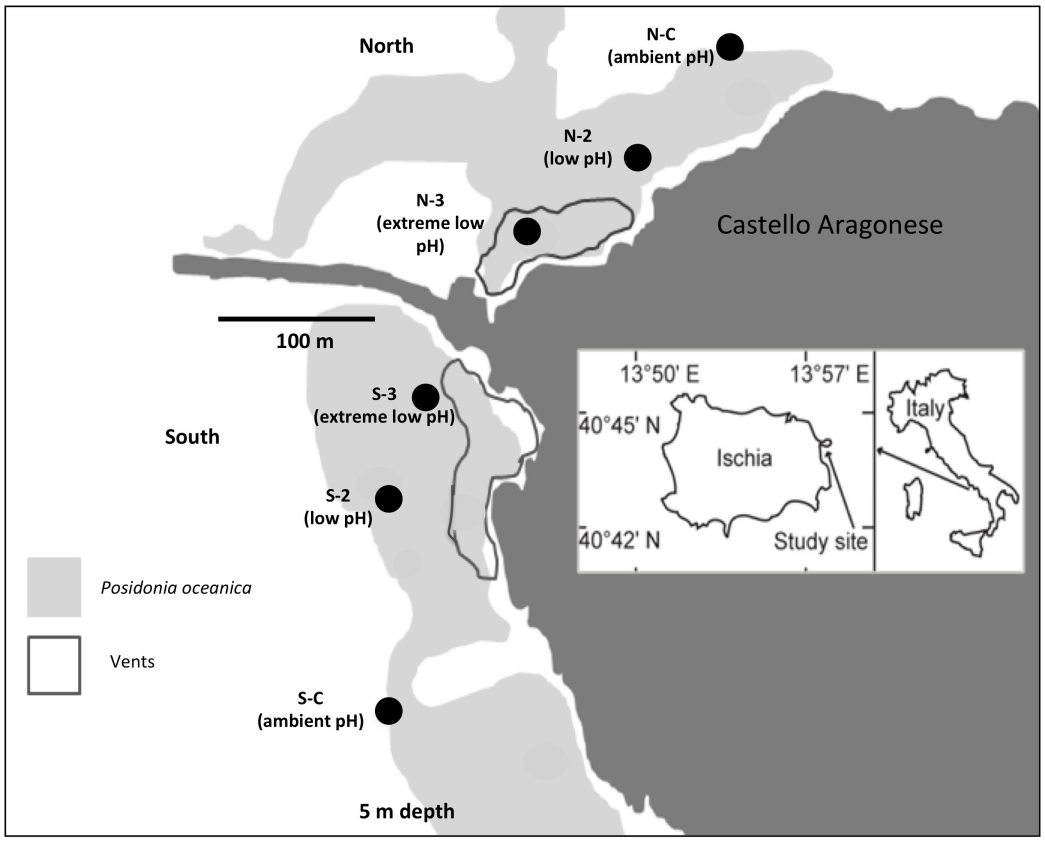


Figure 1.1 Castello Aragonese at Ischia Island (Naples, Italy) with the sampling sites (black dots) at both the south side (S-C, S-2, S-3) and north side (N-C, N-2, N-3) gradients, corresponding to seawater carbonate data in Table 1.1; *Posidonia* seagrass meadows (gray patches), venting sites (black solid lines), and land indicated by the dark gray patches. Modified from Gambi, M.C.

Both the north and south gradients have been divided into three sites for many of the previous studies based there, and these sites are referred to as N3, N2, NC and S3, S2, SC, where "3" represents the extreme-low pH, "2" the low pH conditions, and "C" the control, ambient pH. The "N" and "S" represent north and south sides of the gradient (Figure 1.1) (see also Cigliano et al., 2010; Hall-Spencer et al., 2008; Ricevuto et al., 2012, 2014). Furthermore, a number of these past studies have assessed the variability in water parameters and carbonate chemistry throughout the last six years (Cigliano et al., 2010; Hall-Spencer et al., 2008; Kroeker et al., 2011; Ricevuto et al., 2014). A dataset combining the water parameters from each study at each site is shown in Table 1.1. Little differences among sites are found in the other parameters: temperature does not deviate between sites, nor does salinity (36), alkalinity (2.5 mequiv kg^{-1}), or the tidal range (between 0.30-0.50 m *per* day) (Ricevuto et al., 2014).

Table 1.1 Seawater physico-chemistry parameters from each pH site (mean ± SD); averaged from a published compilation of six time-series datasets between 2008-2015 in (Ricevuto et al., 2014).

Station	mean pH	pCO_2(µatm)	Ω aragonite	Ω calcite	TA (equival Kg-1)
Extreme low, S3	6.99 ± 0.34	8830.87 ± 1942.55	0.75 ± 0.50	0.99 ± 0.65	2499.83 ± 23.99
Low, S2	7.61 ± 0.26	2031.19 ± 1,411.65	1.49 ± 0.61	2.52 ± 0.95	2523.68 ± 9.66
Ambient, SC	8.03 ± 0.08	455.61 ± 94.01	3.36 ± 0.34	5.17 ± 0.47	2499.35 ± 6.94
Extreme low, N3	7.39 ± 0.25	4302.71 ± 5769.224	1.41 ± 0.71	1.94 ± 0.96	2549.45 ± 25.264
Low, N2	7.65 ± 0.29	2639.82 ± 7993.29	2.07 ± 0.70	2.91 ± 1.23	2514.49 ± 7.76
Ambient, NC	8.03 ± 0.05	468.21 ± 63.85	3.41 ± 0.20	5.20 ± 0.28	2499.67 ± 4.68

Both the northern and southern sites are characterized by the presence of seagrass *Posidonia oceanica* meadows at 0.5-3 m of depth, as well as rocky algal-reef formations, (Fig. 1.2) (Buia et al., 2003). These areas host a diversity of benthic assemblages, which are dominated by common invertebrates such as crustaceans, polychaetes and echinoderms (Garrard et al., 2014; Kroeker et al., 2011). The organisms inhabiting this area represent a model system for examining the emergent ecosystem responses to ocean acidification in benthic marine communities common to most of the ocean (Hall-Spencer et al., 2008). For example, spatial changes in species distribution, diversity and abundance in relation to the most intense venting sites (low pH) have suggested that under future OA conditions, marine ecosystems are likely to also experience severe reductions in the diversity and abundance of calcifying species (Cigliano et al., 2010; Hall-Spencer et al., 2008; Kroeker et al., 2011; Ricevuto et al., 2012).

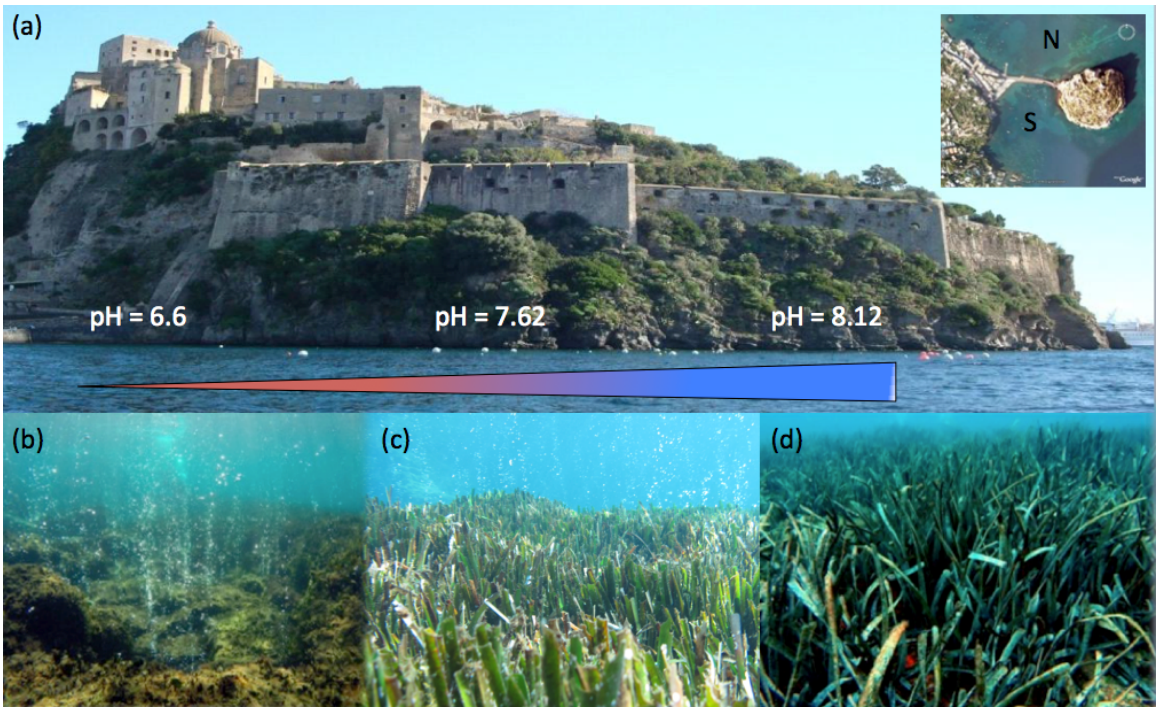


Figure 1.2 Castello Aragonese Study Area in Ischia, Naples – Italy; (a) south side and areal view of the Castello islet, (b-d) underwater habitats in the southern gradient, corresponding to the above pH.

Beyond serving as a good proxy for understanding future ecosystem responses, the history of the Castello vents also endorses its use as an analog for exploring species' evolutionary responses to OA/low pH conditions. Archaeological and historical evidence suggests that this underwater area was above sea level in the fourth century B. C., but that the region underwent a tectonic lowering and was flooded by about 130-150 A.D. (Lombardi et al., 2011a). As such, subsurface vent activity has likely been present for no more than 1,800 years. Furthermore, the intensity of the venting has probably varied in recent decades; as they were not as active and intense in the early 1980s as they are currently (Gambi M.C., *pers. obs.)*. Therefore, the organisms inhabiting this vent site may be employing the mechanisms needed to evolutionary persist in low pH habitats within timescales highly representative of those necessary for coping with future ocean acidification in the next century (Hairston et al., 2005; Visser, 2008).

1.7 Study Assemblage

In this thesis, the biological focus within the Castello vent site of Ischia is its native polychaete assemblage. These polychaetes are considered to be good models to study adaptation potential to OA (Giangrande et al., 2014) for two main reasons. First, they are one of the most abundant taxa thriving in this area, and both sessile calcifying and semi-sessile non-calcifying polychaete species have been previously identified and studied in these vents (Cigliano et al., 2010; Kroeker et al., 2011). Second, polychaetes, as a class, have demonstrated a remarkable ability to adapt to extreme environments (Fauchald,

1977; McMullin et al., 2000). The group has a long evolutionary past: polychaetes have been dated back to the Cambrian Era 500 million years ago, with some fossil evidence even recording larvae matching polychaete structures in the Pre-Cambrian Eras (Chen et al., 2000). As such, these marine metazoans have developed a variety of forms and functions for overcoming difficult conditions within geological timeframes, and are highly useful for understanding evolutionary outcomes and consequences. For these reasons, I use the polychaetes in the Castello vents of Ischia as a proxy for future evolutionary forecasts in regard to OA.

1.8 Thesis aims and objectives

The main aim of this thesis was to determine how marine metazoans might persist as OA conditions intensify using polychaetes from a natural system representative of global OA projections for or before the year 2100.

This aim was achieved by addressing three objectives:

(1) to identify OA-tolerant phenotypes by assessing the distributions, abundances and life history traits of marine polychaetes persisting in the low pH Castello vent environment (Chapter 2 and 5).

(2) to determine the relative importance of phenotypic plasticity and/or local adaptation as mechanisms responsible for the low pH tolerance found in a population of the polychaete species *Simplaria* sp. (Chapter 3 and 4).

(3) to create a framework for identifying marine invertebrate species that are more likely to persist under the environmental conditions predicted to occur in the world's oceans (Chapter 5).

The chapters in this thesis are structured as follows:

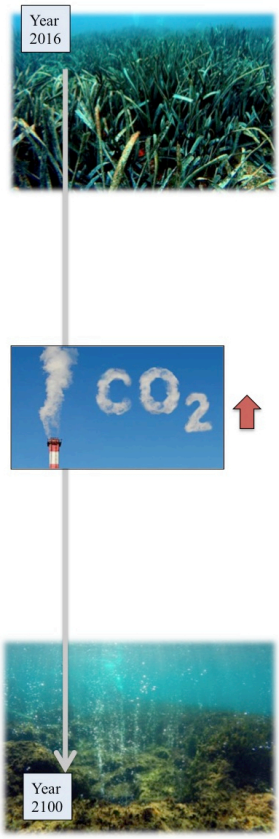
In Chapter 2 I report the results (a) for a field survey that I have performed to characterize the distributions of calcifying polychaete species along the pH gradients of the Castello vent system and (b) for a comparative species recruitment experiment that I have performed in order to investigate how differences in life history traits between two closely-related species (*Pileolaria militaris* Claparède 1870 and *Simplaria* sp.) vary with respect to differing pH-related distributions.

In Chapter 3 I investigate the relative importance of local adaptation and/or phenotypic plasticity in the persistence of the *Simplaria* sp. polychaete population the naturally low pH Castello ecosystem using reciprocal transplant experiments with two F1 generation polychaete populations, one from a low pH site and the other from a control pH site. Experiments assessed early life stage responses and adult life stage responses in the laboratory.

In Chapter 4 I consider the relative importance of local adaptation and/or plasticity in a field-based *in situ* reciprocal transplant experiment, using second-generation offspring from the same polychaete populations described in Chapter 3.

In Chapter 5 I perform breeding experiments to characterize developmental modes associated with low pH tolerance. I then explore the patterns between the species that are comparably dominant in low pH areas and their life history traits to create a framework that can be broadly used to identify other types of marine metazoans that are more likely to be able to adapt to, and survive, under the predicted environmental conditions.

In my final chapter (Chapter 6), I provide a general summary of my thesis including its implications for our understanding of marine metazoans' evolutionary potential to ocean acidification.



The Problem:
Ocean acidification is rapidly changing all marine environments, and consequently the reaction of marine metazoans to such changes is presumed to be detrimental, altering marine resources and biodiversity. Our current understanding of how this will occur is limited, and does not account for evolutionary responses.

The Solution: Improve our predictive power

How? Using the polychaete assemblage at the low pH vent model site of Ischia, Italy as a proxy for future responses as a tool to explore the following questions:

(1) Are there specific traits or phenotypes that allow species to live in low pH habitats?
  a. What are OA-tolerant phenotypes?
    • Chapter 2 & 5
(2) How important is phenotypic plasticity or adaptation in promoting low pH persistence?
  a. Have the polychaetes from low pH sites adapted to this low pH in order to persist?
  b. Have they responded through phenotypic plasticity?
    • Chapter 3 & 4
(3) What extant species or populations in today's oceans will be able to survive in future oceans?
  a. How can we use current knowledge and datasets to create a framework to identify, now, which marine invertebrates species will be less vulnerable to OA?
    • Chapter 5

Figure 1.3 Schematic of thesis outline.

2 Distribution patterns of functional traits in calcifying polychaete (Spirorbinae) populations settled along a natural pH gradient.

Abstract

Low pH/ high pCO_2 vent systems are excellent 'natural laboratories' for studying the long-term consequences of exposure to low pH conditions, and therefore identifying species tolerance and sensitivity to future ocean acidification (OA). Furthermore, identifying traits associated with tolerant species is a proven method to improve our broad-scale biological predictions of long-term consequences (i.e. functional trait analysis). The calcifying polychaetes (Annelida, Serpulidae) settled in and around the Castello low pH vent system provide a unique biological model for such an analysis. In order to determine if and how the traits associated with this polychaete assemblage varied along the pH gradient, I first observed the distribution and abundance patterns of all the calcifying polychaetes associated with *Posidonia oceanica* seagrass across two natural pH gradients – one along an exposed coastline (mean range 7.39 - 8.03) and one in a relatively sheltered bay (mean range 6.99 -8.03). I then used laboratory trials with two species from different pH sites, to compare how fecundity and settlement traits vary along the pH gradient. From the field survey, I found two dominant, closely related species of polychaete: *Pileolaria militaris* Claparède, 1870 and *Simplaria* sp. (Serpulidae, Spirorbinae), with *Simplaria* sp. a possible morphotype of *Simplaria pseudomilitaris* (C hiriot-Quiévreux, 1965) having distinct operculum spines. Both species were less abundant under extreme low pH conditions (pH: 6.6). *Simplaria* sp. was most abundant at sites with a mean pH of 7.7 and was the only species present at the site with the lowest pH (mean pH ~ 6.6). The following laboratory trials revealed that *Simplaria* sp. from the low pH site had significantly higher reproductive output and juvenile survival in low pH conditions than *P. militaris* from ambient sites, in ambient conditions (mean pH ~ 8.03). Settlement of *Simplaria* sp. from the low pH site was also comparatively fast. These results indicate that the ability of *Simplaria* sp. to tolerate low pH may, in part, be explained by its successful reproduction and rapid settlement under OA conditions.

2.1 Introduction

Our understanding of how organisms are able to persist in locally disturbed environments can be improved using functional trait-based analyses with closely related species. These analyses involve identifying the underlying traits associated with species from a specific environmental condition, by linking environmental gradient surveys (e.g. characterization of species presence or absence and abundant patterns along natural pH and temperature gradients) to biological trait changes along the gradients (e.g. size, reproductive habit, fecundity) (McGill et al., 2006). Some trait analyses in marine systems have successfully demonstrated how traits, or phenotypes, such as body size and stress sensitivity can co-vary with extinction risk (Solan et al., 2004), or how genetically fixed heat tolerance traits can co-vary with seawater warming (Watt and Dean, 2000). Similarly, trait analyses in locally disturbed low pH sites may prove to be useful tools to identify traits associated with species' sensitivities and tolerances to low pH- high pCO_2, which would in turn help to inform predictions on the biological implications of the exposure to OA conditions (Law, 2007; Massot et al., 2008; Root et al., 2003; Solan et al., 2004; Williams et al., 2008).

The locally disturbed low pH site this study focuses on is the low pH Castello CO_2 vent system (see section 1.6). Abundance and distribution surveys at this site have underscored a distinct correlation between decreased pH and the loss of calcifying species (Hall-Spencer et al., 2008; Kroeker et al., 2011). However, these surveys have identified a small number of species, or taxonomic groups, that inhabit the low pH areas, regardless of their status as calcifiers (Cigliano et al., 2010; Ricevuto et al., 2012; Gambi et al., 2016). Some of these species include amphipods, tanaids, isopods (Kroeker et al., 2011), polychaetes (Giangrande et al., 2014), bryozoans (Lombardi et al., 2011b; Rodolfo-Metalpa et al., 2010), and limpets (Rodolfo-Metalpa et al., 2011). The impacts of low pH on these species have been associated with various effects, such as increased growth and defense mechanisms (i.e. bryozoans), and increased metabolic energy (i.e. limpets). Yet, the larval behavior and life histories of these species is an important and generally missing component of these responses.

Understanding the larval behaviors and histories of 'tolerant' species is important as any tolerance patterns that are revealed as adults may be confounded by the possibility that early life stages (i.e. larval phases) were not subjected to the same conditions. Early life stages are suspected to be highly susceptible to the decreased long-term survival of many marine invertebrates (Byrne, 2011a; Byrne 2011b; Kurihara, 2008). For instance, OA induced mass mortality of larvae in oyster hatcheries on the west coast of the USA have emphasized the importance of early developmental responses of marine invertebrates to OA conditons (Barton et al., 2015, 2012). Laboratory studies have also demonstrated numerous examples of sensitivies to low pH as larvae and juveniles (Byrne, 2011a; Dupont et al., 2009; Ross et al., 2011). Despite these findings, the larval traits associated with the vent site's invertebrate inhabitants are not well documented.

Depending on the parental location at the time of reproduction and type of larvae, the organisms found in open environments such as the Castello vents may have been exposed to low pH for multiple generations, or they may have migrated in as pelagic larvae (Ricevuto et al., 2014). It is possible that many recruits haphazardly settle in these areas and cannot persist. This is supported by observations that early stages of succession have showed relatively little difference in coverage of calcifying organisms between the low and control zones (Crook et al., 2016). However, follow-up assessments accounting for post-settlement developmental periods (e.g. 14 months) correspond to extreme declines (70 %) in the presence of calcifying organisms (Crook et al., 2016; see also Kroeker et al., 2013b). Conversely, the recruits that are able to settle and grow into reproductive adults (i.e. the other 30 %) in these conditions demonstrate traits that at the very least are accompanied by post-recruitment low pH persistence (*via* plasticity), and possibly traits that have genetically adapted to the low pH (local adaptation). This points to the possibility that either early and/or adult life stage traits in the species found along natural pH gradients vary between pH level or habitat (Grassle and Grassle, 1974; Levin, 1984).

This study aimed to identify the calcifying polychaetes living in and around the Castello CO_2 vents, and then determine if and how the traits associated with these species varied along the pH gradient using a functional trait approach (McGill et al., 2006). Calcareous polychaetes (Annelida, Serpulidae) are the study subject used for this study. Generally, polychaetes represent one of the few taxonomic groups having a diverse suite of life history traits that can vary among closely related species (Macdonald, 2003). Another attribute beneficial for their use in this study are their dual life stages with both free-swimming larval phases and sessile calcareous adult stages (Kupriyanova et al., 2001; Kupriyanova et al., 2006), traits also common to many other marine invertebrates (Thorson, 1950). Calcareous polychaetes have been documented growing both in and around the *Posidonia oceanica* seagrass meadows within the Castello CO_2 vents (Donnarumma et al., 2014, Garrard et al., 2014). However, no species-level identification or distribution analyses have been investigated within this group to date.

The objectives of this study are to first observe the distribution and abundance patterns of all the calcifying polychaetes associated with *Posidonia oceanica* seagrass across two natural pH gradients – one along an exposed coastline (mean range 7.39 - 8.03) and one in a relatively sheltered bay on the south side of the Castello islet (mean range 6.99 -8.03). Laboratory trials are then performed with the two dominant, closely related species, from different pH sites, to compare how fecundity and settlement traits vary along the pH gradient.

2.2 Materials & Methods

2.2.1 Field survey

2.2.1.1 Study area

Six sampling sites were chosen along the natural pH gradient to represent three pH levels along both the north and the south sides of the Castello islet, where underwater CO_2 volcanic emissions interact with a *Posidonia oceanica* seagrass habitat and lower the pH. These sites are referred to as N3, N2, NC and S3, S2, SC, where "3" represents the extreme-low pH, "2" the low pH conditions, and "C" the control, ambient pH. The "N" and "S" represent north and south sides of the gradient (see Figure 1.1 and Section 1.6 for further site information).

The locations of the ambient sites (NC and SC) were chosen for the comparable depths and conditions of the *Posidonia* meadows to the other sites along the gradient. The ambient site in the south side (SC) corresponds to the control selected for a study on *Posidonia* and epibionts on seagrass mimics by Donnarumma et al. (2014), while the ambient site in the north side was established for this specific study. The gradient and sampling site names were identified with their corresponding carbonate seawater parameters in Table 1.1. Seawater parameters represent a culmination of all available seawater data in the last six years to convey the most comprehensive and realistic time-series data for the study sites discussed here.

2.2.1.2 Specimen sampling

Sampling was performed in September 2015 *via* SCUBA diving. Four quadrats (replicates) of 40 x 40 cm were haphazardly placed at least 2 m apart on the seagrass canopy in each pH site. Within each quadrat, the leaves of ten *Posidonia* shoots were haphazardly cut at the base of the rhizome and put in separate plastic bags. In the two extreme low pH sites (N3 and S3), initial visual inspection showed a highly reduced number of worms settled on leaves. Consequently, the number of sampled shoots was increased by cutting only the external leaf (oldest leaf) of 30 shoots within each of the four quadrats in both N3 and S3. This provided a more reliable estimate of the worm abundance and helped to preserve the sensitive seagrass meadow from over-handling and impact by sampling. Leaves of each shoot were transferred in plastic bags containing seawater to the laboratory at the Benthic Ecology Center of Ischia (Stazione Zoologica Anton Dohrn; approx. 4 km from the Castello vent system) within 1 h of field sampling. They were then preserved in 4 % neutralized formalin for 24 h before being rinsed with fresh water and transferred into 70 % EtOH for long-term preservation in order to avoid any tube corrosion due to formalin.

2.2.1.3 Specimen identification

The number of calcifying polychaetes on the *Posidonia* leaves of each shoot was determined by viewing each preserved leaf from each replicate/quadrat under a dissecting microscope (AZ100, Nikon, Milan, Italy; magnification ranges of 10x up to 50x). All species were identified from their tube orientation, operculum and chaetae morphology (Figure 2.1). A distinction was made between adults and juveniles of the two closely related spirorbid species (*Simplaria* sp. and *Pileolaria militaris* Claparède 1870) in each replicate. Juvenile spirorbid tubes were defined as incomplete tube spirals, corresponding to an age of approximately one week post-settlement (Kupriyanova et al., 2001). Not all spirorbids could be identified due to the loss of taxonomic features (i.e. loss of operculum). In order to account for the unidentified specimens and best represent each species in all sites without bias, I applied a correction, by finding the ratio between the number of specimens identified to the total number of specimens found, and multiplied this by the total for each replicate in all sites; for ratios and count data see Appendix 9.3.

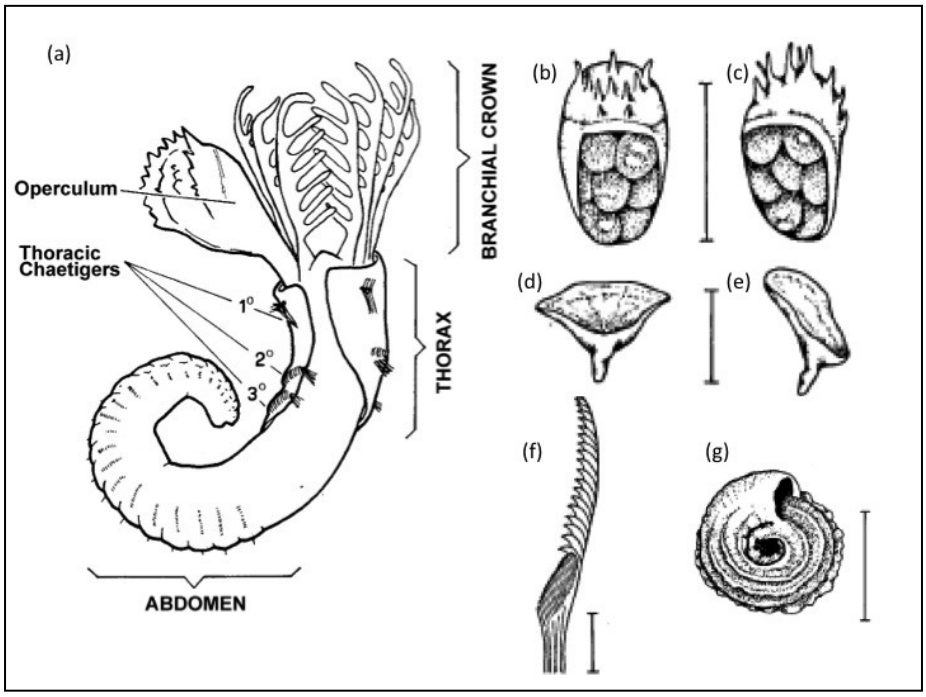


Figure 2.1 General morphology of (a) Spirorbinae and taxonomic features of (b-g) *Pileolaria quasimilitaris sensu* Bailey 1970. (b-c) Face and side view of adult operculum chamber, (d-e) face and side views of primary operculum (juvenile stage), (f) hooked seta of third thoracic segment; the main taxonomic character of *Pileolaria quasimilitaris* that is not present in our study's specimens, (g) tube. Scale bars: b-c = 0.15 mm, d-e = 0.25 mm, f = 0.01 mm, g = 1 mm (Adapted from Bailey (1970)).

2.2.1.4 Seagrass morphology

In order to account for potential changes in available settlement area influencing the abundance of the polychaetes, the mean shoot density in each site from Donnarumma et al. (2014) was related to the abundances found in this survey for each site. This average number of polychaetes at each site accounting for the full *Posidonia* coverage was calculated by multiplying the shoot density to the settlement area (percentage of *Posidonia* shoots colonized by spirorbids * average number of spirorbids *per* shoot). Only leaves longer than 5 cm were considered in this calculation. In the extreme low pH sites (S3 and N3), where sampling included only external leaves, the estimation followed the same procedure, as visual field inspections confirmed that the external leaves were the only colonized portion of the entire shoot.

2.2.2 Laboratory trials: fecundity and settlement

2.2.2.1 Specimen collection and preparation

One day after the field survey, *Posidonia* leaves originating from S2 and NC were collected for the laboratory trial *via* SCUBA diving. These sites were chosen because they have the greatest pH difference (Table 1.1), and both contain adequately sized polychaete populations (Lucey N.M. and Gambi M.C., *pers. obs*.). Leaf sections (0.5 mm x 0.5 mm) with attached polychaete tubes were cut in the field and placed in placed fabric bags, keeping the low pH and ambient originating individuals separated and in their original seawater pH conditions.

The material was then transported by boat to the Villa Dohrn-Benthic Ecology Center of the Stazione Zoologica in Ischia (approx. 4 km from the Castello CO_2 vent's system; transport time < 1 h) and maintained inside 10 L coolers with fresh seawater from each of the collection sites. Samples were kept in seawater matching the pH level of their respective field origin at the Benthic Ecology Center of Ischia until being transported 3 d later to the ENEA Laboratory in La Spezia, Italy. During transport, site specific containers with unfiltered seawater (volume = 1300 ml; T = 21.96 ± 1.29 °C; pH : ambient = 8.03 ± 0.08, low = 7.61 ± 0.26; S = 36; density = approx. 100 *per* container) were kept in styrofoam coolers packed with ice to maintain a consistent water temperature. During the 8 h transport, temperature and pH were recorded twice using a pH meter with integrated thermometer (SG2, Mettler-Toledo Analytical, Milan, Italy). The mean pH in the containers remained at 8.03 (ambient samples), or increased from 7.61 to 8.01 (low pH samples). The temperature decreased from 21.96 to 19.00 °C for 1 h in all containers. On arrival at the ENEA laboratory, containers were immediately placed in pre-conditioned temperature baths (T = 22.00 °C, S = 36). Temperature was controlled *via* two thermal baths connected to a temperature conditioner (TR 15, TECO, Naples,

Italy) with heaters (V2-Them 300, São Julião do Tojal, Portugal). To enhance a homogeneous mixing of the water, and thus thermal stability of the system, submersible circulation pumps (Aquapump HJ-311, Mondial fauna, Milan, Italy) were also used. Containers were aerated with either ambient air (pCO_2 ~ 380 μatm, for pH = 8.22), or CO_2-enriched air (pCO_2 ~ 1000 μatm, for pH = 7.70). CO_2 gas was slowly released into a Buchner flask to enable mixing using a CO_2 regulator (6000 CO_2, BOC, La Spezia, Italy).

Once at the ENEA laboratory, leaves were trimmed to eliminate as much leaf material surrounding the spirorbid tubes as possible, to help avoiding undesired fermentation. Individual worms were then identified and sorted by their sinistral tube spiral direction and operculum morphology, the main taxonomic characters that are considered in living individuals to identify the genus and species adult individuals were identified to the species level (Bailey 1970). During this 2 - 4 d process individuals were held at the pH conditions based on field measurements at their respective field sites during sampling (ambient or low pH).

2.2.2.2 Trial set-up and trait observations

For the trial, the polychaete species characterizing each pH site were based on the observation that the low pH site's sample was dominated by *Simplaria* sp. and the ambient pH site's sample was dominated by *P. militaris*. Therefore, samples of *P. militaris* from NC and *Simplaria* sp. from S2 were randomly chosen for the trial. Adults were placed in separate plastic petri dishes preconditioned with a biofilm from a 2 d non-filtered seawater soak (1 individual *per* petri dish). The pH in the dishes was set at 7.61 for *Simplaria* sp. individuals, representing the average value found in the S2 field site considering all available time-series data Table 1.1. Similarly, dishes with *P. militaris* individuals were maintained at the ambient pH value, 8.1. All other seawater parameters matched the field values (Table 2.1). Sets of six dishes with 3 ml of pH-conditioned seawater were uncovered and placed in larger covered six aquaria baths holding 20 ml of seawater. Dishes were randomly moved between the covered aquaria every two days.

All adults were monitored once a day under a light microscope (AZ100, Nikon; magnification ranges of 25x up to 50x) for the presence of embryos in the opercular brooding chamber (Figure 2.2b). When adults released their first brood from their brood chamber, they were monitored daily, together with their offspring in the same dish, for the following 14 d. The number of offspring from each parent (brood size) was counted after the first day of brood release from the operculum. The number of settled larvae was counted each day, along with any deaths or additional broods. The tubes of the living parents were photographed with a digital camera (Nikon Sight DS-U1, Nikon, Milan, Italy) mounted on a light microscope (AZ100, Nikon), and tested as a trait covariate to account for any bias between parental size and offspring traits (i.e. brood size, mortality, brood survival). Photographs were analyzed with ImageJ software (Rasband WS, US

National Institutes of Health, Bethesda, MD, USA) to obtain tube area (mm^2) (Abràmoff et al., 2004).

2.2.2.3 Seawater parameters and polychaete husbandry

To achieve the correct pH, the covered aquaria containing the petri dishes were connected to air supplies that passed enriched (elevated pCO_2 air) and normal air into the 20 ml of seawater (*via* bubbling). Lowered pH in the petri dishes was attained through surface CO_2 diffusion within the covered aquaria (Gattuso, 2011). Levels of pCO_2 in the air supplied to these aquaria were measured continuously throughout the exposure period with a CO_2 gas analyzer (Li-820, Li-Cor Biosciences, Lincoln, NE, USA). In order to maintain stable thermal conditions, all covered aquaria were placed in the thermal baths described above.

Seawater pH, temperature, and salinity were measured in each petri dish daily with an integrated pH and temperature meter (SG2, Italy) and refractomer (V2, TMC, São Julião do Tojal, Portugal). The pH meter was calibrated daily with pH buffer standards (4.01, 7.0, 9.21; Mettler-Toledo, Leicester, UK). Seawater samples (250 ml) were taken at the beginning and end of the trial from the stock seawater prepared for each treatment. Samples were fixed with $HgCl_2$ (0.02 %) to eliminate microbial activity (Dickson, 1994), stored in borosilicate flasks (250 ml), and maintained in dark, dry conditions until total alkalinity (A_T) was determined using gran titration method (Dickson, 1994; Dickson et al., 2007). Carbonate-system parameters of pCO_2 (μatm), total carbon dioxide (TCO_2, mol kg^{-1}), bicarbonate concentration (HCO_3^- mol kg^{-1}), calcite saturation (Ω_{ca}), and aragonite saturation (Ω_{ara}) were calculated from A_T, pH, temperature and salinity using the package SeaCarb v.2.4.8 in software R (Lavigne and Gattuso, 2013). Water-chemistry parameters for each dish during the 14 d experimental phase, as well as discreet field data from each pH site are presented in Table 2.1.

Table 2.1 Seawater physico-chemistry parameters (a) at the field collection sites, and (b) corresponding laboratory trail pH treatments (mean + SD), measured (**in bold**) or calculated using the SeaCarb program* over the total trial period for each habitat.

	Ambient pH (SC)	Low pH (S2)
(a) Field site data		
pH	8.04 ± 0.09	7.84 ± 0.24
Temperature (°C)	23.4 ± 0.7	23.8 ± 0.7
Salinity	37.9 ± 0.3	37.9 ± 0.3
TA (µmol/ kg)	2563 ± 3	2560 ± 7
pCO_2 (µatm)	567 ± 100	1075 ± 943
DIC (mol/kg)	0.002 ± 1.02E-04	0.002 ± 1.72E-04
Ω calcite	4.75 ± 0.53	3.52 ± 1.11
Ω aragonite	3.13 ± 0.35	2.32 ± 0.73
(b) Laboratory trials		
pH *(d)*	8.08 ± 0.47	7.54 ± 0.53
Temperature (°C) *(d)*	22.31 ± 0.57	22.17 ± 0.83
Salinity *(d)*	36.38 ± 2.11	36.67 ± 2.87
TA (µmol/ kg) *(m)*	2350.71 ± 53.70	2291.53 ± 122.55
$[CO_2]$ (mol/kg)	9.65E-06 ± 3.10E-06	2.11E-05 ± 6.62E-06
pCO_2 (µatm)	327.88 ± 108.21	721.73 ± 228.33
$[HCO_3^-]$ (mol/kg)	0.002 ± 8.38E-05	0.002 ± 1.57E-04
$[CO_3^{2-}]$ (mol/kg)	2.49E-04 ± 4.75E-05	1.42E-04 ± 2.55E-05
DIC (mol/kg)	0.002 ± 4.601E-05	0.002 ± 1.47E-04
Ω calcite	5.82 ± 1.07	3.33 ± 0.60
Ω aragonite	3.82 ± 0.70	2.19 ± 0.39

*** Note:** Lavigne and Gattuso 2013**.**

Seawater in each petri dish was changed every other day by removing water with a syringe and injecting new seawater prepared for each treatment. This preparation involved collecting seawater from La Spezia bay, La Spezia, Italy, and cleaning it with a 0.1µm filter and UV sterilization system (V2ecton 600, TMC, São Julião do Tojal, Portugal) for 5 d before being transferred to sterile 2 L flasks. One flask was prepared for each treatment and placed in the temperature bath described above with bubbling elevated pCO_2 air, or normal air, depending on the treatment. Additionally, a food mixture of rotifers, *Artemia* sp. and microalgae for filter feeders was added at a concentration of 3 ml feed *per* 300 L seawater to the new seawater immediately before water changes (Gamma Nutraplus Reef Feed, TMC, São Julião do Tojal, Portugal). Seawater in petri dishes was mixed three times *per* day by gently tilting aquaria to promote feeding.

2.2.3 Data analysis

2.2.3.1 Field survey data

Two data sets generated from the field survey were subject to analysis: (a) the abundance of all calcifying polychaete species at every life stage along each of the six pH sites of the gradient (distribution); (b) the abundance of *Simplaria* sp. and *P. militaris* adults along the pH gradient. Initial data exploration using Cleveland dot- and boxplots revealed no outliers in both datasets (total abundance and individual species abundances). Conditional boxplots revealed heteroscedasticity of the variances among the pH sites for both datasets, and histograms indicated violation of normality (Zuur et al., 2010). Non-linear patterns within the species-level dataset also existed (Zuur et al., 2010). As a consequence, a Welch's ANOVA with a Games – Howell post-hoc test was used for both datasets to assess how the number of calcifying polychaetes varied along the pH gradient, with 'side' (north/south) and 'pH site' as fixed factors. This test is robust to non-parametric distribution of count data and heteroscedasticity of the variances.

Additionally, dataset (b) was analyzed by employing generalized additive models (GAMs; Wood, 2006, 2011b; Zuur, 2009) to describe the abundance of each species with respect to nominal 'pH' and to compare each species' abundance along both gradient 'sides'. GAMs were used in order to account for non-linear patterns in this count data. Nominal mean water pH for each gradient side and site was based on the one month average of September field site data from (Kroeker et al., 2011) to accurately represent seasonal pH values during the survey. The models were fitted using the mgcv (Wood, 2011b) and nlme (Pinheiro et al., 2015) packages in R. The explanatory variables considered in the analysis were gradient 'side' (north or south), pH and interactions between side (factor) and pH (fitted as a smoother),

$$\text{Abundance}_{ij} = \alpha + f(\text{pH}_i)\text{: factor (side}_{ij}) + \varepsilon_{ij} \quad (1)$$

where α is an intercept; f is the smoothing function; and ε is independently, normally distributed noise with expectation 0 and variance (σ^2). The interaction between pH (smoother) and side (categorical variable) was fitted using the 'by' command in the mgcv package. It applies a pH smoother on the data for each gradient side. This interaction was not significant for *P. militaris*, therefore the following model was fitted:

$$\text{Abundance}_{ij} = \alpha + f(\text{pH}_i) + \text{factor (gradient side}_j) + \varepsilon_{ij} \quad (2)$$

The appropriate degrees of freedom of the smoothers were selected automatically using cross validation (Wood, 2006, 2011b). Statements about changes in abundance are based on the significance of the main effect 'gradient side', and not on the interaction between gradient side and pH. The model was optimized by first looking for the optimal random structure and then for the optimal fixed structure (Zuur et al., 2007). The principal tool was comparison of Akaike information criteria for each model. Residual plots were used to assess the mean-variance relationships; models for both species indicated no violation of the assumption for homogeneity of the variances. Over-dispersion was also calculated for each model (sum of Pearson residuals^2 / residual d.f.). High over-dispersion, particularly in *Simplaria* sp., required the use of negative binomial distribution with a log link (Pinheiro et al., 2015; Zuur, 2009; Zuur et al., 2007). The optimization function of the models (k parameter) was adjusted for our specific dataset at six.

2.2.3.2 Laboratory trials

One-way ANOVA tests were performed on each functional trait to test for differences between *P. militaris* from an ambient pH site (NC) and *Simplaria* sp. from a low pH site (S2). Traits analyzed included: brood size of each parent; time of larval release to settlement (days); and percentage brood mortality *per* parent on day seven and 14. Data were tested for normality of distribution and homogeneity of variance using Cleveland dot- and boxplots. Conditional boxplots indicated homogeneity of the variances among the pH species groups, and histograms indicated no violation of normality of distribution (Zuur et al., 2010).

All statistical analyses were performed by using the statistical software R (v.3.1.3; R Core Team, 2015).

2.3 Results

2.3.1 Field survey

2.3.1.1 Species identification

All of the taxa found in the *Posidonia* seagrass meadows in the Castello natural CO_2 gradients belonged to the Spirorbinae sub-family, within the Serpulidae family. The four main species were *Pileolaria militaris* Claparde, 1870, *Simplaria* sp., *Janua pagenstecheri* (Quatrefages, 1865), and *J. pseudocorrugata* (Bush, 1904). A total of forty-eight undetermined Serpulinae were also encountered in the control and low pH

sites. The taxonomy of the *Simplaria* sp. found did not match any known records, and is therefore discussed in further detail below.

The tubes and operculum of both the adults and juveniles of the *Simplaria* sp. specimens found in this study closely resemble that of *Simplaria pseudomilitaris* (Thiriot-Quiévreux, 1965), a taxon first described in Marseille, France, and later identified in the Port of Ischia, Italy (Terlizzi et al., 2000). Morphological similarities of the two are their sinistral coiled (clockwise) tube orientation, similar tube diameter (between 1.5-2 mm), latitudinal tube ridges, and 2-3 indistinct longitudinal tube ridges. The operculum also has a single opercular plate with ornamentation (protuberances, or spines, projecting from top of operculum). The operculum has been described having an elliptical cap with a partially encircling distal papillated rim that is absent on the substratum side, yet feature is not in agreement with my specimens, where the rim completely surrounds the distal papillated rim. This feature is, however, in better agreement with a description of *S. pseudomilitaris* from the west coast of the USA made by Beckwitt (1981), who further noted the high variation in operculum morphology in the species. The primary trait that is found in the *Simplaria* sp. specimens of this study that is not in agreement with the *S. pseudomilitaris* descriptions from the literature is the extent of 'ornamentation' on the operculum plate (e.g. Bianchi, 1981; Fig. 60).

The morphology of the *Simplaria* sp. here also closely matches *Pileolaria quasimilitaris* with respect to larval and operculum morphology (see Figure 2.1), a taxon first described in the Caribbean Sea (Bailey, 1970). In particular, *P. quasimilitaris* has distally projecting calcareous spines on the operculum that form a complete crown. However, it still does not completely agree as there are up to three indeterminate rows of long, slender spines observed in the operculum crown center in this study's specimens *versus* the two which were originally described in *P. quasimilitaris*; see Figure 2.1b, Figure 2.2c, Figure 9.4. Two other difference between these two species are in the tubes and chaetae: this study's specimens have latitudinal ridges and 2-3 indistinct longitudinal ridges, and no sickle chaete on the third thoracic fascicles, *versus* many the many longitudinal ridges and knobs of *P. quasimilitaris* and presence of these sickle chaetae (Figure 2.2g and Figure 2.2c).

The key taxonomic feature for the genus *Pileolaria* is the presence of sickle chaetae on the third thoracic fascicles (Knight-Jones et al., 1974). In the sister genus *Simplaria* erected by Knight-Jones (1984), all of the characters of the genus *Pileolaria* are found, except the sickle chaetae in the third thoracic fascicles. After examining over 40 indiv. of *Simplaria* sp. specimens from this study, no sickle chaetae were found. In the original description of *P. quasimilitaris* by Bailey (1970) the chaetae of the third thoracic fascicle are defined as "hooked" chaetae. The morphology of sickle chaetae, is, however,

quite variable (Knight-Jones and Fordy, 1979) and "hooked" chaetae, *sensu* Bailey (1970) can be considered as sickle chaetae. Regardless, the specimens of this study also lacked hooked chaetae in the third thoracic fascicles.

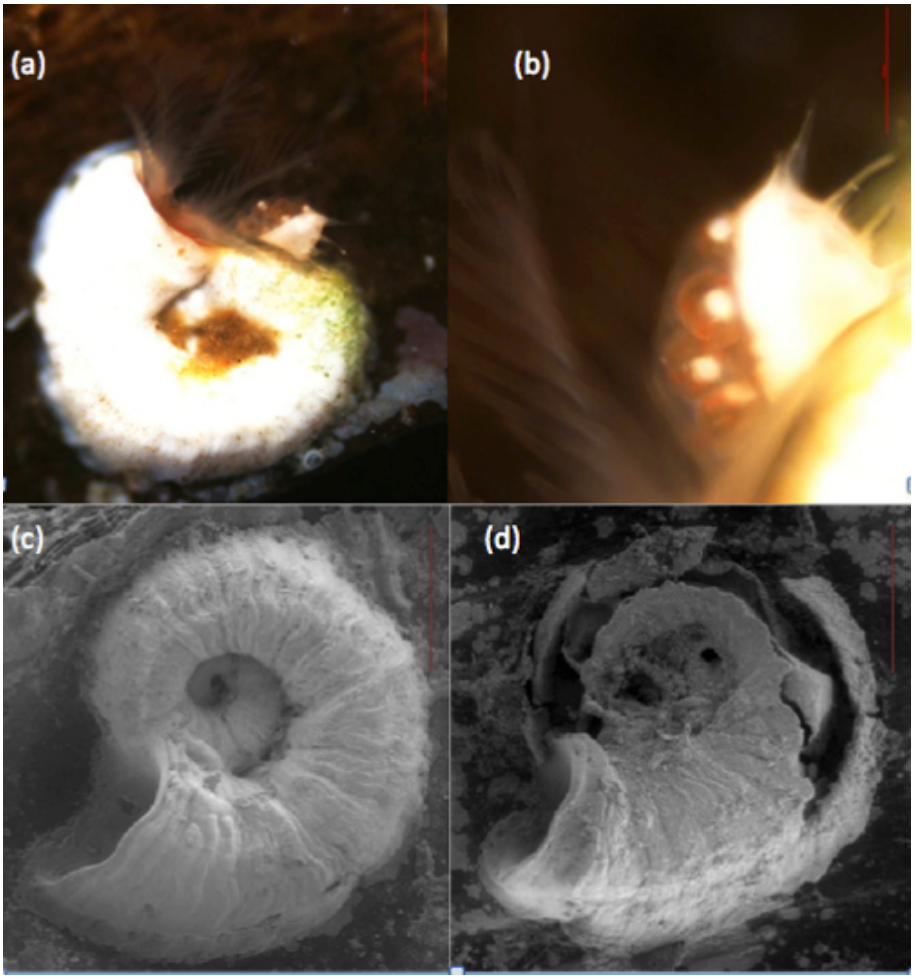


Figure 2.2 *Simplaria* sp. images: (a) live individual from southern, low pH station S2, (b) magnified opercular brood chamber with incubating embryos; SEM images of a (c) tube collected from ambient, control pH conditions, and a (d) tube collected from southern extreme low pH station S3 with notable tube corrosion; a-d scale = 0.5 mm.

It is possible that the *Simplaria* sp. in this study is either (a) a possible morphotype of *P. quasimilitaris* (lacking sickle chaetae due to the peculiar habitat conditions), (b) a morphotype of *Simplaria pseudomilitaris* having more abundant, longer, pronounced distally projecting calcareous spines covering the operculum plate, (c) or a new species from the genus of *Simplaria*. Analysis of additional material from both *Simplaria pseudomilitaris* and *Pileolaria quasimilitaris* is necessary to determine the correct species' status, yet this is beyond the scope of this study. Therefore, the

specimens of this study are designated to the genus *Simplaria* sp., which accounts for the taxonomic inconsistencies described above.

2.3.1.2 Species abundance and distribution

Total polychaete abundances on the *Posidonia* leaves along the pH gradient from the Castello CO_2 vents ranged from 53 to 4,733 individuals (total sum of replicates within each sampling site). There was a decrease in the mean abundance from the ambient pH sites (SC and NC) to the extreme low pH sites (S3 and N3) along both the north and the south side gradients; with a decline from 341 to 13 individuals in the south (SC to S3), and from 1,183 to 14 individuals in the north (NC to N3) ($F_{5, 92.97} = 75.110$, $p < 0.0001$, Figure 2.3). The means in both the northern and southern extreme-low pH sites (N3 and S3) were comparable ($p > 0.05$, Figure 2.3). However, overall mean abundance was three times lower in the southern side compared to the north ($p < 0.05$, Figure 2.3). Additionally, in the north, there was a strong linear relationship between abundance and pH condition (Figure 2.3). On the south side, there was not a linear trend, as mean abundance peaked in the low pH site (S2: 144 individuals), compared to the ambient site (SC), with almost 100 more individuals in S2 compared to SC ($p > 0.05$, Figure 2.3).

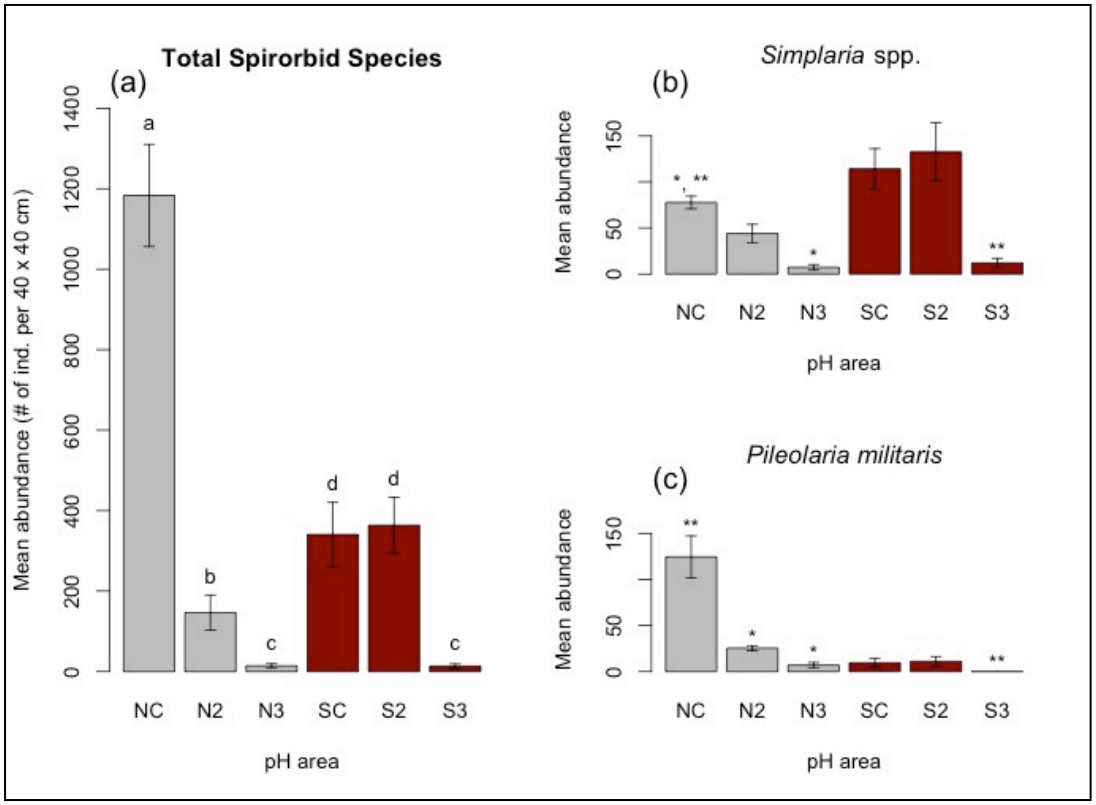


Figure 2.3 Mean abundance (± S.E.) of spirorbids sampled from south sites (SC, S2, S3) and north sites (NC, N1, N2), colored in red and gray respectively, and with 'C' indicating ambient pH, '2' low pH and '3' extreme low pH: (a) Total spirorbid abundance (all species combined) with different letters indicating significant differences among sites. (b) *Simplaria* sp. abundance and (c) *P. militaris* abundance, both with asterix (* or **) representing significant differences among sites.

The same patterns were observed in both the north and south when different settlement area availabilities, or *Posidonia* shoot densities, were accounted for within each sampling site. Despite higher shoot densities, indicative of increased settlement area, in the low pH sites compared to the ambient sites (mean 1,000 shoots m^2 in S3 compared to the mean 467 shoots m^2 in SC, and 719 to 380 m^2 (N3-NC), the polychaete densities remained very scarce in the extreme low pH sites (Figure 2.4).

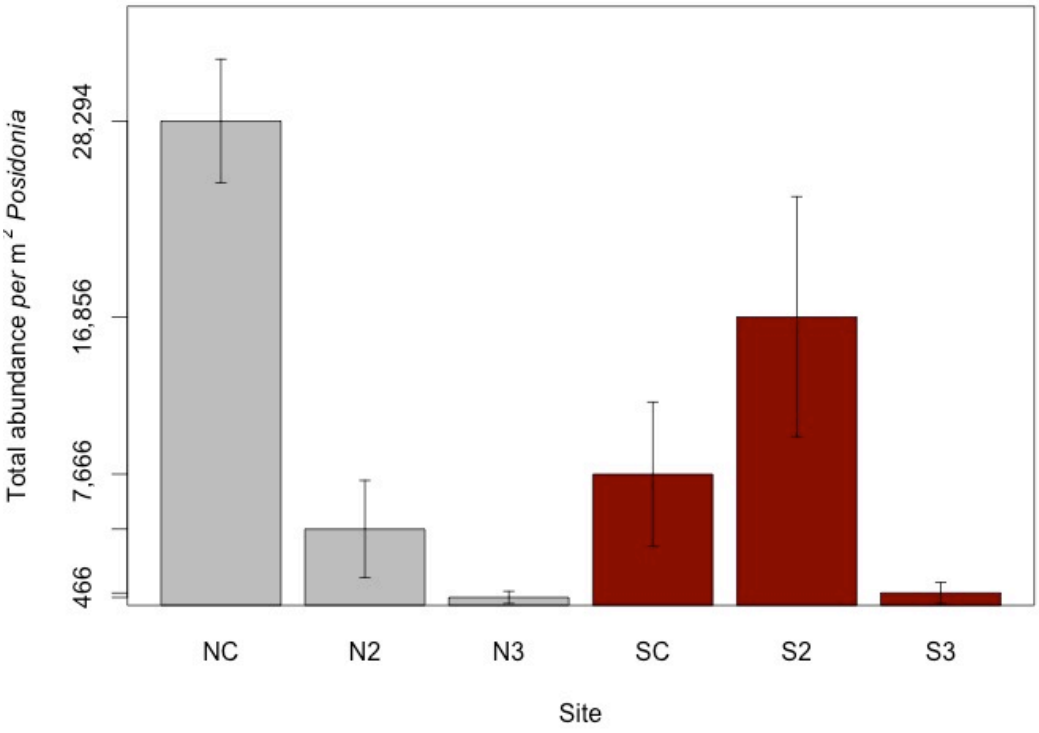


Figure 2.4 Spirorbid abundance related to *Posidonia* shoot density: mean number of spirorbids calculated as total species sampled *per* replicate plot area, multiplied by *Posidonia* shoot density (m^2), with S.D. as error bars.

The species-specific analysis shows that the non-linear pattern in the southern side of the total species analysis is caused by *Simplaria* sp. (Figure 2.3). In the species-specific analysis, the total abundance along the pH sites ranged from 0 to 498 individuals in *P. militaris* and from 48 to 532 individuals in *Simplaria* sp. While the overall number of individuals for both species was comparable, their distribution patterns differed. As in the total species analysis, abundances significantly declined with decreasing pH when considering all sample sites (*P. militaris*: $F_{4, 11} = 9.370$, $p = 0.006$, *Simplaria* sp.: $F_{5,78} = 24.270$, $p = 0.0005$ (Figure 2.3b, Figure 2.3c). The mean abundance of *P. militaris* was highest in the north compared to the south (52 *vs.* 7, respectively), and linearly decreased from the ambient to extreme low pH in the north (NC to N3), and low to extreme low in the south (S2 to S3) (Figure 2.3c). *Simplaria* sp. mean abundance was higher in the south than in the north. The *Simplaria* sp. abundance in the low pH south site (S2) was not significantly different to the mean abundance in the south ambient site (SC), but it was different in the north between the NC ambient and N3 extreme low pH site. Additionally,

Simplaria sp. was the only species found in extreme low pH (S3), the site with the lowest mean pH (S3 pH: 6.99 ± 0.34).

Comparisons of the smoothers (non-parametric curves) generated by the additive mixed models for the two gradient sides of both species confirmed that abundance decreases in both species with decreasing nominal pH across each gradient ($p < 0.001$ for both *P. militaris* and *Simplaria* sp., Figure 2.5). For *P. militaris*, there were significant linear declines in abundance with decreasing pH along both north and south gradients, however the northern side had significantly more individuals compared to the southern side. In contrast, *Simplaria* sp. abundances in the north and south were not significantly different in pH greater than 7.9 (Figure 2.5).

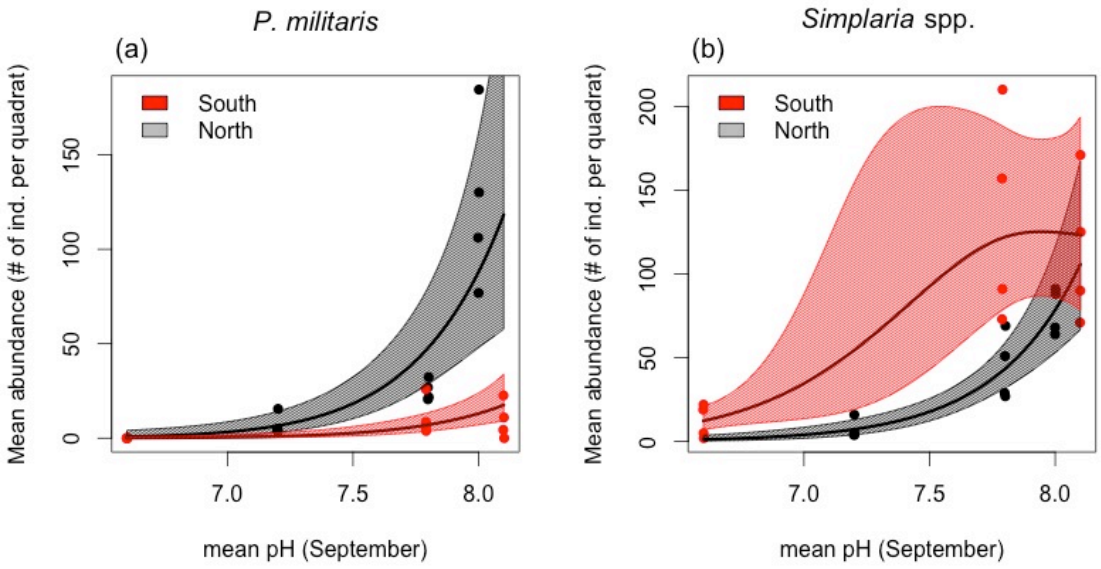


Figure 2.5 Trends in spirorbid species mean abundance (a) *P. militaris* and (b) *Simplaria* sp. Black dots: mean number of individuals found in each replicate along the northern gradient. Red dots: mean number of individuals found in each replicate along the southern gradient. Black lines are the smoothers for each gradient side; red and gray bands along smoother lines are 95 % CIs.

2.3.2 Laboratory trials: fecundity and settlement

In order to assess fecundity and settlement trait differences between low and ambient pH regimes, I compared the two closely related species: *Simplaria* sp., which dominated the low pH site (S2) to the *P. militaris,* which dominated the ambient pH site (NC). The average number of offspring *per* brood from low pH *Simplaria* sp. parents was significantly higher than broods from ambient *P. militaris* parents: means 8.08 ± 1.54 *vs*. 3.61 ± 0.44 ($F_{1, 28} = 10.800$, $p = 0.003$, Fig. 2.6). Also, settlement success was significantly higher in the low pH *Simplaria* sp. compared to ambient *P. militaris*: 86.49

± 6.85 % compared to 13.43 ± 6.29 %, respectively ($F_{1, 28} = 58.800, p < 0.001$, Fig. 2.6). Additionally, *Simplaria* sp. broods released into low pH water, showed metamorphosis and settlement of all offspring within 1 h, whereas less than 13% of the *P. militaris* offspring in the ambient seawater conditions settled in the first 24 h. Parent tube size did not affect brood size in these individuals ($p \geq 0.05$).

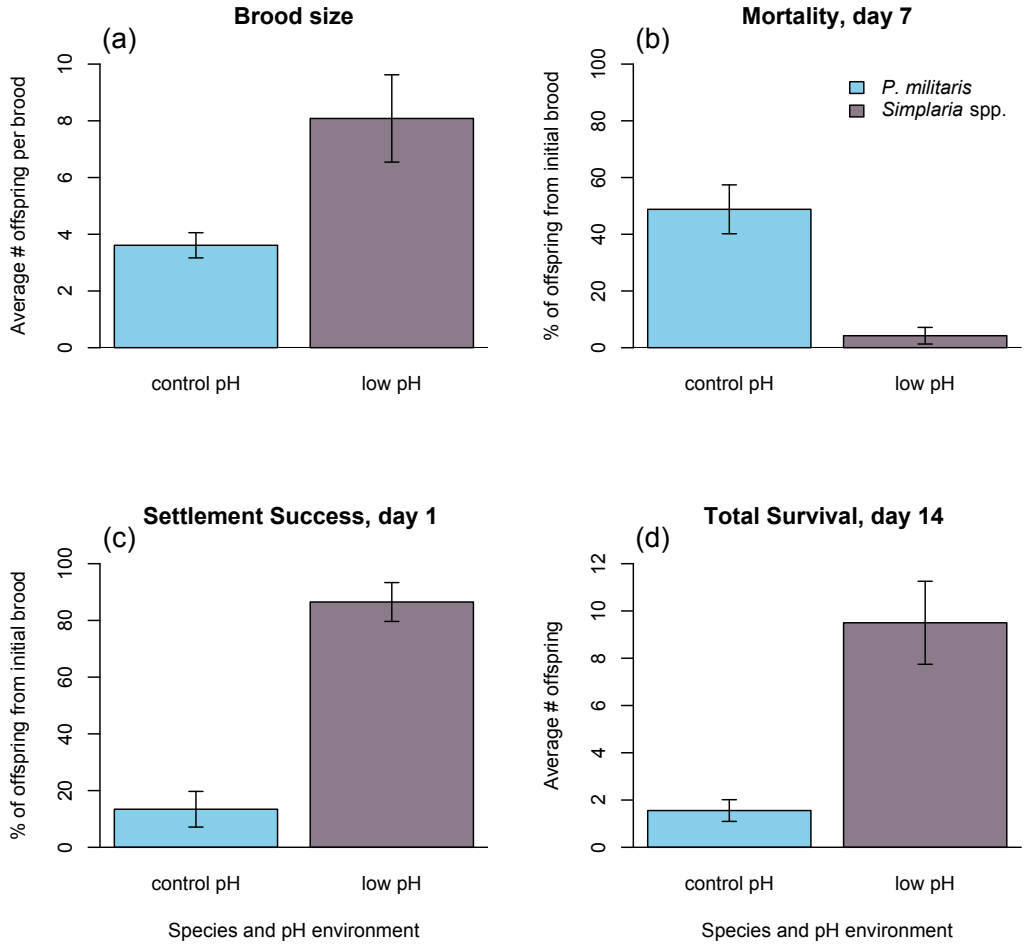


Figure 2.6 Fecundity traits and offspring survival from *Simplaria* sp. and *P. militaris* parents cultured in low and ambient pH conditions respectively, to match their field-originating pH values (7.6 and 8.1); purple and blue bars respectively. (a) Brood size is expressed as the mean number of offspring in the first brood release, (b) mortality as a percent of the beginning brood dead 7 d after initial brood release, and (c) settlement success as the percent of metamorphosed living offspring from each brood 1 day after brood release, (d) total survival as the mean number of offspring living 14 d after the initial brood release, plus any additional offspring released during the 14 d of exposure. Error bars show SE; each trait had significantly different means ($p < 0.05$) between species groups.

Mortality rates 7 d after the first brood release was 4.23 ± 2.93 % in the low pH *Simplaria* sp. and 48.8 ± 8.61 % in the ambient *P. militaris* ($F_{1, 28} = 16.770$, $p < 0.001$, Fig. 2.6). Net survival after 14 d, including additional offspring from subsequent broods, was significantly higher (6.3 times) in the low pH *Simplaria* sp.: means 1.56 ± 0.46 *vs.* 9.5 ± 1.76 offspring *per* parent, respectively ($F_{1, 28} = 26.900$, $p < 0.001$, Fig. 2.6). Furthermore, between day 7 and 14, 10 out of 12 parents released a second brood in the *Simplaria* sp. group, but only 4 out of 18 parents from the *P. militaris* group produced a second brood.

2.4 Discussion

This study aimed to identify the calcifying polychaete species found in and around the Castello vent system, and then determine if the traits associated with them varied along the pH gradient. The first finding from this work was that the calcifying polychaetes comprised of two closely related dominant taxa from the Spirorbinae subfamily, with *Simplaria* sp. having the highest abundance in low pH conditions and *P. militaris* having highest abundance in ambient pH conditions. No traits associated with these two species' general morphology, general reproductive strategy (i.e. both are brooding species), or settlement habitat (i.e. shallow *Posidonia* blades) differed, and thus are not likely to be responsible for driving the higher abundance of *Simplaria* sp. in low pH conditions with respect to *P. militaris*. Conversely, the laboratory trail results indicated that the *Simplaria* sp. had specific reproductive trait differences such as higher fecundity (i.e. larger broods), settlement success, as well as increased juvenile survival compared to *P. militaris*. These fitness-related life history traits are likely to be responsible for the increased abundance of *Simplaria* sp. in low pH, and its higher pH tolerance. These trait differences also suggest that the *Simplaria* sp. at this site may be locally adapted to the low pH system. The differences and similarities in the species' traits are detailed below.

2.4.1 Trait differences between species

The higher abundances of *Simplaria* sp. in low pH (S2) appear to be linked to the species' ability to produce more viable offspring, which quickly metamorphose and settle, compared to that of its close relative, *P. militaris*. A critical component of low pH tolerance is demonstrated by the low-pH *Simplaria* sp. ability to produce many larvae that metamorphose in minutes, compared to the multiple days required of *P. militaris* individuals in ambient seawater conditions. Larval stages of many polychaetes and more generally, marine invertebrates, have been deemed sensitive to low pH (Byrne et al., 2013; Lewis et al., 2012; Milazzo et al., 2014). However, organisms with shorter pelagic larval phases avoid prolonged periods in which their potentially sensitive larval life stages are subjected to the (low pH) environment (Dupont et al., 2010b). This pattern has

also been documented in naturally low pH habitats for: the mussel, *Mytilus edulis* (Thomsen and Melzner, 2010), gastropod, *Crepipatella dilatata* (Chaparro et al., 2008), and polychaetes, *Platynereis massiliensis* (Lucey et al., 2015).

Simplaria sp. appears to have also overcome the challenges of calcification associated with metamorphosis and early juvenile growth in low pH, which Lane et al. (2013) highlight with the serpulid tubeworm, *Hydroides elegans*. Their findings indicated adverse affects associated with metamorphosis and early juvenile growth under comparable pH values (Lane et al., 2012). This may be a result of specific species' physiological ability to secrete an initial tube. In spirorbids, and other species with lecithotrophic larvae, there are specialized glands that are responsible for secreting the primary tube, resulting in successful metamorphosis (see Figure 2.7; white spots in the embryos) (Kupriyanova et al., 2001). The contents of the primary shell gland appear to be extruded *via* the anus and the calcareous secretion is molded by the movements of the larva into a tube capable of housing the entire settled larva in less than 5 min, as in *Simplaria pseudomilitaris* and *Simplaria potswaldi* (Knight-Jones, 1978) (Beckwitt, 1980; Potswald, 1978; Qian, 1999). The secretion from the primary shell gland does not persist past metamorphosis, as the collar unfolds revealing the dorsal collar gland. The dorsal collar gland remains active after metamorphosis, cements the tube to the substratum and is the mechanism for adult tube generation (Nott, 1973). Furthermore, these primary shell glands were noticeably different between the spirorbid species in the trial, with *Simplaria* sp. embryos and larvae having highly defined, larger glands compared to *P. militaris*. Results indicate that this physiological trait difference may be important to the relative metamorphosis and settlement success of the *Simplaria* sp. in low pH.

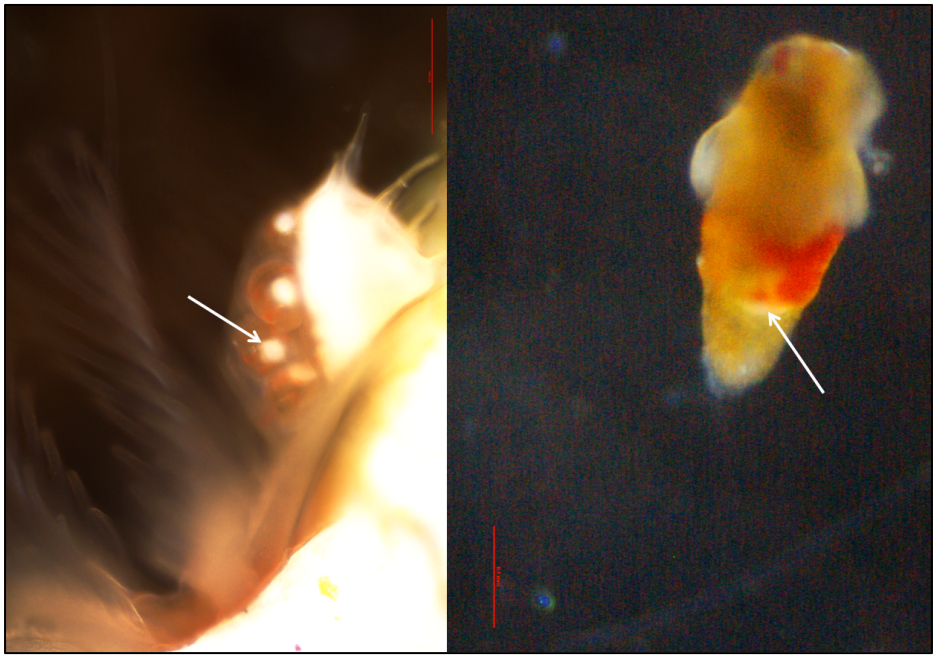


Figure 2.7. Calcified glands indicated by white arrows in embryos (left; scale 0.5 mm) and in competent trochophore larvae (right; scale 0.1 mm)

Lower overall offspring mortality observed in *Simplaria* sp. during the first two weeks of life also correlates to a higher likelihood of overall population success, compared to the ambient pH species *P. militaris*. This success is further supported by the field survey's finding of adults with embryos of *Simplaria* sp. in every site along the whole gradient regardless of pH. However, the overall low numbers of individuals found in the extreme low pH site alludes to a vulnerability occurring in many *Simplaria* sp. individuals due to low pH. In this in partial agreement with Saderne and Wahl (2013), growth rates and recruitment of spirorbid *Spirorbis spirorbis* individuals at extreme low pH/ high pCO_2 levels (3150 ± 446 µatm) were significantly reduced, whereas at more realistic pH levels for end of the century projections, individuals did not show any adverse effects (Saderne and Wahl, 2013). These pH values closely correspond to the low (S2) and extreme low (S3) pH values in this study. Similar trends and pH 'tipping' points have also been demonstrated in larval mussels' development, *Mytilus edulis* (Ventura et al., 2016).

The *Simplaria* sp. traits described above are generally characteristic of 'r- selected' species commonly associated with opportunistic and/or invasive species (Grassle and Grassle, 1974). These traits include the initial ability to occupy a site despite the environmental constraints, and rapid proliferation (Giangrande et al., 2005), followed by the ability to increase population sizes rapidly, mature quickly, and compensate for

typical high mortality (Grassle and Grassle, 1977, 1974). While the *Simplaria* sp. needs further taxonomic analysis, the possibility that it is an introduced morphotype of either *Simplaria pseudomilitaris* or *Pileolaria quasimilitaris*, agrees with the opportunistic nature of the traits found in the *Simplaria* sp. vent population. Invasive taxa are not rare in the area of Ischia and the Gulf of Naples, especially among polychaetes (Occhipinti-Ambrogi et al., 2010). Recently an invasive Sabellidae originally from the Caribbean Sea, *Branchiomma bairdi* (McIntosh, 1885), was recorded in the southern sites of the Castello vent's system (Arias et al., 2013). Past records of *P. quasimilitaris* place the species throughout various islands of the West Indies at very shallow depths (0.25-2 m) in various vegetated substrates, including the alga *Halimeda*, the seagrass *Thalassia testudinum* and the mangroves (*Rhizophora*) (Bailey, 1970). These habitats present a plausible match for *Posidonia* leaf canopy ecology in the vents and add evidence that *Simplaria* sp. may be an opportunistic, introduced species. Likewise, *Simplaria pseudomilitaris,* originally described in southern France, may have been introduced to the Ischia Harbor relatively recently, as the first record for it was in 2000 (Terlizzi et al., 2000). Past records also indicate that this species is commonly found attached boat hulls, and may have been how it arrived in the vent site, shared by the Castello harbor (Beckwitt, 1981).

2.4.2 Trait similarities between species

Generally, Spirorbinae are small, filter feeders that spend their adult lives inside self-built spiraled tubes that are permanently attached to a substrate (Gee, 1964; Potswald, 1968; Tanur et al., 2010). They are common members of the benthic community, especially in early substrate colonization or as epibionts on other organisms (Rouse and Pleijel, 2001). As closely related polychaetes from this Spirorbinae subfamily, many traits related to their general morphology, reproductive strategy, and ecologies are indistinguishable.

Simplaria sp. and *P. militaris* are both are spermcasters, as well as sequential hermaphrodites. They both incubate their embryos in a single opercular brood chamber, as seen in Figure 2.1b and Figure 2.2b (Kupriyanova et al., 2001). These chambers have similar morphologies, containing a pore at the base for embryos to exit from, a mechanism that avoids extra energy costs associated with other spirorbids that shed their brood chambers after each brood (Macdonald, 2003). This opercular pore morphology is thought to be an evolutionary novelty; permitting greater reproductive output and allowing these opercular brooders to radiate more rapidly than any other clade in the family Serpulidae (Macdonald, 2003). While these intrinsic traits do not differ between the species, the flexibility of the brood chamber does allude to diversity in the species'

specific reproductive traits, quantitatively demonstrated in the laboratory trails through production of different quantities of embryos.

Another important similarity is their habitat preference. The presence of spirorbids is dependent on the *Posidonia* seagrass substratum that they settle on. The worms are not obligate epibionts of *Posidonia* since they colonize other plants, as well as artificial substrates, but in this area they do depend on the seagrass substrate for both settlement and a suitable microhabitat facilitating feeding and shelter. Therefore, all changes in the seagrass availability and growth may affect the spirorbid abundance (i.e. shoot density, leaf length, leaf grazing and removal). Recent studies at this vent habitat have documented changes in seagrass along the gradient, and found higher shoot densities and shorter leaves in low pH stations due to intense grazing due to *Sarpa salpa*, 'Sarp', compared to lower density long-leaved shoots in ambient sites (Donnarumma et al., 2014). Garrard et al. (2014) found that shoot densities increase with reducing pH by 58 % and 82 %, in the north and south sides, respectively. This increased density in the extreme low pH areas gives the spirorbids a larger potential settlement area. Consequently, the *Posidonia* growth response to increased CO_2 may be indirectly supporting the spirorbid population. The seagrass may also be creating a micro-environment that buffers the low pH effect on a meaningful scale for the small-sized organisms like spirorbids (Garrard et al., 2014). High levels of photosynthesis are thought to provide a refuge from low pH conditions during the day; seagrass can create a localized change in pH up to 1 unit higher according to Hendriks et al. (2014). An example of this effect was observed in the spirorbid *Spirorbis spirorbis* settled on the algae *Fucus serratus.* When the spirorbids were exposed to high pCO_2, a reduction in the growth rate was observed, whereas the calcification response measured during irradiation hours was 40 % higher with respect to that recorded during dark hours. Spirorbid presence in low pH could therefore be attributable to a pH buffering effect from photosynthetic and respiratory processes of the host alga on the carbonate system, if there is a positive net effect throughout the diurnal cycle (Saderne and Wahl, 2013).

Despite the possibilities of increased shoot density enhancing substrate availability and pH tolerance due to canopy buffering, the calculation of spirorbid abundance indicated no differences in the abundance patterns seen in both species related to shoot density (Figure 2.4). Furthermore, this calculation did not account for the reduction in overall canopy height in the low pH and extremely low sites (leaf length change primarily from the direct grazing pressure of salps) (Deudero et al., 2008). One explanation of this intense grazing is that the plants in the acidified zones have higher nutritional value (lower C: N ratio; Ricevuto et al. (2015)), and/or are more palatable due to fewer calcifying epibionts or less phenolic content (Deudero et al., 2008). The increased grazing in *Posidonia* meadows under highly acidified conditions could explain

the decreased spirorbid abundance, as intense fish grazing removes the epiphytic invertebrates. In addition to this potential indirect seagrass-driven effect, the extreme low pH may be acting synergistically to affect spirorbid distributions by increasing mortality by compromising its calcification or general physiological processes (Wittmann and Pörtner, 2013).

The tube structure is an important component of organismal protection from predation (Tanur et al., 2010). There was observed tube damage in both the extreme low pH sites (S3 and N3), indicating a compounded risk to the organism (Figure 9.4). This tube damage was identified mainly in the center of the tubes, the oldest and biologically inactive section, in all individuals from S3, as well as increased tube thickness around the tube opening. Generally, the collar glands in these species produce a loose fabric of calcareous minerals (Peck et al., 2015; Tanur et al., 2010) and an acid muco-polysaccharide matrix (Vinn et al., 2008). This is molded around the tube mouth, or opening of the tube, when the organism is in a feeding position, a process beginning after metamorphosis (Mill, 1978). Seawater chemistry supports this possibility as the aragonite and calcite saturation states in S3 were below 1 (0.75 ± 0.50 and 0.99 ± 0.65, respectively) (Ricevuto et al., 2014). Furthermore, in S2 where little tube damage is noted, saturation states are above 1. This tube damage also agrees with documented reduced ability of another calcifying polychaete, *Hydroides elegans,* to calcify at similar low pH levels, which led to weaken tube integrity (Chan et al., 2012).

The tube damage may also be explained by the connective tissue composition of these tubes. The connective tissues in the tube matrix and their products are generally water soluble, and the solubility is typically increased at lower pH (Tømmeraas and Melander, 2008). Peck et al. (2015) found that other spirorbid species lost tube material in response to lowered pH, and that this loss appeared to be due to changes in the binding matrix and not crystal dissolution. Mineralogical and geochemical investigation is necessary to determine what role the binding matrix plays in maintaining normal tube structure at different pH levels or saturation states, and evaluate the quantity of aragonite and calcite minerals in each species' tube from both sites, and at different developmental stages (Tanur et al., 2010).

2.4.3 *Simplaria* sp. population differences

The significant differences in the distribution of *Simplaria* sp. along the northern gradient compared to that in the southern gradient and high abundance at the low pH site indicate population level differences within the *Simplaria* sp. These noteworthy differences could be driven by local adaptation and/or phenotypic plasticity (see Section 1.4; Calosi et al., 2013; Sanford and Kelly, 2011; Somero, 2010). The combination of life-history strategies held by *Simplaria* sp. support the possibility of local adaptation to

low pH: rapid colonization, growth and maturation, short larval dispersal periods restricting population connectivity, and the retention of offspring near their natal habitats (Reznick and Ghalambor, 2001). Alternatively, responses may be caused by phenotypic plasticity, where an individual's exposure to a low pH environment changes its phenotypic response (Calosi et al. 2013). Plasticity can improve the fitness of individuals, or subsequent generations (i.e. offspring from laboratory trials) (Chevin et al., 2010; Thor and Dupont, 2014), and is increasingly considered an important component of phenotypic (trait) changes in the wild (Chevin et al., 2010; Pigliucci, 2005). It is also associated with opportunistic behaviors seen in introduced taxa (Davidson et al., 2011). Future investigation onto the drivers of the observed *Simplaria* sp. population differences is carried out in Chapter 3 & 4 (i.e. are plasticity and local adaptation mechanisms driving the different distributions of *Simplaria* in the north and south).

2.5 Conclusion

This study identified the calcifying polychaetes and their distribution on *Posidonia oceanica* leaves along the pH gradients of a low pH CO_2 vent system, and found a putative novel spirorbid species, or morphotype, in the low pH vent site (*Simplaria* sp.). This species exhibits higher pH tolerance through increased reproductive output, rapid larval settlement, and recruitment success compared to its closely related taxon, *Pileolaria militaris*. Additionally, these traits appear to be indicative of how certain organisms are able to persist in low pH habitats when others are not. These results also indicate the existence of population-level differences within the *Simplaria* sp. distribution, where a sub-population in the south side, bay-protected low pH vent site, has comparably higher abundances and tolerance to OA, also occurring in very low pH conditions. By incorporating aspects of community ecology and trait biology into research with an emphasis on mechanisms supporting long-term population persistence, this study shows how it is possible to better our predictive ability of future marine life under increasing ocean acidification.

Chapter 3

3 Adaptation of a calcifying marine polychaete from a low pH environment through genetic accommodation

Abstract

Adaptation to altered environmental conditions, such as those associated with ocean acidification, can arise when selection acts on phenotypic variation. This phenotypic variation can arise through standing genetic variation (genes) or phenotypic plasticity (environment-driven). Here I investigated the relative importance of phenotypic plasticity and adaptation in the persistence of a calcifying polychaete, *Simplaria* sp., in a natural low pH environment. I used two laboratory-based reciprocal-transplant experiments to assess the response of several fitness-related traits in two important life stages: (1) early life: survival, metamorphosis, settlement and juvenile tube construction; and (2) adult life: survival, tube size, growth, and tube attachment. Both experiments used two *Simplaria* sp. populations, one originating from low pH (7.7), and the other from control pH (8.1). These populations were tested under the pH conditions from their own and the other populations' pH. Significant interactions between genotype * environment in most early life stage traits suggest that the population of *Simplaria* sp. originating from low pH vent sites had locally adapted to the low pH conditions. The low pH population had increased overall survival during the first 7 days of life, was able to rapidly metamorphose, and construct juvenile tubes that were not as prone to dissolution in low pH water – compared to the population from control conditions. Adaptive benefits of slowed juvenile tube construction were also indicated in the low pH population, as they were coupled to decreased tube dissolution and increased survival. This was compared to the control population, which constructed tubes relatively fast, but also incurred increased tube dissolution and mortality. As 5.5-month-old adults, the low pH population also exhibited a number of traits indicative of increased fitness: higher tube growth rates (change in surface area), increased ability to secure tube structure to substrate (peripheral flange area), higher animal growth rates (operculum length) – compared to the control population, regardless of pH exposure, as indicated by significant population effects (E). These results suggest that the influence of natural exposure to low pH may have increased the ability of the low pH population to alter its genotype through the evolutionary process of genetic accommodation (a shift in the reaction norm after

exposure to a novel environmental stimulus, i.e. promoting evolutionary diversification in response to low pH).

3.1 Introduction

The evolutionary persistence of marine organisms will be determined by their ability to cope with the changes associated with ongoing ocean acidification (OA) (Munday et al., 2013; Sunday et al., 2013, Reusch 2013). Inability to cope with these conditions will likely result in increased mortality, and possibly extinction (Bell, 2013). Research investigating the adaptive capacity of marine biota to OA has only recently been targeted as an important component of future projections (Bell and Collins, 2008; Chevin et al., 2010; Merilä and Hendry, 2014), and identifying the evolutionary processes marine organisms may follow in future OA-impacted oceans is just beginning to take hold (Kelly and Hofmann, 2012; Munday et al., 2013; Sunday et al., 2013).

One way to test explicitly if and how species might be able to respond to future oceanic conditions is through studies of local adaptation along natural pH gradients (Bell 2008, Sanford and Kelly 2011). Reciprocal transplant experiments in such gradients can be used to determine levels of adaptation among populations living in low pH areas, and whether the persistence of the population is enabled by specific forms of adaptation to low pH (Ayrinhac et al., 2004; Etterson and Shaw, 2001). In this approach, individuals are taken from different field habitats and held in their respective (and their own) 'habitat' conditions for multiple generations. Following this grow-out period, their progeny are relocated to the *in situ* source and test habitats, after which their fitness (e.g. survival) is quantified (Falconer and Mackay, 1996; Kawecki and Ebert, 2004). The performance of local genotypes can then be explored using reaction norms and analysis of variance to test for the relative importance of local adaptation (significant differences between trait means between populations), plasticity (significant effects of environment), or genotype * environment interactions (Nuismer and Gandon, 2008). The resulting reaction norms from such experiments can then be used to infer if genotypes differ in their plasticity (West-Eberhard, 2003). Reaction norms bridge the gap between phenotypic plasticity and quantitative genetic studies of natural selection by connecting quantitative phenotypic plasticity and genotype (Dam, 2013; Murren et al., 2014; Pfennig et al., 2010).

By examining fitness-influencing (phenotypic) traits through reaction norms, important distinctions between genotype and plasticity can help to elucidate the role of plasticity in promoting genetic adaptation. There is a great amount of uncertainty about how adaptation manifests itself in novel environments, but plasticity is likely one of the most important mechanisms in its promotion (West-Eberhard, 2003). Plasticity is also thought to be an initial mechanism many marine organisms will utilize in order to primarily cope with the novel OA oceanic environment (Charmantier et al., 2009; Merilä

and Hendry, 2014). The extent to which plasticity acts solely as a coping mechanism (i.e. trait) or as a mechanism of genetic adaptation, however, are still wrapped in contention (Ghalambor et al., 2015, 2007; Pigliucci, 2005; West-Eberhard, 2003). Unwrapping this contention is particularly relevant now, in terms of the novelty of the rapidly changing and restructuring OA is causing in global oceanic environments (Palumbi, 2001).

The aim of this study was to determine whether adaptation or plasticity appear to underpin the persistence of populations found living in low pH conditions around the CO_2 vent. I used the tubeworm *Simplaria* sp. at a natural low pH environment as a model. *Simplaria* sp. is found settled on *Posidonia* seagrass growing in and around the low pH habitat (see Section 1.5 for in-depth description). Moreover, it is the only species of calcifying polychaete that is able to develop to maturation in the low and extreme low pH vent sites (6.6-7.7; Chapter 1). It is likely a species with a broad distribution and high capacity for pH tolerance through plasticity (Kuo and Sanford, 2009; Chapter 2). Although, it is also a brooding species with limited to non-existent larval dispersal (Beckwitt, 1980), suggesting it has a high capacity for local adaptation (Reznick and Ghalambor, 2001).

Like many other marine organisms, the metamorphosing *Simplaria* sp. exhibits a diverse range of characteristics appropriate to functioning during its different life stages (Kupriyanova et al., 2001; Strathmann, 1990). Early life stages include embryos that are protected in brood chambers attached to the parent, free-swimming, non-feeding trochophore larvae, metamorphs that settle in the benthic environment, and juveniles that commence tube formation (Kupriyanova, 2003). Adult worms are sessile filter feeders that build calcareous tubes (Rzhavsky, 1994). Due to its taxonomic uncertainty, however, very little is known about this species' life history.

Early developmental stages in other calcifying marine invertebrates have been shown to be at higher risk to OA impact than adult stages due to difficulties producing and maintaining calcified larval structures and maintaining juvenile tubes/shells (Kleypas et al. 2006; Byrne 2011a; Byrne 2011b; polychaetes: Lane et al. 2012; Chan et al. 2011). The traits identified as important to maintaining organismal fitness during these early life stages include, but are not limited to, sperm mobility, larval mobility, larval development, metamorphosis, and settlement (Byrne, 2011a; Dupont and Thorndyke, 2009; Kurihara, 2008), whereas the adult phases of marine calcifiers are supposed to experience changes related to either calcification, growth, and reproduction (Ries et al., 2009; Wood et al., 2008). However, one problem with these studies is that they do not link responses from each life stage together. If such sensitivities are in fact driven by fitness responses in certain life stages, it is possible that the *Simplaria* sp. could exhibit adaptive traits in one specific life stage and not another, or in both, or neither.

Considering that the life stages of *Simplaria* sp. may be subjected to different selective pressures, and thus display different adaptive outcomes, identifying fitness-related responses at both major life stages will help to assert realistic adaptive responses (Dupont and Thorndyke, 2009). Consequently, I compared the low pH *Simplaria* sp. population from the Castello vent system to a nearby control population using two-laboratory based reciprocal transplant experiments. The first experiment will establish whether local adaptation or plasticity is indicated during early-life stages by reciprocally transplanting brooding adults, and measuring larval release rates, time to metamorphose, juvenile tube formation and dissolution, and survival. The second experiment will also establish whether there is evidence for local adaptation or plasticity, only using 5.5-month-old individuals (adults) and measuring survival and tube growth proxies (i.e. growth rates, tube morphologies).

3.2 Material and Methods

3.2.1 Experimental design

Two laboratory based reciprocal transplant experiments were performed for both early and adult life stages (Fig. 3.1; Experimental Design). The first experiment assessed the relative importance of the population of origin and experimental pH during early-life stage development by measuring fitness-related traits of offspring. Wild-caught adults without embryos (i.e. with unfertilized eggs) from each field habitat (low pH and control) were collected and reciprocally transplanted into both pH conditions in the laboratory (treatments: control pH→control pH, control pH→low pH, low pH→control pH, low pH→low pH). Adults were maintained in experimental conditions while embryos developed in their brood chambers. Embryos were released from parental brood chambers (after 1 -13 d) into the same pH conditions of their parents as larvae. The survival of these larval offspring and their subsequent development was followed for 7 d. The measured fitness-related traits include: larval release rate, settlement rate, time to metamorphose, time to form an initial tube (juvenile tube formation rate), time to form a spiral tube (spiral tube formation rate), tube dissolution 7 d post-larval release (%), and offspring survival 7 d post-larval release (%) (Fig. 3.1; Experiment 1).

The second reciprocal transplant experiment assessed the relative importance of the population of origin and experimental pH during the adult phase. Wild-caught adults from each field habitat (low pH and control) were maintained and spawned in the laboratory under their source pH levels, and their F1 offspring were raised under the same parental source pH levels for 5.5 months. The F1 adults from both populations were reciprocally transplanted into both pH conditions for 30 days (treatments: control→control, control→low pH, low pH→control, low pH→low pH). Traits measured

were survival (%), tube growth (tube surface area), animal growth (operculum length) and tube maintenance *via* attachment (peripheral flange surface area).

Figure 3.1 provides a flow chart of both experimental designs. Details of the experimental set-up and traits analyzed for each experiment are described in the following two sections.

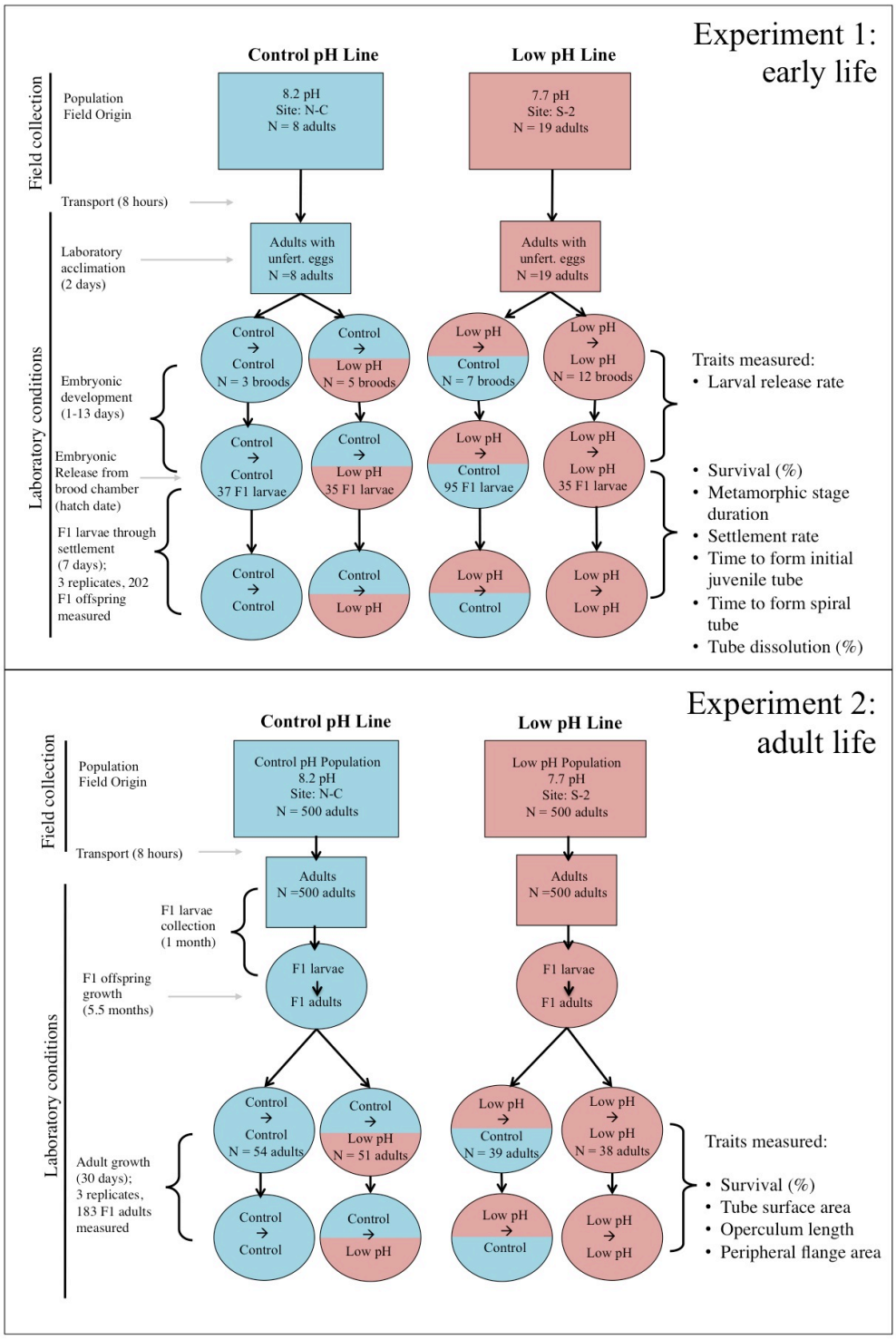


Figure 3.1 Schematic representation of the experimental design for Experiment 1 (early life stage effects) and Experiment 2 (adult life stage effects): field originating parent samples are represented as boxes and F1 generation offspring are represented as circles, colors match the pH conditions of the collection sites and laboratory exposures, with duel-colored circles indicating a change between origin and exposure conditions (blue: control, red: low pH).

3.2.2 Collection and transport of wild-caught adults

Simplaria sp. were collected from two habitats in the *Posidonia oceanica* seagrass meadow of the Castello CO_2 vent area: a low pH site and two control pH sites, which were located at a distances between 100 and 400 m away from the low pH site (Figure 3.2, see Section 1.6 for general information on the Castello vents). The low pH site was selected as the area on the southern side of the CO_2 vents where *Simplaria* sp. were found in higher abundance compared to the control sites: S2 (see Section 2.3). The control sites correspond to the control areas selected for the recent studies on the colonization of *Posidonia* seagrass: SC and NC (Donnarumma et al., 2014; Chapter 2). Adults from both control sites were mixed to increase genetic diversity in the sample, however the northern site (NC) had significantly less *Simplaria* sp. than the southern site (SC) (see Chapter 2; collection details).

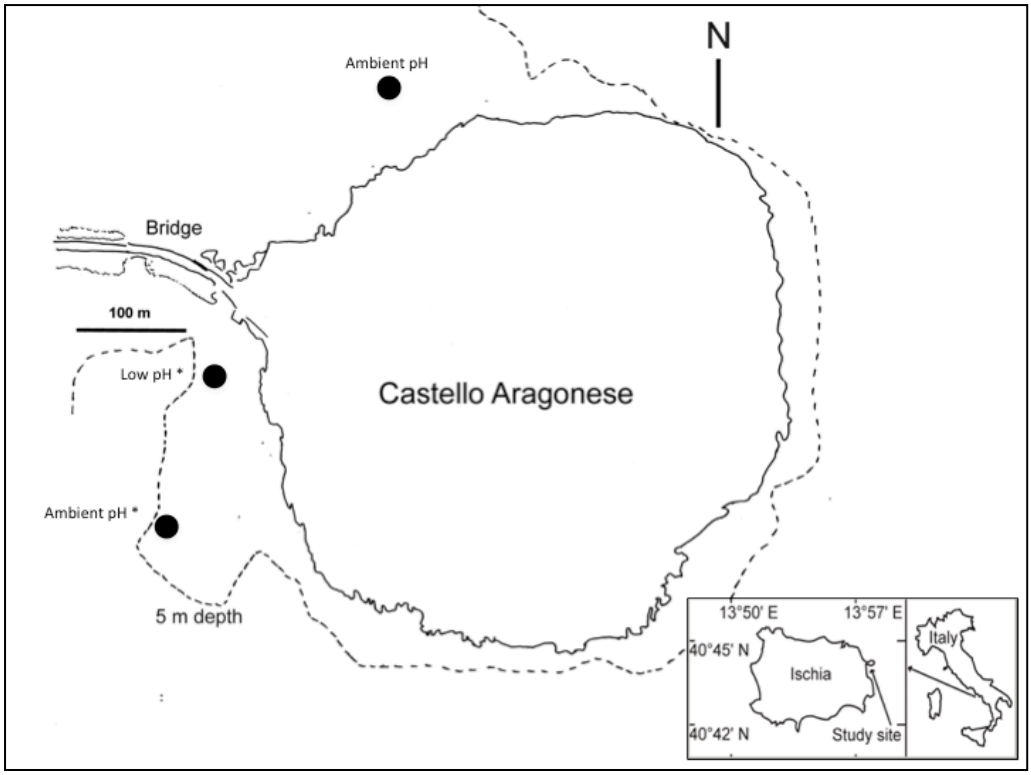


Figure 3.2 Schematic representation of the study area at the Castello Aragonese off of Ischia (Naples, Italy), showing the sampling locations of both control, or ambient pH and low pH collection sites (black dots), and *Posidonia* meadows (black dashed lines).

The two collected population samples were transported from their respective field sites to the ENEA laboratory (see Section 2.2.2 for transportation details). All individuals were held in laboratory seawater at the pH level matching that of their respective field

site source (control or low pH: 8.1, 7.7), while *Simplaria* sp. adults were identified from each site (2 d) (see Section 2.2.1.3; Specimen Identification). After the identification process, adults from each site were divided for each of the two reciprocal-transplant experiments.

3.2.3 Experiment 1: Early life stage effects

Twenty-seven field-collected adults with eggs from both pH habitats were transplanted into an experimental system on October 7th, 2015. The low pH-originating adults were divided and introduced into both low pH (7.7) and control pH (8.1) conditions. The control pH-originating adults were divided and transferred in the same way. Each adult individual was kept in a separate plastic petri dish. Sets of six dishes with 3 ml of pH-conditioned seawater were uncovered and placed in six larger covered aquaria baths (replicates = 3) holding 20 ml of seawater. These dishes were preconditioned with a biofilm from a 2 d non-filtered seawater soak, and randomly moved within each replicate aquarium every 2 d after the experimental start.

All adults were monitored daily for embryonic development in the operculum brooding chamber with a light microscope, (AZ100, Nikon; magnification ranges of 25x up to 50x). The time from when eggs were present in adult operculum (i.e. post egg fertilization) and when they were released from the parent's operculum as larvae (i.e. hatched) was measured as the larval release rate. The resulting offspring were counted and monitored daily for the following 7 d. The number of swimming trochophore larvae, metamorphosed and settled juveniles were counted each day, along with any deaths at each day and all developmental stage changes. Settlement was considered to occur when the larvae began to metamorphose. The time required to fully metamorphose was determined as the time from the initial calcium gland secretion to the time when the animal's collar unfolded (Kupriyanova et al., 2001). Juvenile tube formation was considered to occur when calcification from the juvenile collar glands began to occur. Spiral tube formation was considered to have occurred at the point when the tube shape consisted of a complete 360° spiral and covered the initial calcareous secretion with new tube material. Where possible, juveniles were photographed on d 7 with a digital camera (Nikon Sight DS-U1, Nikon) mounted on a light microscope (AZ100, Nikon). Photographs were analyzed with ImageJ software (Rasband WS, US National Institutes of Health, Bethesda, MD, USA) to obtain tube surface area (mm^2) and tube dissolution (%) (Abràmoff et al., 2004). Percent tube dissolution was visually determined for tubes on petri dish sides. The tubes of the parents were similarly photographed and tested as a trait covariate to determine if parental tube size influenced offspring tube traits.

Water parameters in this experimental system were maintained at values based on the averages and standard deviations of six time-series datasets between 2008-2015 at

each pH site/habitat (Ricevuto et al. 2014; Table 1.1). The low pH treatments were set at (7.69 ± 0.32), and the ambient at 8.1 (8.03 ± 0.08) (Ricevuto et al. 2014). The temperature was set at a constant 22 °C, the average temperature at the Castello vent site the previous year during the same time frame of the planned field experiment (see Chapter 1; 4). Temperatures in this dataset did range from 17.5 to 25.6 °C, but this variability was not accounted for in the laboratory, as the standard deviation of this range was low (± 1.23 °C). Full details on the experimental system, as well as detailed animal husbandry information can be found in Section 2.2.2.

3.2.4 Experiment 2: Adult life stage effects

3.2.4.1 Grow-out period and aquarium system

The adult individuals not used in the above experiment were transferred into a different grow-out system, consisting of two experimental seawater culturing aquarium systems simulating the individuals' respective pH field habitats (N < 500 *per* population). Water parameters in the culturing systems were set at the same values used in the first experiment (Section 3.2.3). Initially, adults on leaf sections were maintained within this grow-out system in the containers they were transported in for a 2 d exposure period. One hundred adult individuals of *Simplaria sp.* were then transferred into one of the five larval catchment containers *per* pH habitat. Each larval catchment container was lined with 16 glass 'catchment slides' pre-treated with a biofilm from a 24 h filtered seawater soak. These slides were positioned along the sides of each larval catchment container as a substrate for F1 juvenile settlement (Figure 3.3a).

Adults were kept in the larval catchment container at a density of 100 indiv. *per* container for one month while they reproduced, and their F1 offspring settled on the catchment slides. The containers were then disassembled and parents removed (Figure 3.3b). Slides with F1 recruits were placed in slide stands in their respective pH grow-out tanks in a vertical position. The low pH and ambient individuals and their seawater were never mixed throughout the entire grow-out period to ensure no inter-population breeding or genetic mixing *via* spermcasting occurred. Overall, this grow-out period lasted over six months (162 d), during which mortality and development to maturation were assessed bimonthly.

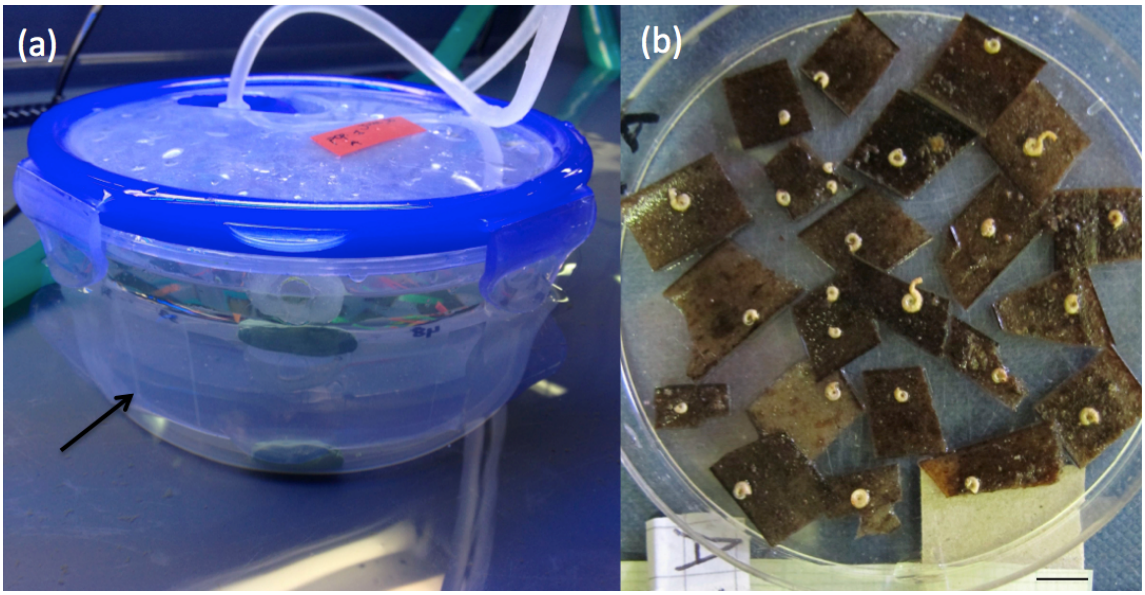


Figure 3.3 (a) Larval catchment containers lined with glass slides indicated by black arrow, (b) Field collected individuals on *Posidonia* leaf sections (parent stock), scale: 10 mm.

In more detail, the grow-out system consisted of two seawater culturing aquarium systems, which were modified from the integrated version of the equilibration flow-through systems used by Widdicombe et al. (2009) and Melatunan et al. (2011). Each pH treatment level consisted of two header tanks (volume = 90 L each) of seawater, supplied from one sump (22 °C) and aerated with either air (pCO_2 ~ 380 µatm, for pH = 8.2), or CO_2-enriched air (pCO_2 ~ 1000 µatm, for pH = 7.7). CO_2 gas was slowly released into a Buchner flask to enable mixing using a CO_2 regulator (6000 CO_2, BOC, La Spezia, Italy). pCO_2 in the air supplied to header tanks was measured continuously throughout the grow-out period with a CO_2 gas analyzer (Li-820, Li-Cor Biosciences, Lincoln, NE, USA) and adjusted manually to the experimental level when necessary.

From each of the four header tanks, seawater was gravity-fed at a constant rate (100 ml min^{-1}) to each of the five larval catchment containers for each population (transparent sealed 1.3 L containers), which were held within larger holding trays (volume = 150 L) where excess seawater was allowed to flow and the settlement slides were set up in slide stands. These slides were distributed evenly throughout the holding trays and mini submersible circulation pumps (HJ-311, Aquapump) created a circular flow around the trays to promote filter feeding and gamete circulation. Slides and larval catchment containers were randomly rotated on a weekly basis. One standard fluorescent white light was positioned above each experimental tank and put on a 14 h : 10 h = day : night schedule to simulate a diurnal cycle. Seawater overflowed from the experimental trays to the respective sumps and was filtered by protein skimmers (V2Skim 600, TMC,

São Julião do Tojal, Portugal). Heaters (V2-Therm 300 W, TMC,) and submersible circulation pumps (Aquapump HJ-311) were also used in each sump to maintain stable temperature conditions and enhance a homogeneous mixing of the seawater that was then recirculated *via* a submersible pump (V2 Power Pump 2200, TMC) to the header tanks (Figure 3.4).

All seawater for this system was collected from La Spezia bay, La Spezia, Italy, approximately every three weeks and filtered using a 0.1 μm and UV sterilization filtration system (V2ecton 120, TMC) for 5 d before being introduced to the systems. Partial water exchanges were made with this filtered seawater, exchanging approximately 400 L *per* system every two weeks. Additionally, a food mixture of rotifers, *Artemia* and microalgae for filter feeders was added to each system at a concentration of 3 ml feed *per* 300 L of seawater twice weekly (Gamma Nutraplus Reef Feed, TMC). On feeding days, the food mixture was injected near the growing individuals and protein skimmers were turned off for a 24 h period to provide adequate time for *ad lib.* feeding. These laboratory-based feeding conditions were chosen to best match the food composition and availability found in the natural *Posidonia* habitats in both pH sites (Ricevuto et al. 2014), preventing any growth restrictions due to diet. Deionized water was added daily as needed to keep the salinity stable around 37 ± 1 to match the published data in Ricevuto et al., (2014), as well as discreet field measurements made during the time of initial specimen collection.

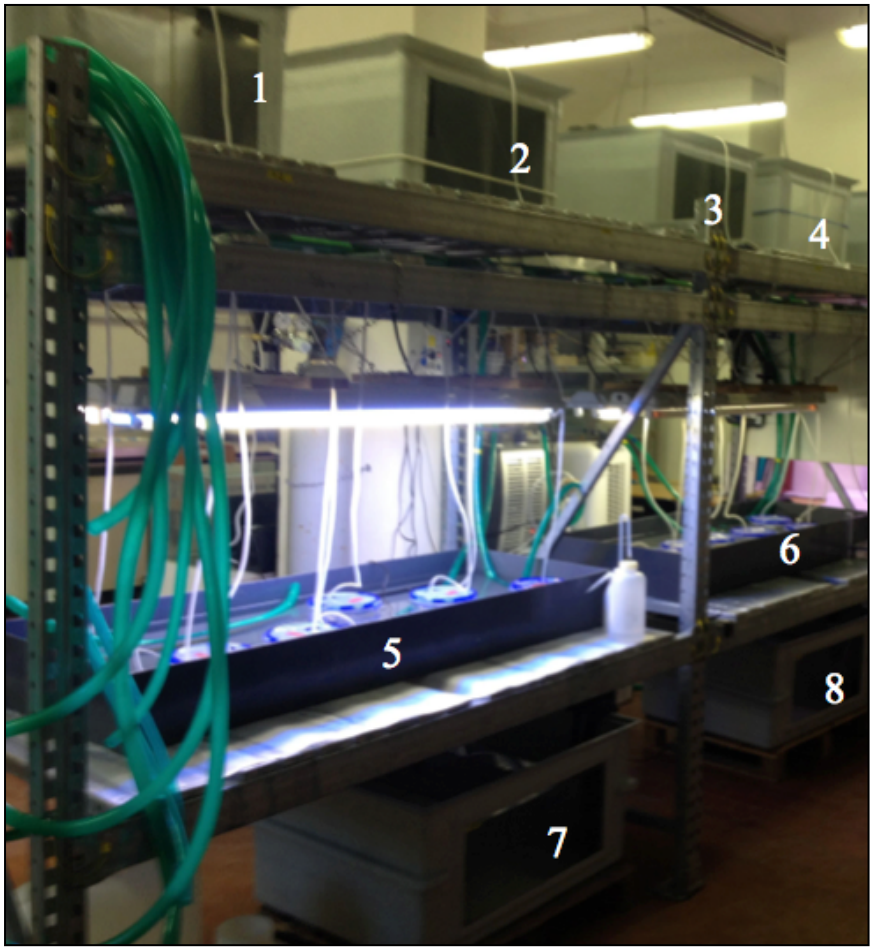


Figure 3.4 Laboratory grow-out system, where 1-4 indicates the header tanks, 5-6 the experimental trays holding the larval catchment containers, and 7-8 filtration tanks.

3.2.4.2 Experimental system and traits measured

After the grow-out period, F1 individuals on glass slides from each pH habitat were randomly chosen from the grow-out system and transferred into the second reciprocal transplant experimental system. The system was modified from the grow-out system described above, with three differences. First, only two header tanks were used to supply pH-conditioned seawater flowing at 23.5 ml min^{-1} to the replicate aquaria (n = 3); one for control pH treatments, and the other for low pH treatments. Secondly, all experimental seawater was mixed and filtered in a separate sump before being pumped up to both header tanks. Thirdly, submersible circulation pumps (Aquapump HJ-311) were used in each replicate aquarium to enhance flow and homogeneous mixing of the water. The weekly feeding regime, partial water changes, light schedule and water monitoring performed in the grow-out period were continued in each replicate aquarium without deviation from the grow-out period.

On days 1, 10, 20 and 30 of the experiment, individuals were photographed with a digital camera (DS-U1, Nikon) mounted on a light microscope (AZ100, Nikon). Photographs were analyzed with ImageJ software (Rasband WS, US National Institutes of Health, Bethesda, MD, USA) to obtain tube surface area (mm^2) (Abràmoff et al. 2004), operculum length, and peripheral flange surface area (Figure 3.5). These tube morphometrics were chosen as proxies to assess the individuals' performance; with tube surface area increase indicative of a calcification or growth response (Vinn et al., 2008), and operculum length increase as an indicator of organism size (Kupriyanova et al., 2001). The peripheral flange area, a secreted sticky calcareous and protein-based matrix, is assumed to be a proxy for settlement success, as it helps in securing the tube to the settlement site (Peck et al., 2015). In all above traits, the net change between the measures made on the first and last experimental day were used. Individuals were viewed upside-down through the glass settlement slide to assess the survivorship of the animal inside the tube, and missing animals were considered dead (Figure 3.5a,c).

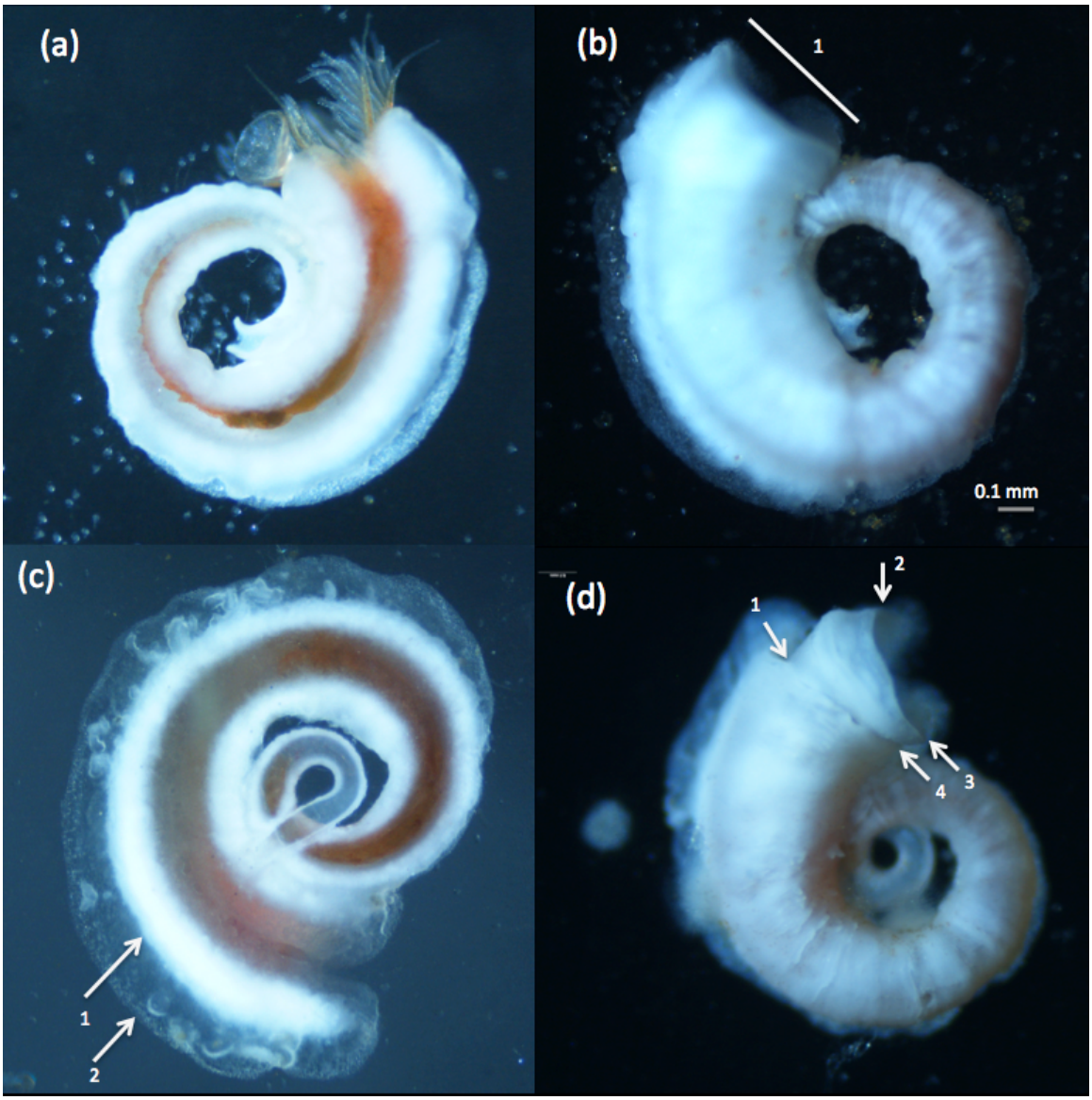


Figure 3.5 Photographs of experimental individuals and the metrics used. (a-b) show 'below' and 'above' views, respectively (note orange body inside tube); b-1 indicates how the operculum diameter was measured; the c-1 arrow indicates the opaque white tube border and the c-2 arrow indicates the translucent peripheral flange border traced to attain the peripheral flange area; arrows in (d) indicate the points that were connected to determine the new operculum growth during the experiment.

3.2.5 Seawater carbonate chemistry

The water parameters in the two experiments, and throughout the grow-out period were established from the time-series data at the control (SC) and low pH (S2) field site (Table 1.1). To validate the use of these parameters I measured temperature, pH and salinity at both field sites with an integrated pH/temperature meter (SG2, Mettler-Toledo) and refractometer (V2, TMC) (n = 3) during the species collection (Table 3.1). I also

collected seawater samples (250 ml) from each site for total alkalinity measurements. These samples were fixed with $HgCl_2$ (0.02 %), stored in borosilicate flasks (250 ml), and maintained in dark, dry conditions until total alkalinity (AT) was determined using gran titration method (Dickson, 1994). Carbonate-system parameters were calculated from AT, pH, temperature and salinity using the package SeaCarb v.2.4.8 in software R (Lavigne and Gattuso, 2013), (Table 3.1). For the first experiment, the laboratory-low pH, grow-out period, and the second experiment, the seawater pH, temperature, and salinity were measured in each replicate petri dish or container daily with an integrated pH meter (SG2, Mettler-Toledo) and refractometer (V2, TMC). The pH meter was calibrated daily with pH buffer standards (4.01, 7.0, 9.21, Mettler-Toledo). Seawater samples (250 ml) were taken monthly or weekly from the same locations for total alkalinity analysis. Water parameters are given for each stage in Table 3.1.

Table 3.1 Seawater physico-chemistry parameters (mean ± SD) at (a) reference field sites, (b) during the first laboratory reciprocal transplant experiment, (c) throughout the grow-out period, and (d) during the second laboratory reciprocal transplant experiment; measured parameters are **in bold**, while others were calculated using SeaCarb * as averages over each experimental period in each treatment. Sampling frequency is denoted superscripts, where 'h': hourly, 'd': daily, 'w': weekly and 'm': monthly.

(a)	Variable	Control pH (SC)	Low pH (S2)
Field Reference **	pH	8.03 ± 0.08	7.69 ± 0.32
	Temperature (°C)	22.06 ± 0.96	22.38 ± 1.08
	Salinity	37.32 ± 0.99	37.43 ± 0.92
	TA (µmol/ kg)	2499.35 ± 6.94	2523.68 ± 9.66
	pCO_2 (µatm)	455.61 ± 94.01	2031.19 ± 1411.65
	Ω calcite	5.17 ± 0.47	2.52 ± 0.95
	Ω aragonite	3.36 ± 0.34	1.49 ± 0.61
(b)	**pH** (d)	8.09 ± 0.37	7.57 ± 0.39
Experiment 1	**Temperature** (°C) *(d)*	22.21 ± 0.68	22.28 ± 1.36
	Salinity *(d)*	36.33 ± 2.05	36.64 ± 3.32
	TA (µmol/ kg) *(m)*	2350.71 ± 53.70	2291.53 ± 122.55
	$[CO_2]$ (mol/kg)	9.65E-06 ± 3.10E-06	2.11E-05 ± 6.62E-06
	pCO_2 (µatm)	327.88 ± 108.21	721.73 ± 228.33
	$[HCO_3^-]$ (mol/kg)	0.002 ± 8.38E-05	0.002 ± 1.57E-04
	$[CO_3^{2-}]$ (mol/kg)	2.49E-04 ± 4.75E-05	1.42E-04 ± 2.55E-05
	DIC (mol/kg)	0.002 ± 4.601E-05	0.002 ± 1.47E-04
	Ω calcite	5.82 ± 1.07	3.33 ± 0.60
	Ω aragonite	3.82 ± 0.70	2.19 ± 0.39
(c)	**pH** **(d)**	8.22 ± 0.07	7.79 ± 0.10
Laboratory: Acclimation and Grow-out Phase	**Temperature** (°C) *(d)*	22.06 ± 0.96	22.38 ± 1.08
	Salinity *(d)*	38.70 ± 0.99	38.57 ± 0.92
	TA (µmol/ kg) *(m)*	2469.78 ± 110.15	2434.51 ± 156.66
	$[CO_2]$ (mol/kg)	7.33E-06 ± 1.43 E-06	2.55E-05 ± 5.90E-06
	pCO_2 (µatm)	244.05 ± 43.54	852.65 ± 197.83
	$[HCO_3^-]$ (mol/kg)	0.002 ± 0.0001	0.002 ± 0.0002
	$[CO_3^{2-}]$ (mol/kg)	3.08E-04 ± 3.70E-05	1.36E-04 ± 1.79E-05
	DIC (mol/kg)	0.002 ± 1.02E-04	0.002 ± 1.72E-04
	Ω calcite	4.70 ± 0.54	2.07 ± 0.27
	Ω aragonite	7.16 ± 0.82	3.16 ± 0.40
(d)	**pH** (d)	8.13 ± 0.06	7.79 ± 0.07
Experiment 2	**Temperature** (°C) *(d)*	21.95 ± 2.93	22.65 ± 0.62
	Salinity *(d)*	38.48 ± 0.62	38.48 ± 0.62
	TA (µmol/ kg) *(weekly)*	2421.43 ± 0.56	2119.35 ± 4.36E-03
	$[CO_2]$ (mol/kg)	9.20E-06 ± 1.37E-06	2.12E-05 ± 4.36E-06
	pCO_2 (µatm)	309.30 ± 46.18	714.80 ± 146.52
	$[HCO_3^-]$ (mol/kg)	0.002 ± 5.77E-05	0.002 ± = 6.59E-05
	$[CO_3^{2-}]$ (mol/kg)	2.63E-04 ± 2.27E-05	1.56E-04 ± 2.49E-05
	DIC (mol/kg)	0.002 ± 3.87E-05	0.002 ± 4.72E-05
	Ω calcite	6.14 ± 0.53	3.64 ± 0.58
	Ω aragonite	4.03 ± 0.35	2.39 ± 0.38
	Flow Rate (ml min^{-1})	23.70 ± 0.81	23.47 ± 0.47

*Lavigne and Gattuso 2013; **Seawater physico-chemistry parameters averaged from six time-series datasets between 2008-2015 from a published compilation in Ricevuto et al., (2014) and include discreet field measurements made at the time of initial specimen collection.

3.2.6 Data analysis

Experiment 1:

To test the effect of 'population' of origin (e.g. potentially different genotypes), pH 'exposure' (e.g. different experimental pH conditions for F1 offspring) and their interaction on: (1) offspring survival; (2) larval release rate; (3) settlement rate; (4) time to metamorphose; (5) juvenile tube formation; (6) spiral tube formation; and (7) juvenile tube dissolution, I constructed general linear models, setting 'population' and 'exposure' as fixed factors. Initially, models included 'parent tube area' set as a covariate to account for differences in offspring traits. Models also initially included 'replicate' set as a random factor nested in 'exposure.' As the factors 'parent tube area' and 'replicate' did not exert a significant effect on the study variables, they were removed from subsequent models (Crawley, 2012). Interactions were retained in all cases. Levine's test was used to determine any heterogeneity of variances. Larval response rate met the assumptions of homogeneity of variance following $\log_{10}$ transformations. When factors did not meet the assumptions of homogeneity, despite data transformations, I plotted the residuals from each analysis against the factor tested and no significant relationships were detected. The experimental design had four treatments, with approximately four replicates, so I assumed that than ANOVA design employed should be tolerant to deviation from the assumption of heteroscedasticity. Post hoc multiple comparisons were evaluated using Tukey's tests (Sokal and Rohlf, 1995).

Experiment 2:

As in experiment one, to test the effect of 'population' of origin (i.e. potentially different genotypes), pH 'exposure' (e.g. different experimental pH conditions for adults) and their interaction on: (1) F1 survival; (2) tube surface area; (3) flange surface area; and (4) operculum length, I constructed general linear models, setting 'population' and 'exposure' as fixed factors. Models also included 'replicate' set as a random factor nested in 'exposure.' Interactions were retained in all cases. Levine's test was used to determine any heterogeneity of variances and post hoc multiple comparisons were evaluated using Tukey's tests (Sokal and Rohlf, 1995).

All statistical analyses were performed using the statistical software R v.3.1.3 (R Core Team, 2015).

3.3 Results

3.3.1 Experiment 1: Early life stage effects

The control pH population had lower offspring survivorship when exposed to low pH, while less of an effect was observed in the low population, as indicated by the presence of a significant interaction between population * exposure (G * E) (Figure 3.7, Table 3.3; $p = 0.005$). Both populations had comparably faster larval release rates when exposed to low pH, with embryos staying for significantly less time in their parental brood chambers before being released as competent larvae to the environment, as indicated by the presence of a significant pH exposure effect (E) (Fig. 3.7, Table 3.3; $p = 0.005$). Additionally, both populations had similar settlement rates regardless of their pH exposure, as indicated by the non-significant effects of population, exposure, and their interaction (Figure 3.7, Table 3.3; $p > 0.06$). The ability to metamorphose rapidly (< 1d) was a trait that was only found in the low pH population, with the control population taking a significantly longer time to fully metamorphose in low pH water, as indicated by a significant interaction between population * exposure (G * E) (Fig. 3.7, Table 3.3; $p = 0.005$). The low pH population took longer to build their initial juvenile tubes following metamorphosis than the control pH population when exposed to low pH, which was also indicated by a significant interaction between population * exposure (G * E) (Fig. 3.7, Table 3.3; $p < 0.001$). Similarly, the time needed to construct their spiral tubes was longer for low pH population compared to the control pH population when exposed to low pH, and in control conditions this trend was reversed, which was again indicated by a significant interaction between population * exposure (G * E) (Fig. 3.7, Table 3.3; $p < 0.001$). No tube dissolution occurred in control conditions, yet in low pH both populations did have notable dissolution as indicated by the significant exposure effect (E) (Fig. 3.7, Table 3.3; $p < 0.001$). However, the low pH population incurred less dissolution (13.58 ± 7.43 %) than the control population (38.88 ± 23.23 %) in low pH, as shown by the significant interaction between population * exposure (G * E) (Figure 3.6, Figure 3.7, Table 3.2; $p = 0.004$).

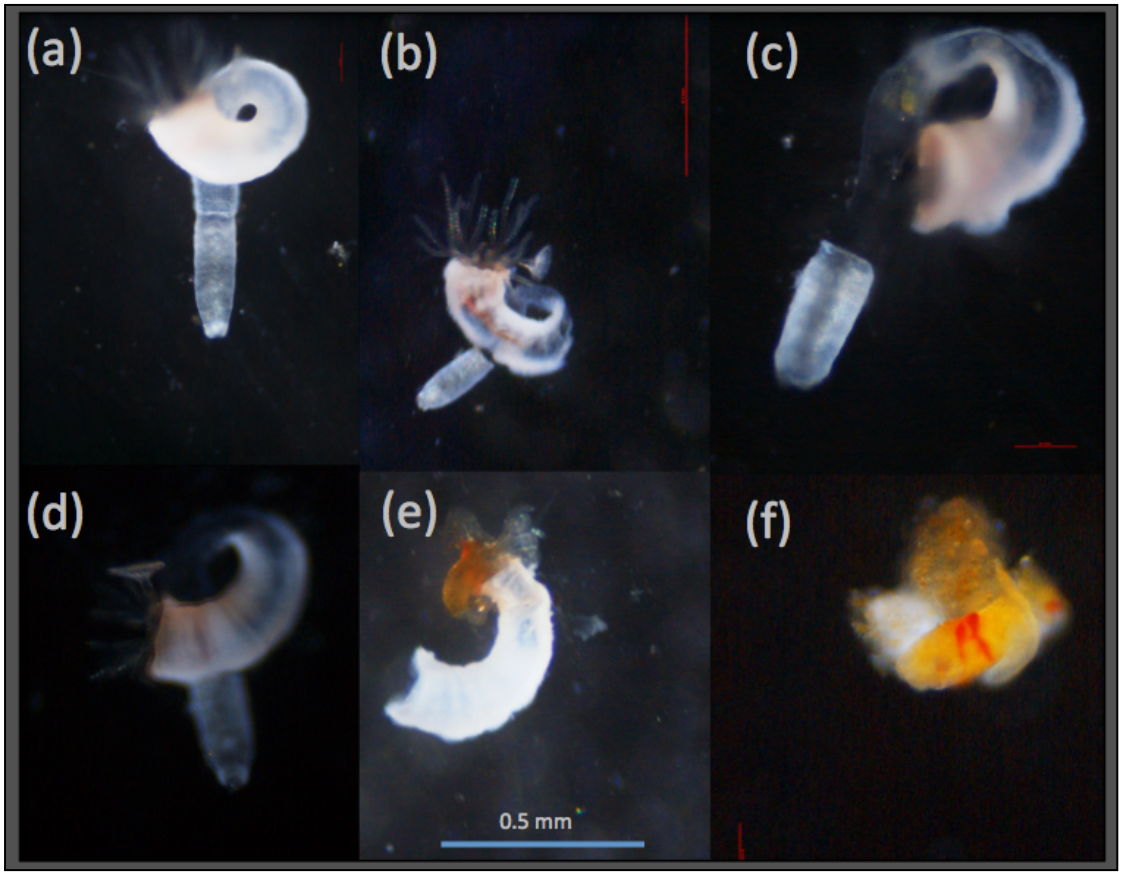


Figure 3.6 Stages of juvenile tube dissolution: (a) normal tube (spiral formation), (b) initial phases of dissolution, (c-d) 25 % tube dissolution, (e) 50 % tube dissolution, (f) 100 % tube dissolution; photographs (a-e) taken 7 days post-metamorphosis and (f) taken at day 5. Scale bar: 0.5 mm

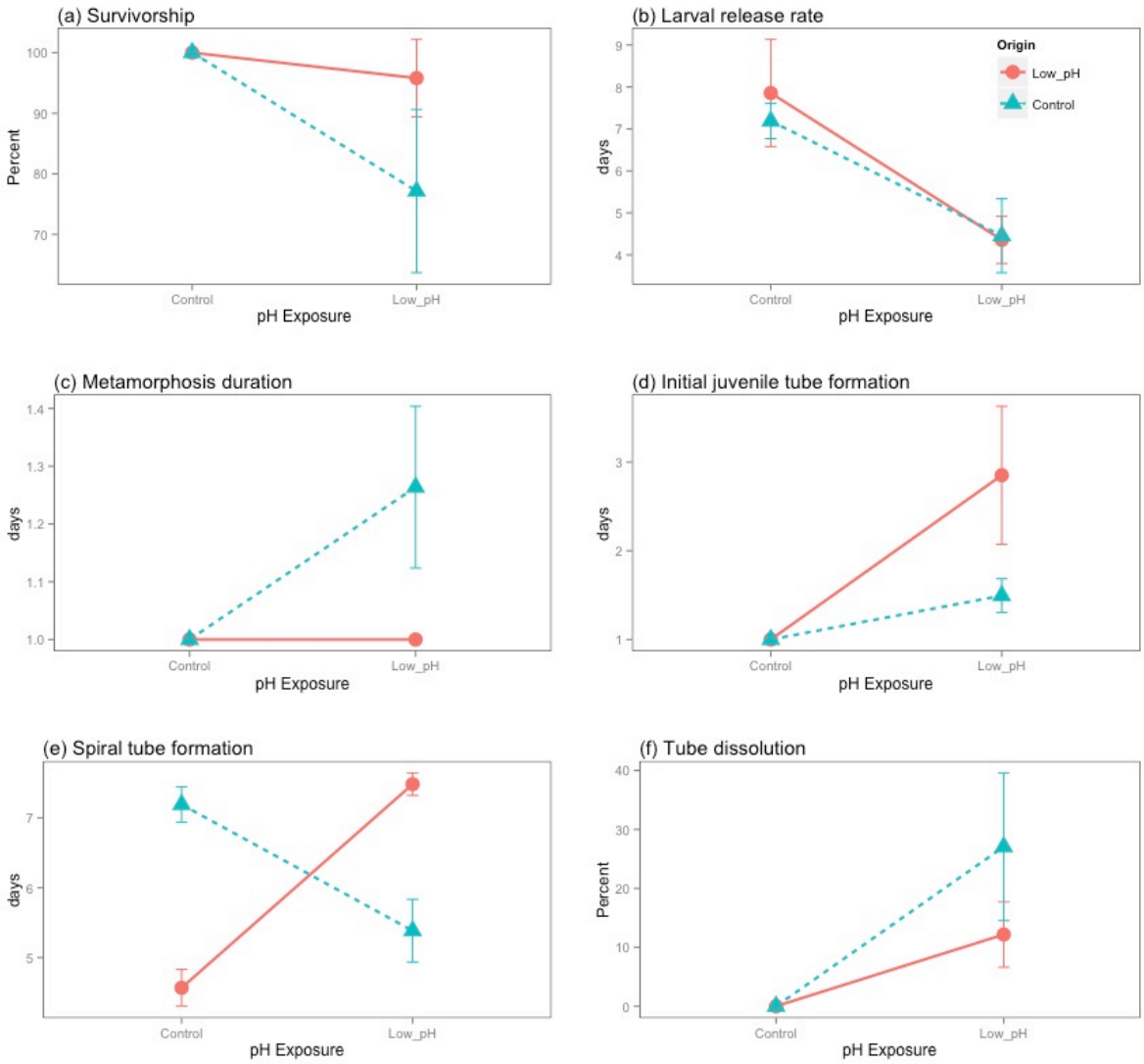


Figure 3.7 Experiment 1 reaction norms for mean traits of *Simplaria* sp. F1 offspring *from* parent populations originating in either low (7.7) or ambient (8.1) pH habitats, red and blue colored lines respectively; and exposed to either low (7.7) or ambient (8.1) pH.

3.3.2 Experiment 2: Adult life stage effects

For adult survival, there were no differences between either population and/or pH exposure, with survivorship during the 30 d experimental period ranging from between 76.2 ± 4.1 % in control conditions and 86.9 ± 2.5 % in low pH conditions (Fig. 3.8, Table 3.2; $p > 0.05$). In terms of tube growth, the low pH population had increased tube growth when exposed to low pH compared to the control population, as indicated by the significant effect of exposure (E) (Fig. 3.8, Table 3.2, $p = 0.043$). The tube growth in low pH population was also approximately three times greater than the control population,

regardless of pH exposure, a significant increase indicated by the significant effect of 'population' (G) (Fig. 3.8, Table 3.2, $p = 0.016$).

Low pH populations also had increased operculum lengths (diameter) and flange areas (peripheral flange surface area) compared to control populations, regardless of pH exposure, as indicated by a significant population (G) effect in both traits (Fig. 3.8, Table 3.2; $p = 0.003$; and $p = 0.009$, respectively). Unexpectedly, the operculum opening narrowed in the individuals from the control population, regardless of pH exposure, with the most severe narrowing in the low pH exposure. Conversely, operculum lengths in the low-pH population increased, with the greatest increase seen when the low pH population was exposed to low pH (Fig. 3.8). A similar trend occurred in the flange area, where the surface area of the peripheral flange was reduced in control populations regardless of pH exposure, with the most severe reduction in the control population when exposed to control pH. The flange area expanded in the low pH-population regardless of exposure, with the greatest increase seen in those exposed to low pH (Fig. 3.8). Furthermore, adult life-stages did not incur any significant interactions between population origin and exposure on any of the traits: percent survival, net tube surface area, net operculum diameter change, or net change in peripheral flange area (Table 3.2; $p > 0.05$ for all traits).

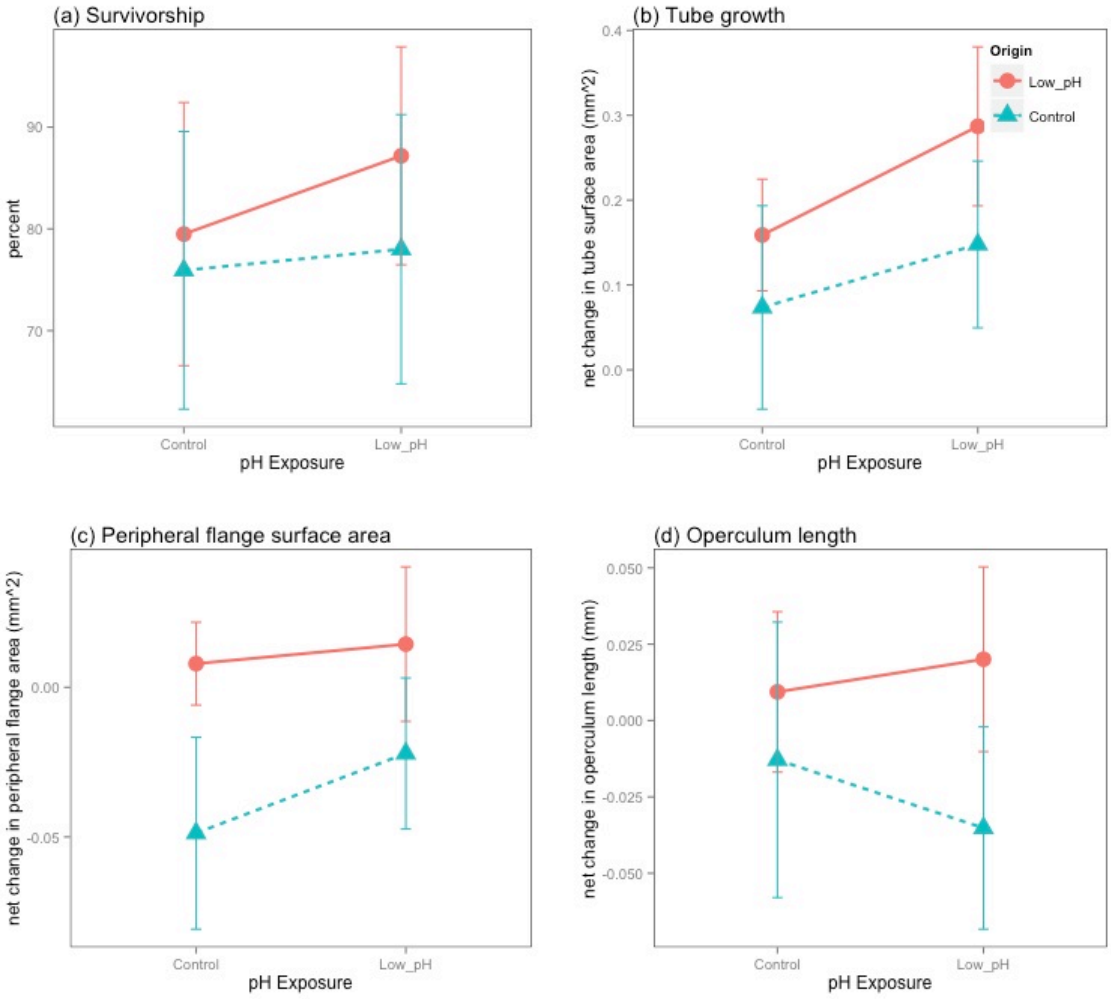


Figure 3.8 Experiment 2 reaction norms for mean traits of *Simplaria* sp. F1 adults *from* parent populations originating in either low (7.7) or ambient (8.1) pH habitats, red and blue colored lines, respectively; and exposed to either low (7.7) or ambient (8.1) pH.

Table 3.2 Results of 2-way ANOVA investigating the effect of 'population' pH and 'exposure' pH, representing the effects of genotype and environmental, as 'G' and 'E', on the early life traits (Experiment 1) and adult life traits (Experiment 2) in the polychaete *Simplaria* sp.

Exp. #	Trait	Factor	df	SS	*MS*	*F*	*P*
Experiment One	Survival	**Population pH (G)**	1	0.317	0.317	6.490	**0.012**
		Exposure pH (E)	1	0.605	0.605	12.393	**0.001**
		Interaction (G * E)	1	0.387	0.387	7.923	**0.005**
	Larval release rate (days)	Population pH (G)	1	0.143	0.143	2.677	0.103
		Exposure pH (E)	1	3.142	3.142	58.993	**<0.001**
		Interaction (G * E)	1	0.011	0.011	0.200	0.655
	Settlement rate (days)	Population pH (G)	1	0.244	0.244	3.654	0.057
		Exposure pH (E)	1	0.225	0.225	3.364	0.068
		Interaction (G * E)	1	0.122	0.122	1.827	0.178
	Metamorphosis duration (days)	**Population pH (G)**	1	1.407	1.407	15.367	**<0.001**
		Exposure pH (E)	1	1.295	1.295	14.147	**<0.001**
		Interaction (G * E)	1	0.704	0.704	7.685	**0.006**
	Initial juvenile tube formation rate (days)	**Population pH (G)**	1	8.480	8.480	8.603	**0.004**
		Exposure pH (E)	1	41.920	41.920	42.549	**< 0.001**
		Interaction (G * E)	1	18.640	18.640	18.915	**< 0.001**
	Spiral tube formation rate (days)	Population pH (G)	1	1.080	1.080	0.868	0.353
		Exposure pH (E)	1	0.930	0.930	0.746	0.389
		Interaction (G * E)	1	225.330	225.330	181.323	**< 0.001**
	Tube dissolution (%)*	Population pH (G)	1	0.000	0.000	0.002	0.969
		Exposure pH (E)	1	0.8698	0.8698	68.504	**< 0.001**
		Interaction (G * E)	1	0.1088	0.1088	8.569	**0.004**
Experiment Two	Survival (%)	Population pH (G)	1	1832	1831.5	1.118	0.292
		Exposure pH (E)	1	914	914	0.558	0.456
		Interaction (G * E)	1	351	351.5	0.215	0.644
	Tube growth (net surface area change, mm^2)	**Population pH (G)**	1	0.523	0.5225	5.464	**0.021**
		Exposure pH (E)	1	0.388	0.3876	4.054	**0.046**
		Interaction (G * E)	1	0.029	0.0288	0.301	0.584
	Peripheral flange (net surface area change, mm^2)	**Population pH (G)**	1	0.058	0.058	9.172	**0.003**
		Exposure pH (E)	1	0.010	0.010	1.513	0.221
		Interaction (G * E)	1	0.002	0.002	0.321	0.572
	Operculum length (net diameter change, mm)	**Population pH (G)**	1	0.0487	0.04874	3.982	**0.048**
		Exposure pH (E)	1	0.002	0.00198	0.161	0.689
		Interaction (G * E)	1	0.0082	0.00823	0.672	0.414

* Replicates also were found to have a significant effect on the interaction.

3.4 Discussion

3.4.1 Adaptation versus plasticity

The aim of this study was to determine whether local adaptation or plasticity were accountable for the persistence of populations of the tubeworm *Simplaria* sp. found living at the naturally low pH habitat. The results here do provide evidence of local adaptation in the low pH population found inside the CO_2 vents. Furthermore, they provide evidence that plasticity was involved in this low pH /vent population's adaptation through genetic accommodation. Genetic accommodation is a process of evolution where a phenotype shifts through either pre-existing variation for plasticity or genetic mutation, and this shift results in a genotype ('population') shift to a new mean value (i.e. increased growth) (Crispo, 2007). The original definition simplifies this somewhat, saying that genetic accommodation is when heritable variation can occur in the same direction as the plastic response, and thus phenotypes that are originally environmentally induced can be selected upon and inherited (Baldwin et al., 1902).

The process of genetic accommodation in natural populations can be identified by a shift of the genotype to a new mean value, with or without changes in plasticity (Crispo, 2007). Understanding the patterns of plasticity in this process requires a better understanding of the two theories/types of genetic accommodation: the Baldwin Effect and Genetic Assimilation (Crispo, 2007; Lande, 2009). The Baldwin Effect is where plasticity allows for survival *via* natural selection, followed by heritable change in the reaction norm. Plasticity accompanies this process. Genetic assimilation, the other type of genetic accommodation, is when the plasticity is eventually lost in the new population. Genetic assimilation occurs when the trait becomes fixed (canalized) and that the plasticity disappears. If there is plasticity in the ancestral population but then no plasticity (and a change in mean trait value) in the decedent population, then the trait has been genetically assimilated (Ehrenreich and Pfennig, 2015). Box 2 below describes the process of genetic accommodation and genetic assimilation, and provides a hypothetical example of the process.

Box 2: The Process of Genetic Accommodation and Genetic Assimilation

Genetic accommodation is any adaptive genetic change in the environmental regulation of a phenotype. For example, a trait may evolve either increased (A) or decreased (B) environmental sensitivity (i.e. phenotypic plasticity). The complete loss of phenotypic plasticity (i.e. increased canalization) is an extreme form of genetic accommodation known as genetic assimilation. Source: (Ehrenreich and Pfennig, 2015)

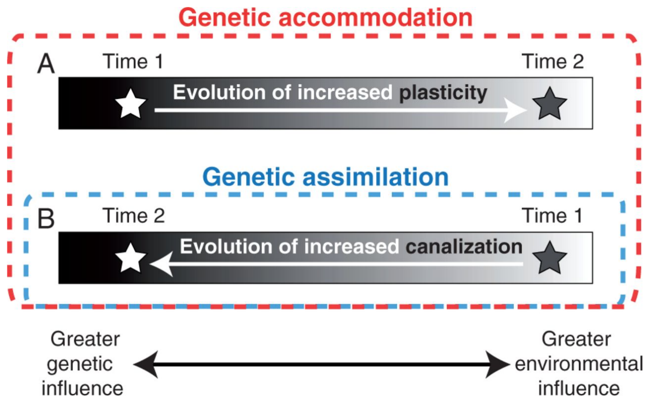


Figure 3.9 Diagram of Genetic Accommodation. Source: Ehrenreich and Pfennig, 2015)

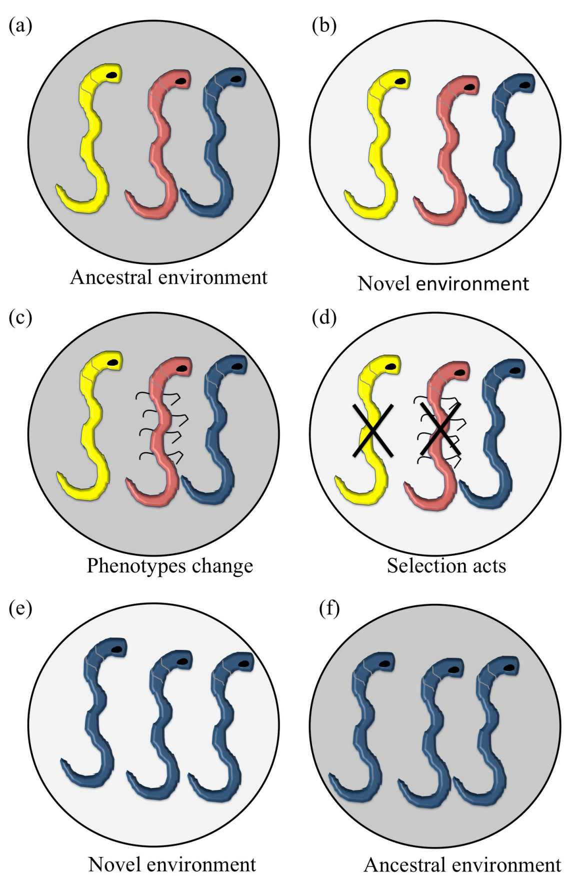


Figure 3.10 Process of Genetic Assimilation. Adapted from (Ehrenreich and Pfennig, 2015)

Phenotypic plasticity, followed by genetic accommodation, and then assimilation may facilitate the evolution of a new, trait regardless of the environment through the following steps illustrated in the schematic on the right. Consider the trait is a new worm shape and different colors represent different genotypes: (a) a genetically variable population (b) experiences a novel environment (indicated here as a change from a shaded to an unshaded background). (c) Consequently, the environment induces novel phenotypes (different worm shapes), but different genotypes respond differently (by producing different- shaped worms). (d) Selection disfavors those genotypes that produce nonadaptive phenotypes (worm shapes) in the novel environment (indicated here by an X). (e) Such selection may result in the evolution of a novel trait (a novel worm shape) that is expressed regardless of the environment. (f) That is, the novel trait is produced even when the environment changes back to original, ancestral state.

In the low pH *Simplaria* population in this study, plasticity was retained in the majority of the traits in both experiments, but at different magnitudes. For the first experiment on early life fitness, the reaction norms of each trait showed comparable slopes for the low pH population, but increased non-adaptive plasticity in the control population. The increased plasticity in the control population was not influential in maintaining the comparatively high level of fitness in the low pH population (Fig. 3.8). The exception to this plasticity trend was in the juvenile growth traits, where this plasticity pattern was reversed in the two populations. If this reduced growth is considered beneficial (as it was positively correlated to less soluble tubes), this pattern can potentially represent evidence of the Baldwin effect, where selection acting on the phenotype results in increases in the level of plasticity, as the most plastic individual possesses the most extreme phenotype and are thus positively selected (Crispo, 2007; Lande, 2009).

Plasticity levels in the second experiment on the adult stages all were similar in both pH populations (same reaction norm slope), with a higher mean value of each trait in the low pH population (genotype). This pattern presents evidence that the traditional Baldwin effect has occurred, where initial plasticity allowed for the survival in a novel environment and genetic adaptation proceeded from there. Together these plasticity trends support the possibility that the low pH population of *Simplaria* sp. is derived from the ancestral control population through the evolutionary process of genetic accommodation (Crispo, 2007; Lande, 2009).

The plasticity patterns observed here can also be used to make assumptions about how early and adult-life stage development differs. The high levels of plasticity in the early life traits of both populations (see reaction norms in Fig. 3.7) are retained but slightly reduced later in life (see reaction norms in Fig. 3.8). This indicates that early-life stage plasticity may be a highly important component of the populations' ability to colonize the low pH environment, or persist in a fluctuating environment through early life stage plasticity (Crispo, 2007). The only trait that did not follow these directional patterns was tube growth. In the low pH population, juvenile tube formation switched from a highly non-adaptive plastic response to an adaptive plastic response later in life.

3.4.2 Early life fitness considerations

The first weeks of most marine invertebrates life are considered critical to long-term population persistence (Marshall et al., 2010). Long-term, multigenerational *in situ* exposure is expected to promote increased fitness in these initial stages of life when they are also exposed to low pH conditions (Parker et al., 2012; Suckling et al., 2014). In agreement with these expectations, this study found that adaptive responses do appear to be promoting increased early life-stage fitness in the low pH population of *Simplaria* sp., as indicated by the significant interaction (G * E) found on: survival, metamorphosis,

juvenile tube formation, and tube dissolution (Fig. 3.7). The low pH *Simplaria* sp. population is able to metamorphose rapidly, and build tubes that are less prone to dissolution, with increased survivorship throughout swimming larval phases, metamorphosis, settlement and tube producing juvenile forms, compared to the nearby population from control conditions.

The results also indicate that there has been shift in the low population's genotype that resulted in slowed juvenile tube formation in low pH water. The time needed to form juvenile and spiral tubes, two progressive stages of tube construction, was significantly increased in the low pH population when compared to the control pH population under low pH. Tube formation, which in this case is relatable to tube size, is often considered a proxy for fitness in quantitative genetic breeding experiments, where larger larvae are considered more fit (Sunday et al., 2011; Kelly et al., 2013). Interestingly, the disadvantageous side effects of building initially small juvenile tube structures seem to be a trade-off for forming tubes that are less susceptible to dissolution. Also, this slowed juvenile tube production does not appear to affect survival and is reversible later in life, with adult tube growth increased in the low pH populations, comparatively. One hypothesis may be that the juveniles are constructing less soluble tubes by changing their mineralogy to be better suited to low pH, either by shifting from the use of a more soluble calcium carbonate polymorph, such as from aragonite or amorphous calcium carbonate, to one less soluble, such as calcite, as seen in the mussel *Mytilus edulis* (Fitzer et al., 2014). With evidence that slowed tube generation coupled with decreased dissolution has little effect on overall population survival, I assume these traits act as advantageous adaptations in the early life stages of low pH – originating *Simplaria* sp.

The results here demonstrate that without long-term exposure, sensitivities to low pH will likely negatively impact populations through increased mortality, and inability to build juvenile tubes capable of withstanding dissolution. Furthermore, dissolution may have underestimated the impact of low pH on control individuals. Juveniles from the control pH population exposed to low pH suffered mean tube dissolution as high as 40 %. While a significant difference between populations was found, it is important to note that the mean tube dissolution in low pH populations was less than half of those from control populations. As illustrated in Figure 3.6, at 50 % tube dissolution the polychaete is unable to remain in its tube, and mortality is inevitable without a tube. Unfortunately, mortality was not directly correlated to tube dissolution between 25 - 50 % during the 7 d early life experiment, however observations during the subsequent grow-out period indicated that these levels are positively correlated with mortality. While the correlation between tube dissolution and mortality needs to be proven, these observations do provide evidence that mortality may have been conservatively estimated for the control populations exposed to low pH.

3.4.3 Adult fitness considerations

The tube surface area, or tube size, is often considered a proxy for polychaete size (Kupriyanova, 2003), but for many other marine invertebrates, it is also a proxy for metabolism and/or calcification (Findlay et al., 2011; Wood et al., 2011a). Therefore the increase in adult tube size here appears to be a positive adaptation aiding fitness through increased growth, increased metabolism and/or calcification. Conversely, these findings can also be a product of a comparably slow development period during the grow-out period that the low pH population experienced. It is also possible that these individuals grow at different rates depending on their developmental stage *versus* their age (Podolsky and Moran, 2006). Here, similar ages were compared, but the low pH population was just attaining maturation at the time of the experiment, whereas the control population had matured (on average) before the experimental start. This possible caveat may have influenced the change in tube size.

Like tube size, the operculum diameter is considered to be a function of the polychaetes' body size (Langer et al., 2009; Rzhavsky, 1994). In this study decreasing operculum diameters were unexpectedly observed in the individuals originating from the control population, regardless of the pH exposure, suggesting the control individuals incurred a decreased body mass. Conversely, the low pH originating populations, regardless of pH exposure, broadened their operculum opening during the 30 d period. One possibility for the morphological changes in the control population is that they represent a protective effort to create a tube closure, limiting the worm bodies' exposure to the water, a response that is accentuated by low pH exposure. While this phenomenon has never been observed in calcified polychaetes to date, other non-calcifying polychaetes have been observed to modify or close their organic tubes in response to adverse conditions such as water temperature extremes or low pH. For example, the tube building polychaete *P. sulfonicola* has been found to secrete a 2-mm-thick layer of FeS_2 below its tubes, which forms a barrier between hot vent water and cold sea water (McMullin et al., 2000). Another example is in the polychaete, *Platynereis dumerilii*, which seals off its mucus tube opening when waters become too cold (Southward, 1923). Comparatively, the low pH population response of increasing length of its operculum may indicate an adaptive ability to increase its body size, and thus increase its reproductive output. This effect is substantiated by the high fecundity and reproductive output in the low pH population during preparation for the second experiment (see Appendix 9.1). However, this is in contrast to recent and historical accounts that reduced body size is advantageous for reviving extinctions (Garilli et al., 2015; Harries and Knorr, 2009). Alternatively, the low pH population may have locally adapted to adverse water conditions of, or associated with, the low pH environment with a comparatively decreased protective effort. This adaptive response may be based on the physico-chemical properties of the water, or it may be related to predatory cues triggered by

associated low pH microbial communities (Knight-Jones and Fordy, 1979; Kupriyanova et al., 2001). There is long history of microbial communities associated with polychaetes, and a common finding that the balance of these relationships hinges on environmental stability (reviewed in Kupriyanova et al. 2001). Changes in the water parameters can tip this balance, and be detrimental to the associated polychaetes (e.g. salinity change, pH, temperature). I hypothesize that the inadvertent introduction of microbial communities from the low pH population into the shared experimental seawater revealed an adaptive response in the low pH population, modifying the operculum diameter, that was not evident in the control population. In response to predators, other types of marine invertebrates have developed shells with smaller apertures that were linked to increased predatory protection (Vaughn, 2007). The costs and benefits of modifying the operculum diameter remains unclear, as does the potential that an associated microbial community is playing a role in this pattern.

The peripheral flange area expansion was also different between populations. In the low pH population, the flange area increased; in the control pH population it decreased. The flange area acts as a support for holding the main tube onto the settlement surface, and tube detachments result in mortality (Bianchi, 1981). Detachment risk is presumed to be increased under low pH, as other sessile invertebrates have demonstrated increased detachment under low pH conditions (i.e. weakened mussel byssal threads; (Donnell et al., 2013). With long-term exposure, the peripheral flange expansion of the low pH *Simplaria* sp. population is similar to that observed by Rodolfo-Metalpa et al. (2011), which found that limpets from a low pH environment also increased their shell area near the site of attachment – a response that they inferred was potentially adaptive toward increasing calcification capacity, but could also be attributed to attachment success.

Indirect ecological effects may explain the peripheral flange response. For instance, microbial populations coating the settlement areas have been specifically associated with *in situ* low pH conditions (Lidbury et al., 2012; Morrow et al., 2015). They were also observed during the grow-out period. Other studies have also noted that there are increased biofilms associated with low pH environments (Crook et al., 2016), however the compositions of these microbial communities have not been investigated in depth. Investigating the relationship between the microbial communities derived from the low pH and the attaching capacity of invertebrate settlers may help to determine the reason for the increased peripheral flange response observed in *Simplaria* sp. By increasing flange surface area, it is possible that the individuals from low pH populations are modifying their tube morphology to limit their susceptibility to tube detachments. These findings raise intriguing questions regarding the nature and extent of low pH microbial communities and how associated biofilms might be changing the cues needed for normal metamorphosis and settlement of benthic invertebrates (Hadfield et al., 2014).

3.5 Conclusion

This study identified a population of the calcifying polychaete, *Simplaria* sp., from a natural low pH vent system that has locally adapted through genetic accommodation to ocean acidification conditions projected for the end of the century (pH 7.7). This population incurred increased overall survival during the first 7 days of life, was able to rapidly metamorphose and construct juvenile tubes that were less soluble in low pH waters (i.e. not as prone to dissolution)- compared to the population from control conditions. Results also revealed a clear trade-off between juvenile tube construction and tube dissolution, where slower tube construction was coupled with decreased tube dissolution, resulting in increased survival. As 5.5-month-old adults, the low pH population exhibited comparable increases in tube growth. Tubes also developed larger peripheral flanges and tube openings indicating increased fitness, potentially through enhanced substrate attachment and bigger body sizes, regardless of pH exposure. Overall, this study indicates low pH exposure can cause phenotypic changes putatively resulting in genetic adaptations, which can improve the population's fitness in low pH.

Chapter 4

4 An *in situ* assessment of local adaptation in a calcifying polychaete from a shallow CO_2 vent system

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4.1 Abstract

Ocean acidification (OA) is likely to exert selective pressure on natural populations. Our ability to predict which marine species will adapt to OA, and what underlies this adaptive potential, are of high conservation and resource management priority. Using a naturally low pH vent site in the Mediterranean Sea (Castello Aragonese, Ischia) mirroring projected future OA conditions, I carried out a reciprocal transplant experiment to investigate the relative importance of phenotypic plasticity and local adaptation in two populations of the sessile, calcifying polychaete *Simplaria* sp. (Annelida, Serpulidae, Spirorbinae): one residing in low pH and the other from a nearby ambient (i.e. high) pH site. I measured a suite of fitness related traits (i.e. survival, reproductive output, maturation, population growth) and tube growth rates in laboratory-bred F2 generation individuals from both populations reciprocally transplanted back into both ambient and low pH *in situ* habitats. Both populations showed lower expression in all traits, but increased tube growth rates, when exposed to low pH compared to high pH conditions, regardless of their site of origin, suggesting that local adaptation to low pH conditions has not occurred. I also found comparable levels of plasticity in the two populations investigated, suggesting no influence of long-term exposure to low pH on the ability of these populations to adjust their phenotype. Despite high variation in trait values among sites and the relatively extreme conditions at sites close to the vents (pH < 7.36), response trends were consistent across traits. Hence, my data suggest that, for *Simplaria* and possibly other calcifiers, neither local adaptations nor sufficient phenotypic plasticity levels appear to suffice in order to compensate for the negative impacts of OA on long-term survival. My work also underlines the utility of field

experiments in natural environments subjected to high level of pCO_2 for elucidating the potential for adaptation to future scenarios of OA.

4.2 Introduction

Ocean acidification (OA) is the process by which anthropogenically-derived atmospheric carbon dioxide (CO_2) is absorbed into surface seawater, lowering the pH and concentration of carbonate ions in the global ocean (Caldeira and Wickett, 2003; Doney et al., 2009b). These changes have a large potential to impact marine biodiversity, as many marine species are expected to be affected detrimentally (Kroeker et al., 2013a; Mostofa et al., 2016; Wittmann and Pörtner, 2013). Adaptation may be the most realistic option for survival, but our understanding of the scope for marine species to adapt to ongoing global change over realistic, multi-decadal time-scales is limited (Carroll et al., 2007; Hendry and Kinnison, 1999; Kelly and Hofmann, 2012). One way to test explicitly if and how species might be able to respond to future oceanic conditions is through studies of local adaptation along natural pH gradients (Sanford and Kelly, 2011).

The scope for local adaptation in response to environmental stressors has previously been investigated using natural environmental gradients and the responses from their residing populations (Dam, 2013; Gaston et al., 2009; Kawecki and Ebert, 2004; Reznick and Ghalambor, 2001; Sanford and Kelly, 2011). These studies have provided us with an understanding on how and why natural populations succeed or fail to adapt to particular stressful conditions, and demonstrate realistic ecological scenarios for species' adaptation to OA (Calosi et al., 2013; Lewis et al., 2013; Maas et al., 2012; Rodolfo-Metalpa et al., 2011). The majority of examples of contemporary adaptation have shown that new populations are established by colonization events, where a subset of a metapopulation is subjected to a modified environmental patch within the pre-existing range of the species (Reznick and Ghalambor, 2001). Individuals that initially colonize and proliferate in these new environments can become isolated from their ancestral population, and may later radiate and establish a new metapopulation from which distinct populations and species arise through gene flow and repeated colonization (e.g. Losos and Schluter, (2000); see also 'The Rockall Paradox' Johannesson, (1988)). This type of colonization is likely to be the most effective for the investigation of adaptation to OA, as future chemistry changes will be global, encompassing many species' pre-existing ranges, and initially intensifying current low pH areas (i.e. estuaries, fiords, coastal areas, and upwelling areas) (Barton et al., 2012; Hofmann et al., 2014).

There is limited work on local adaptation in response to OA (see: Bozinovic et al., 2011; Sanford and Kelly, 2011). The evidence presented so far indicates that local adaptation to low pH may have occurred in populations inhabiting naturally, low-pH upwelling areas. For example, populations of the purple sea urchin, *Strongylocentrotus purpuratus*, found in persistent upwelling waters on the west coast of the USA appear to

be less sensitive than those that are not exposed to low pH waters (Kelly et al., 2013; Pespeni et al., 2013b). A similar distinction implying locally adapted populations is further exemplified in studies of highly calcified coccolithophore *Emiliania huxleyi* strains that dominate low pH upwelling habitats in Chile (Beaufort et al., 2011; Smith et al., 2012). Supporting this idea, laboratory studies have also demonstrated that the *E. huxleyi* upwelling strains have a high degree of adaptation potential compared to strains that are not found in high CO_2 conditions (Iglesias-Rodriguez et al. 2008); for counter evidence see Langer et al. (2009).

OA adaptation studies can also be established in venting areas where volcanic CO_2 bubbles through the seafloor and locally lowers pH (Fabricius et al., 2011; Hall-Spencer et al., 2008; Lidbury et al., 2012). A vent site off the island of Ischia, Naples in the south of Italy is one such example. Underwater CO_2 volcanic emissions interact with a *Posidonia oceanica* seagrass habitat off the coast of the Castello Aragonese penisula. The CO_2 bubbles from the sea floor and drives the seawater pH down to equal to- or lower than- business-as-usual IPCC projections for 2100 (pH 6.5-7.8) (IPCC, 2014), resulting in a low pH ecosystem (Hall-Spencer et al., 2008; Kroeker et al., 2011). As such, the site has been used as an analogue for ecosystems' responses to the ongoing OA projected to occur in the next century (Hall-Spencer et al., 2008; Kroeker et al., 2011; Lombardi et al., 2011b).

In most cases species abundance declines in low pH (Cigliano et al., 2010; Hall-Spencer et al., 2008; Kroeker et al., 2011). However, a few studies have identified species with higher abundances in low pH areas, primarily amphipods and brooding polychaetes (Garrard et al., 2014; Giangrande et al., 2014; Kroeker et al., 2011; Fabricius et al., 2014; Lucey et al., 2015; Ricevuto et al., 2014). This is the case in the low pH Castello CO_2 vent system where several species persist in high abundance and provide an opportunity for testing for the presence of local adaptation (Rodolfo-Metalpa et al. 2011, Calosi et al. 2013; Lucey et al. 2015).

Reciprocal transplant experiments can be used to determine levels of adaptation among populations living in low pH areas, and whether the persistence of the population is enabled by forms of adaptation to low pH (Ayrinhac et al., 2004; Etterson and Shaw, 2001). In this approach, individuals are taken from different field habitats and held in their respective 'habitat' conditions for multiple generations. Following this grow-out period, their progeny are relocated to the *in situ* source and test habitats, after which their fitness (e.g. survival) is quantified (Falconer and Mackay, 1996; Kawecki and Ebert, 2004). The performance of local genotypes can then be explored using reaction norms and analysis of variance to test for local adaptation (significant differences between trait means between populations), plasticity (significant effects of environment) or genotype by environment interactions (Nuismer and Gandon, 2008). To our knowledge, no studies

so far have used the reciprocal transplant approach to test for local adaptation to low pH habitats in this context.

Consequently, the aim of this study was to use a reciprocal transplant approach to investigate whether there was evidence for local adaptation and/or plasticity in response to natural exposure to low pH conditions representative of future OA in the Castello CO_2 vents. The study species was a Spirorbinae polychaete (Serpulidae), *Simplaria* sp., which is able to subsist in the naturally low pH vent habitat (Chapter 2). Generally, Spirorbinae are small, filter feeders that spend their adult lives within self-built spiraled tubes that are permanently attached to a substrate (Gee, 1964; Potswald, 1968; Tanur et al., 2010). They are common members of the benthic community, especially in early substrate colonization or as epibionts on other organisms (Rouse and Pleijel, 2001). These polychaetes are responsive to rapid evolutionary change through adaptation (Macdonald, 2003). A key aspect of their suitability for adaptation studies is their life history: they incubate their embryos in specialized operculum brood chambers (Bailey, 1970; Macdonald, 2003), and release non-feeding gregarious lecithotrophic larvae that settle quickly by parent worms, limiting their dispersal (Knight-Jones, 1951; Beckwitt, 1981; Kupriyanova et al., 2001). This can result in patchy distributions and significant genetic differences among populations found less than 10 m apart from seemingly identical habitats, with no apparent barriers to mutual colonization (Beckwitt, 1981). Additionally, it is thought that these brooders have radiated more rapidly than any other clade in the family (Macdonald, 2003). Spirorbinae also serve as an excellent taxon for multi-generational studies as they can be easily cultured under laboratory conditions and have relatively short generation times (~ 90 d, Kupriyanova et al. 2001).

Previous work on calcifiers in the Castello CO_2 vents identified *Simplaria* sp. as the dominant and most abundant calcifying polychaete species living in moderately low pH (~ 7.7) areas, with respect to population sizes in ambient seawater sites nearby (Chapter 2). It is also the only species of Spirorbinae polychaete growing on the *Posidonia oceanica* seagrass leaves in an aragonite-calcite tube to maturation within the pH range 6.6-7.7 (Chapter 2). With this study, I tested if the population of *Simplaria* sp. from the low pH site (7.7) would have a significantly greater tolerance to low pH *via* plasticity and/or local adaptation in comparison to that settled in ambient pH (8.1) originating populations. This also allowed me to show how the reciprocal transplant approach can be utilized to find evidence for, or lack of, adaptation to environmental drivers, which may be helpful in informing future conservation and resource management actions within the context of the global change.

4.3 Materials and Methods

4.3.1 Field site and experimental design

Local population samples from within the larger calcifying polychaete *Simplaria* sp. metapopulation living around the Castello CO_2 vent system were collected from two habitats in the *Posidonia oceanica* seagrass meadow: a low pH site (7.69 pH) and two ambient, control pH sites (8.03 pH). The low pH site was selected as the area within the CO_2 vents where *Simplaria* sp. was found in higher abundance compared to the control sites (Chapter 2). The ambient sites were located between 100 and 400 m away from the low pH site and correspond to the control areas selected for a recent study on the colonization of *Posidonia* seagrass mimics (Donnarumma et al., 2014). There is the chance that whole populations could be rafted on floating objects (i.e. boat hulls or detached *Posidiona* blades moving between sites *via* currents). However, research on the genetic structure of colonies of *Simplaria pseudomilitaris*, a likely morphotype of *Simplaria* sp., estimated that significant genetic differentiation between populations often occurs on a scale of a few meters (approx. 10 m), supporting the probability of genetic separation between collection sites in the *Simplaria* sp. in this study (Beckwitt, 1981; Kupriyanova et al., 2001). The locations for these sites correspond to those in Chapter 3, and are shown in Figure 3.1.

In order to investigate the presence of local adaptation to low pH conditions within the Castello CO_2 vent system, I used a reciprocal transplant experiment to compare the fitness responses of second generation offspring raised from grandparents from both pH habitats grown in their source conditions before being reciprocally transplanted into both field habitats (see Fig. 4.1 for experimental schematic), (Kawecki and Ebert, 2004).

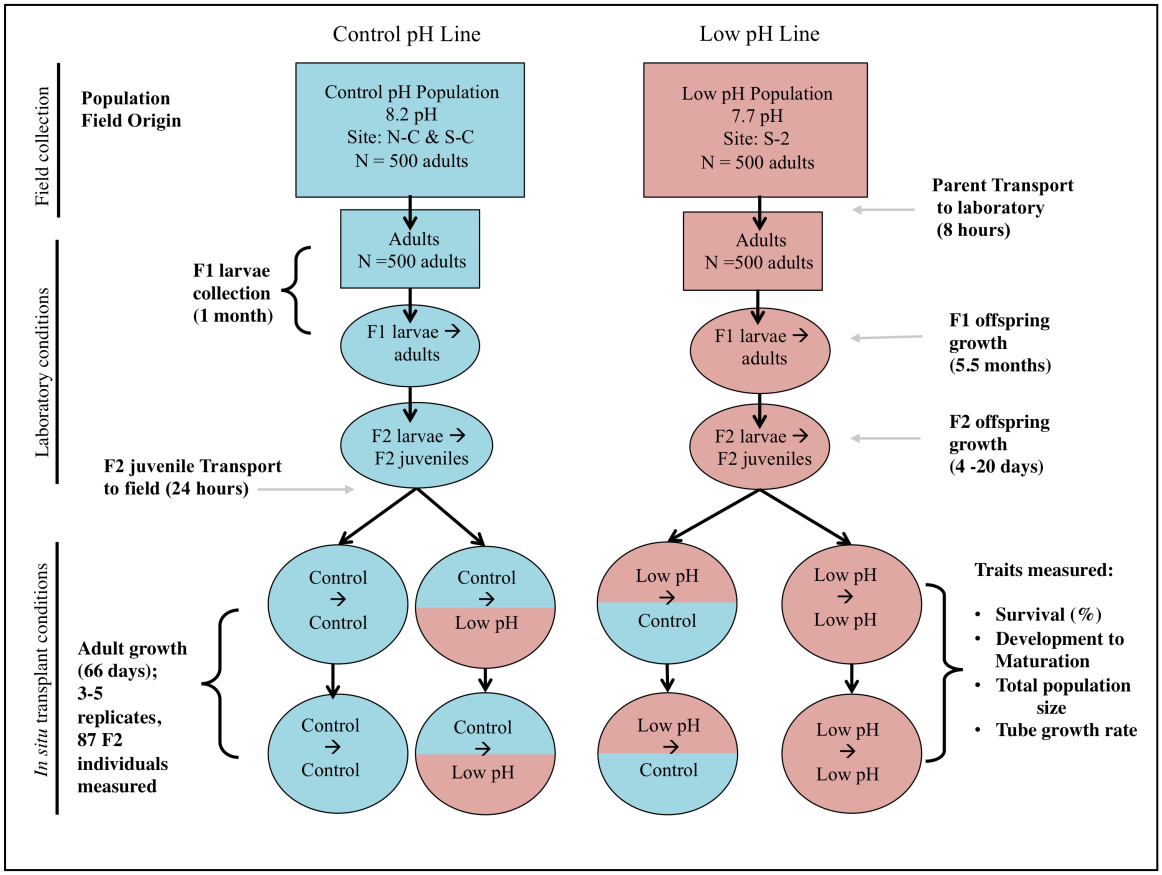

Figure 4.1 Experimental design schematic illustrating the pH environment of both populations; field originating parent samples are represented as boxes and offspring are represented as circles, colors match the pH conditions of the collection sites, laboratory, and *in situ* transplant exposures, with duel-colored circles indicating a change between origin and exposure conditions (blue: control, red: low pH).

Each starting population consisted of 500 adult individuals, a quantity regarded as suitable to minimize inbreeding or genetic drift effects in the subsequent generations (Colin and Dam, 2005; Dam, 2013). Adults collected from the two control pH field sites were mixed together to achieve the maximum genetic diversity within the control population, and provide comparably sized low and control-pH starting populations (Nacci et al., 1999). The individuals that were used in the reciprocal transplant experiment were 4-20 d old F2-generation recruits bred in laboratory grow-out conditions resembling the local pH habitats from which their respective field-collected grandparents were found (Fig. 4.1). Using F2-individuals from controlled laboratory conditions minimized any field-based plasticity and transgenerational effects that may have occurred if field-collected or F1-generation individuals were used (in accordance with similar experimental designs; Dam 2013). All F2 recruits transplanted into ambient pH were transplanted to the field site closest to the low pH site (indicated by * in Fig. 4.1). Population-level replication by pH habitat was not feasible for two reasons. Firstly, I was

required to comply with site-based restrictions using minimal transplants within the sensitive site. Boating traffic related to the summer season causes heavy disturbances in many of the alternative transplant sites. Additionally, a preliminary abundance and distribution survey before the collection of adults for this experiment found that the species was not highly abundant in either of the control or low pH areas (see Chapter 2), therefore, any statistically relevant replication within the site would have jeopardized the *Simplaria* sp. population through oversampling.

The experimental F2 individuals from both populations were left in the field for a 66 d. period before being recovered, preserved and assessed for survival, development, reproductive output, population growth, and tube growth rates. This time frame was chosen to allow adequate time for growth to maturation, and was based on life span projections of similar species (Kupriyanova et al., 2001).

4.3.2 Reciprocal transplant experiment set-up

Detailed methods on the collection of the initial adult individuals (grandparents), the subsequent laboratory grow-out, and the breeding and rearing of the F2 individuals used in this experiment are found in Section 3.2.4. Additionally, the laboratory grow-out system, animal husbandry, and water chemistry conditions for these stages are found in Section 3.2.4.2 and 3.2.5.

In preparation for the field transplant, slides with post-metamorphic individuals from the F2 generation between age 4 and 20 d from both low pH and ambient populations were collected from the laboratory grow-out systems and photographed with a digital camera (Nikon Sight DS-U1, Nikon, Milan, Italy) mounted on a light microscope (AZ100, Nikon). Photographs were analyzed with ImageJ software (Rasband WS, US National Institutes of Health, Bethesda, MD, USA) to obtain tube surface area (mm^2) (Abràmoff et al., 2004).

F1 individuals were removed from these slides, as well as any individuals on the back of the slides. This ensured that there was only one slide face with F2 individuals. Each slide had between 1–14 individuals. While controlling which slides the larvae settled on was not possible, I accounted for the resulting variability by placing slides with both low and high numbers of settlers in traps in each treatment. Furthermore, I attempted to balance the number of individuals *per* treatment by equally dividing the slides between treatment and field stakes (also see Table 4.2). Details of replication levels are given in Table 4.2. The slides were then inserted into settlement traps that consisted of three faces folded together as a triangular prism with 0.45 μm mesh caps secured on both ends. This mesh size was primarily used to attain F3 data by retaining any trochophore larvae within the trap, as the larvae are approximately 50 μm in diameter. The mesh served three additional purposes: (1) preventing any tube loss due to detachment inside the trap, (2)

decreasing the predatory risk from larger crustaceans or fish, and (3) restricting other polychaete larvae from setting inside traps. Laboratory flow-through tests using colored dye were performed to determine if water circulation through the traps differed depending on the presence of mesh caps (i.e. with or without mesh caps). No differences were observed so I assumed that food availability and water parameters inside traps would be comparable to the field measurements. This glass structure was then was inserted into a PVC tube and secured with thin plastic zip-ties (Figure 4.2).

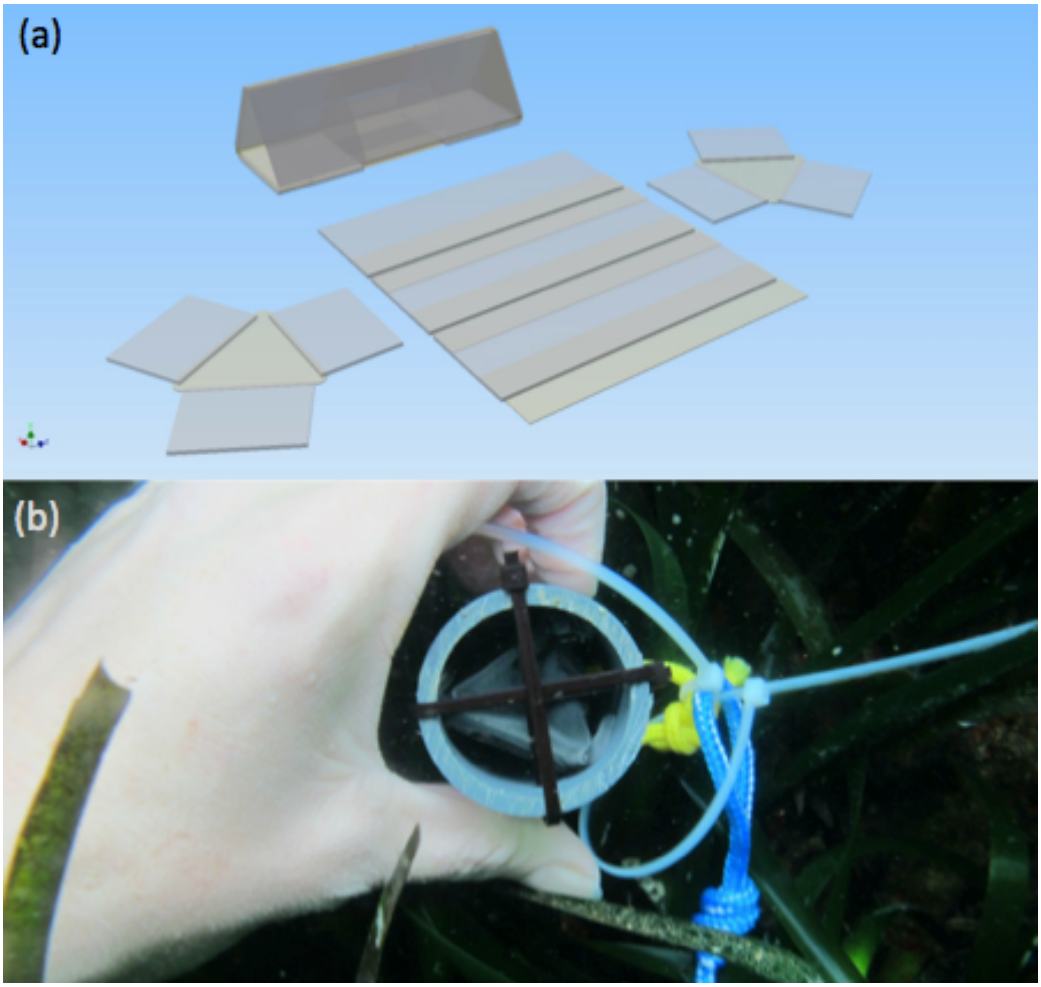

Figure 4.2 (a) Settlement traps holding laboratory-grown F2 individuals on glass slides (drawing by J. Paulus). (b) PVC tubes with animals secured inside the glass triangular prism, capped with mesh and secured to the top of stakes vertically positioned on the seafloor in the *Posidonia* meadow at the sites of both population's origin.

These field traps were transported to the Benthic Ecology Center of Ischia on April 26th, 2015. During the 8 h transport, the individuals were maintained in separate containers with filtered seawater (volume = 1.3 L; T = 22 ± 3 °C; pH: ambient = 8.15 ± 1, low = 7.7 ± 1; S = 36; density: 8 traps container $^{-1}$). All containers were kept in styrofoam

coolers packed with ice to maintain a consistent water temperature (22 ± 3 °C). Upon arrival, traps were put in flowing ambient seawater for one night. Before deployment, mesh caps were visually inspected for detached tubes. Then, three to four traps were attached to rope between 5-10 cm long and secured to the top a 40 cm long iron stakes and transferred by boat in coolers with seawater to the field sites, and brought to the seafloor *via* SCUBA diving. Stakes were pushed into the seagrass mat and vertically positioned on the seafloor in the *Posidonia* meadow at the origin sites of both populations. Attached traps drifted within the seagrass meadow. Ten stakes were prepared for the control and low pH population traps, with 10 traps for each of four treatments: (1) Control → Control, (2) Low → Low, (3) Control → Low, and (4) Low → Control, with 'Control' as ambient, control pH conditions (8.1), and 'Low' as Low pH (7.7). This experimental time frame allowed the F2 individuals to grow into adulthood, and any resulting offspring to settle on the three glass slide surfaces of the trap. The stakes are considered replicates of each treatment nested inside the traps.

4.3.3 Reciprocal transplant experiment collection and characterization of fitness metrics

The stakes with the traps were collected from the field *via* SCUBA diving after the 66 d of *in situ* exposure (on July 2nd). The traps were immediately put into a magnesium chloride solution [75 g L^{-1} seawater] to relax the specimens for 20 min, after which they were transferred to 4 % neutralized formalin for 24 h. Traps were then immersed in fresh water to rinse formalin out, and immediately transferred into 70 % EtOH for long-term preservation. This was done to prevent formalin, although neutralized, from possibly corroding the calcium carbonate tubes of the worms.

The traps were disassembled and the contents were examined to find and identify larvae or detached tubes. Recorded positions of individuals on the slides at the start of the experiment were compared to those at the end of the experiment to identify original F2 individuals. Survival at time of collection was defined by determining the presence of worm bodies within the F2 tubes, seen through the underside of the slides: presence of worm body indicated that the individual was alive throughout the experiment. Photographs of 'live' individual tubes were taken and tube surface area measured using ImageJ software described above. Tube growth rates were calculated as the ratio between final tube surface area over initial tube surface area (mm^2), which also accounted for tube dissolution. Mortality was recorded for any empty tubes or detached tubes found in the mesh without bodies.

To determine the developmental stage of the individuals, tubes were carefully broken open and worm bodies extracted. Individuals were categorized as juveniles, or mature adults with or without embryos. For mature adults with embryos, the operculum was dissected and embryos counted. Reproductive output was measured as the number of additional tubes found inside the trap on any of the three slides plus any embryos found

in the F2 operculum brood chambers. When more than one mature F2 adult was present in a trap with F3 recruits, the number of F3 individuals was divided by the number of surviving mature F2 adults in the trap. When more than one F2 individuals was found dead in a trap with living F3 individuals, offspring were assumed to originate from only one F2 parent. F3 growth was defined as the average tube area of all F3 tubes from one adult, determined by photographing new F3 tubes with ImageJ software as described above. F3 individuals with full spirals were dissected to determine developmental stage.

4.3.4 Seawater carbonate chemistry

During the laboratory grow-out period the seawater pH, temperature, and salinity were measured in each larval catchment container and holding tray daily with an integrated pH meter (SG2, Mettler-Toledo) and refractometer, (V2, TMC) (n = 6). The pH in the low pH and ambient conditions averaged 7.79 ± 0.10 and 8.22 ± 0.07, with a temperatures of 22.38 ± 1.08 °C and 22.06 ± 0.96 °C, respectively, throughout the 5.5-month grow-out period (Table 4.1). Seawater samples (250 ml) were taken monthly from the same holding tray locations for total alkalinity analysis (n = 3). Samples were fixed with $HgCl_2$ (0.02 %), stored in borosilicate flasks (250 ml), and maintained in dark, dry conditions until total alkalinity (A_T) was determined using gran titration method (Dickson, 1994; Dickson et al., 2007). Carbonate-system parameters were calculated from A_T, pH, temperature and salinity using the package SeaCarb v.2.4.8 in software R (Lavigne and Gattuso, 2013), (Table 4.1).

During the field transplant experiment, hourly measurements of pH and temperature at the low pH site were recorded with the pH-meter (Honeywell Seafet pH sensor; Martz et al. 2010), which was deployed next to the experimental transplant from June 17th, 2015 to July 2nd, 2015 (Table 4.1, Figure 4.3). pH values for the ambient site were taken from past datasets (Table 4.1). Discreet field measurements of pH, salinity, temperature, and total alkalinity were taken at the field sites at the time of specimen collection and at the experimental end with an integrated pH meter (SG2, Mettler-Toledo) and refractometer (V2, TMC) (n = 3). For total alkalinity, seawater samples (250 ml) were collected from each site (n = 3), and sampled with the same methodology used for the laboratory seawater chemistry described above.

Table 4.1 Seawater physico-chemistry parameters (mean + SD) measured (**in bold**) or calculated (plain text using the SeaCarb program*) during the laboratory grow-out phase and reciprocal transplant experiment in each pH habitat. Sampling frequency is denoted superscripts, where 'h': hourly, 'd': daily, and 'm': monthly.

	Control pH	Low pH
Laboratory: Acclimation and Grow-out Phase		
pH (d)	8.22 ± 0.07	7.79 ± 0.10
Temperature (°C) *(d)*	22.06 ± 0.96	22.38 ± 1.08
Salinity *(d)*	38.70 ± 0.99	38.57 ± 0.92
TA (mol/ kg) *(m)*	2469.78 ± 110.15	2434.51 ± 156.66
$[CO_2]$ (µmol/kg)	732.92 ± 142.69	2545.18 ± 590.09
pCO_2 (ppm)	244.05 ± 43.54	852.65 ± 197.83
$[HCO_3^-]$ (µmol/kg)	1704.45 ± 107.19	2097.39 ± 173.84
$[CO_3^{2-}]$ (µmol/kg)	307.99 ± 36.98	135.79 ± 17.88
DIC (mol/kg)	0.002 ± 1.02E-04	0.002 ± 1.72E-04
Ω calcite	4.70 ± 0.54	2.07 ± 0.27
Ω aragonite	7.16 ± 0.82	3.16 ± 0.40
*Field: Reciprocal Transplant Experiment***		
pH *(h)*	8.05 ± 0.05	7.36 ± 0.35
Temperature (°C) *(h)*	24.01 ± 0.51	24.01 ± 0.51
Salinity *(m)*	37.41 ± 1.34	37.41 ± 1.34
TA (mol/ kg) *(m)*	2401.52 ± 91.70	2283.72 ± 222.54
$[CO_2]$ (µmol/kg)	1183.30 ± 183.66	3132.11 ± 1484.10[1]
pCO_2 (ppm)	402.82 ± 61.74	5267.93 ± 7332.39
$[HCO_3^-]$ (µmol/kg)	1848.59 ± 106.07	2101.41 ± 272.14
$[CO_3^{2-}]$ (µmol/kg)	222.76 ± 22.09	73.02 ± 55.99
DIC (mol/kg)	0.002 ± 9.98E-05	0.002 ± 3.59E-04
Ω calcite	3.44 ± 0.30	1.13 ± 0.87
Ω aragonite	5.24 ± 0.46	1.73 ± 1.33

* Lavigne and Gattuso 2013; ** Note: Low pH and temperature field site monitoring spanned from June 17th to July 2nd; ambient pH site data taken from Donnarumma et al. (2014) during similar time periods. Field based TA measurements also include data from collection period and time-series data from Ricevuto et al. (2014). Outlier removed [1].

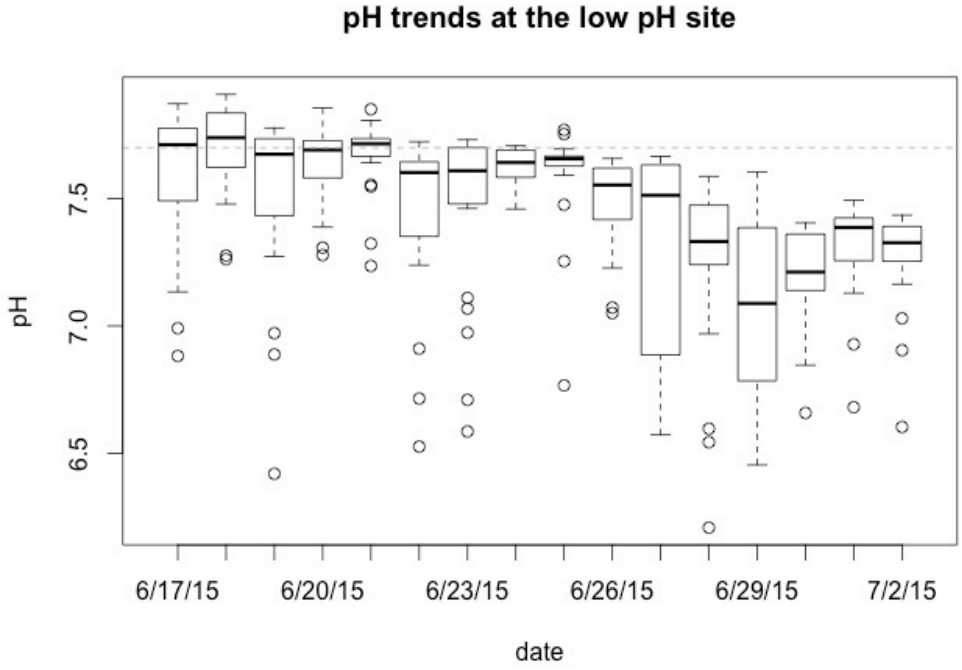

Figure 4.3 Boxplots representing the daily median, spread and skewness of pH measurements throughout each day during the reciprocal transplant experiment at the low pH transplant site, with the dashed horizontal line depicting the expected average pH (~7.7). Measurements taken hourly by a Honeywell Seafet pH sensor stationed approximately 2-4 meters from the transplants on the seafloor.

4.3.5 Data analysis

To test the relative importance of 'population' (i.e. potentially different genotypes), 'exposure' (i.e. different pH conditions) and their interaction on: (1) F2 survival; (2) maturation; (3) reproductive output, as the number of F3 recruits and embryos *per* F2 parent; (4) total population growth, as the total number of F2 survivors, embryos and F3 recruits; and (5) F2 tube growth rate, I constructed generalized linear models (GLM), setting 'population' and 'exposure' as fixed factors. Initial 'tube area' was set as the covariate, to account for differences in starting size and/or age. Initially, models included 'trap' set as a random factor nested in 'stake', which was also set as a random factor nested in 'exposure.' As the factors 'stake' and 'trap' did not exert a significant effect on the study variables, they were removed from subsequent models (Crawley, 2012). Interactions and the covariate were retained in all cases. For the traits (1) survival and (2) development to maturation, I used GLMs with binomial errors. Preliminary data analysis (Zuur et al., 2010) indicated over-dispersion for the traits (3) reproductive output and (4) total population growth, which I corrected by using a Poisson GLM, and also corrected the standard errors using a quasi-GLM model.

Two replicate stakes placed in the control habitat went missing, likely due to accidental boat anchor removal, leaving the experimental design with three stakes in the ambient field habitat, and five in the low pH field habitat. Regardless, the experimental design included four treatments with a minimum of three stake replicates, and between 12 and 33 individual worm replicates *per* treatment (see Table 4.2), and the models employed accounted for this heteroscedasticity (Sokal and Rohlf, 1995). Additionally, in each final model, non-significant terms are retained to show all interactive effects. In order to further validate the final models, non-significant terms were sequentially dropped until the minimal adequate model was reached (Crawley, 2012), and the fit of these simplified models was assessed by plotting residuals against fitted values to check for mean residual deviation of zero and constant variance.

Table 4.2 Quantity of total individuals in each reciprocal transplant treatment, and the number of corresponding traps and stakes *per* treatment.

Treatments:	Low pH → Low pH	Low pH → Control	Control → Control	Control → Low pH
Individuals (#)	25	12	16	33
Traps *per* treatment (#)	7	6	6	10
Stakes *per* treatment (#)	5	3	3	5

Differences between tube areas from F3 recruits in the control habitats from both populations were analyzed using a one-way ANOVA. No interaction could be tested with F3 tube area, as there was an insufficient quantity of individuals from the low pH population at the experiment end.

All statistical analyses were performed using the statistical software R v.3.1.3 (R Core Team, 2015).

4.4 Results

I observed comparable, significant reductions in the survival, development to maturity and total population growth in both *Simplaria* sp. populations transplanted to the low pH, and found no significant effect of 'population' or the interaction between 'population' and 'exposure' (Fig. 4.4, 4.5, Table 4.3). I also found a reduction in the number of F3 embryos and recruits produced (reproductive output) following exposure to the low pH habitat in both populations, yet this decline was only marginally significant (Fig. 4.4, Table 4.3). The low pH population was, however, able to reproduce more than once during the field period, whereas the control pH population did not. For both

populations, tube growth rates were twice as high when they were exposed to the low pH habitat – a difference that was significant (Fig. 4.5, Table 4.3). There was no significant difference between populations, however, and reaction norms were the same for all traits (Fig. 4.4, Table 4.4). In each of the traits described above except for survival, the initial tube area was a significant covariate, with low pH populations having smaller tubes, compared to control pH population. This covariate significance increased when the 'trap' effect was added to the analysis, but did not affect any other factors. This size difference likely had negative implications for every trait, except tube growth rate (i.e. decreased survival).

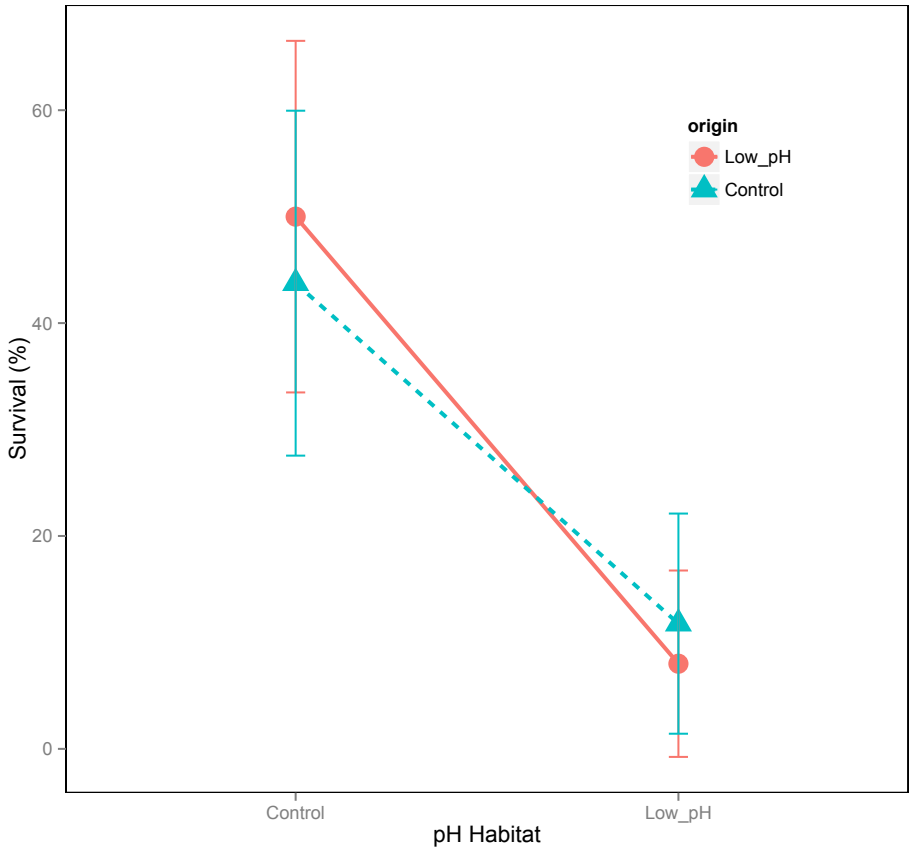

Figure 4.4 Reaction norm of percent survivorship in second-generation (F2) *Simplaria* sp. individuals from both the low pH population (red solid line) and ambient pH population (blue dotted line), transplanted into both pH habitats (8.1 and 7.7). Points are mean ± SE.

Table 4.3 Results of GLMs investigating the effect of population (genotype = G) and habitat (environment = E) on survival, maturation, reproductive output, total population growth and tube growth rate in the calcifying spirorbid *Simplaria* sp. (with initial tube area as a covariate).

Trait		**Estimate**	**SE**	**Z value**	***p***
Survival	Intercept	-1.495	0.797	-1.876	0.061
	Population (G)	0.647	0.804	0.806	0.421
	Habitat (E)	-2.083	0.822	-2.534	**0.011**
	Interaction (G * E)	-0.383	1.245	-0.308	0.758
	Initial tube area (cov)	10.826	5.669	1.910	0.056
Maturation	**Intercept**	-2.704	1.002	-2.699	**0.007**
	Population (G)	0.702	0.889	0.790	0.430
	Habitat (E)	-2.111	1.001	-2.109	**0.035**
	Interaction (G * E)	-0.400	1.557	-0.257	0.797
	Initial tube area (cov)	16.322	7.567	2.157	**0.031**
Reproductive Output	Intercept	-0.341	0.666	-0.513	0.610
	Population (G)	0.856	0.639	1.340	0.184
	Habitat (E)	-4.233	2.286	-1.852	**0.068**
	Interaction (G * E)	2.030	2.450	0.828	0.410
	Initial tube area (cov)	6.867	2.421	2.836	**0.006**
Population Growth	Intercept	-0.052	0.540	-0.096	0.924
	Population (G)	0.813	0.524	1.553	0.124
	Habitat (E)	-2.823	0.986	-2.864	**0.005**
	Interaction (G * E)	0.530	1.246	0.426	0.671
	Initial tube area (cov)	6.378	2.022	3.154	**0.002**
F2 Tube Growth Rate	**Intercept**	2.415	0.184	13.110	**< 0.005**
	Population (G)	0.077	0.205	0.376	0.707
	Habitat (E)	0.676	0.195	3.462	**< 0.005**
	Interaction (G * E)	-0.270	0.308	-0.876	0.381
	Initial tube area (cov)	-2.996	0.988	-3.033	**0.002**

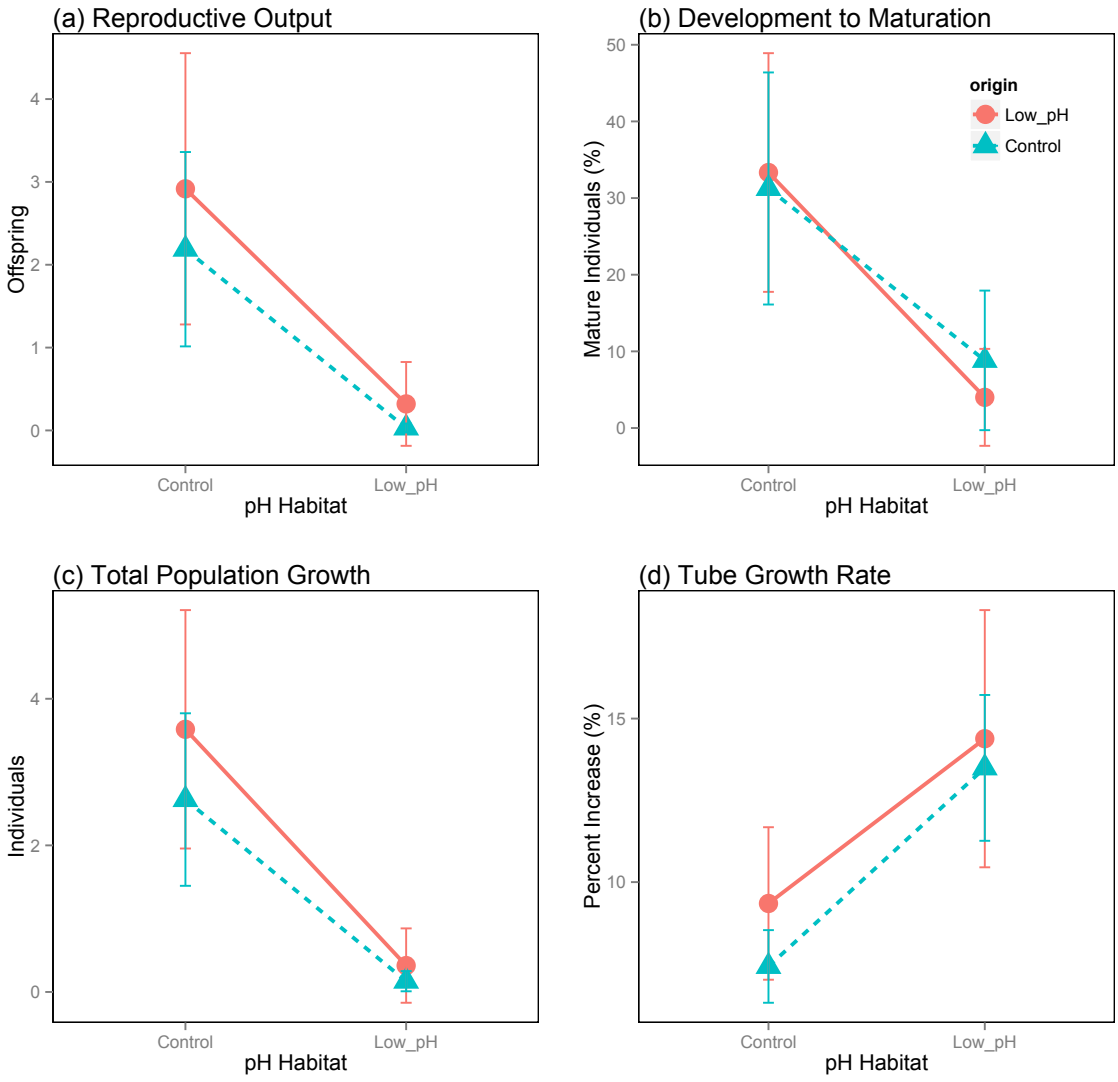

Figure 4.5 Reaction norms for fitness related traits assessed in second-generation (F2) *Simplaria* sp. individuals from both the low pH population (red solid line) and ambient pH population (blue dotted line), transplanted into both pH habitats (8.1 and 7.7): (A) reproductive output from all individuals, (B) percent of individuals developing to maturity in the field, and (C) total population size, as living individuals plus their embryos and settled juveniles of individuals, and (D) percent increase in tube growth rates. Points are mean ± SE.

Additionally, the F3 recruits grew to similar sizes in the control habitat regardless of parent population identity, with F3 recruits from low pH populations having a mean tube surface area of 0.33 ± 0.06 mm^2, and recruits from control pH populations having a mean tube surface area of 0.28 ± 0.06 mm^2, as indicated by a non-significant size effect ($F_{1, 7} = 0.381$, $p = 0.539$). In both low pH treatments, only one F3 tube was found, indicating that low pH severely inhibits tube production regardless of population identity. This tube was from the low pH population.

4.5 Discussion

This reciprocal transplant experiment provides no evidence that local adaptation in the *Simplaria* sp. population living under low pH conditions has occurred. Furthermore, I show that worms' phenotypic plastic responses could not compensate for the negative effects of exposure to low pH by improving fitness. This suggests that multigenerational exposure to low pH conditions within the CO_2 vents has not imposed selection for *Simplaria* sp. genotypes that are tolerant to extreme pH variability and low pH (~7.36). Moreover, the conditions at the low pH vent site seem responsible for reducing the mean fitness of individuals originating from both low and control pH habitats. These results stand in contrast to previous research on polychaetes and other marine calcifiers showing extensive, rapidly evolving adaptive divergence when exposed to pollution, elevated temperature, changes in pCO_2, and even multi-stressors (Grassle and Grassle, 1977; McMullin et al., 2000; Lohbeck et al., 2012; Pansch et al., 2014; Schluter et al., 2014; Rodríguez-Romero et al., 2015). Below I discuss the possible constraints to local adaptation in this low pH habitat, and emphasize the complexities involved in predicting evolutionary patterns, as well as the need for further work with more replication to further validate these findings (Bell and Collins, 2008). I then consider how the *Simplaria* sp. response might reflect the inherent inability of certain species to adapt to the stressful ocean acidification conditions expected to occur (Dupont and Pörtner, 2013).

4.5.1 Local adaptation constraints

Many studies have indicated that phenotypic plasticity might enhance the process of adaptation by moving the phenotype closer to the fitness optimum for genetic selection, e.g. adaptive plasticity (Pigliucci, 2005; Ghalambor et al., 2007). Here the responses do not seem to strongly support previous findings where plasticity was considered to be a precursor to adaptation (Merilä and Sheldon, 2000; Réale et al., 2003; Rodriguez-Romero et al., 2015). Neither of the *Simplaria* sp. populations investigated here have different plastic responses in any of the fitness traits, indicating an inability to change fitness outcomes through plasticity. However, the general depressed fitness in low pH of both populations compared to those in ambient pH habitats indicates a possible non-adaptive plastic response at the metapopulation scale (Huey and Berrigan, 1996; Calosi et al., 2013; Turner et al., 2015). Non-adaptive plasticity is where phenotypic changes do not directly contribute to increased fitness under the changed conditions, and it has primarily been associated with limiting population persistence (Chevin et al., 2010). However, current work has recently revived the idea that non-adaptive plasticity may also facilitate adaptive genetic changes by increasing the strength of natural selection (Ghalambor et al., 2015; Merila, 2015).

In contrast to the uncertain role of plasticity, bottleneck and genetic drift effects are mechanisms thought to hinder adaptation and speciation (Coyne and Orr, 2004). The

brooding nature of the *Simplaria* sp. could naturally result in founder effect/ genetic drift effects compared to species with greater dispersal potential (Beckwitt, 1980). However, genetic drift may eventually result in adaptation (Gavrilets and Hastings, 1996), as demonstrated by the numerous brooding species found with local adaptations (Sanford and Kelly, 2011). While no adaptation was evident at the time of this study, expanded distribution surveys of *Simplaria* sp. and experimental replication at alternate vent sites, as well as along varying temporal periods in the same site, would help to determine if genetic drift is affecting this population, and if improved fitness through genetic drift is possible.

Increased magnitude and frequency of the selective driver through time, in this case pH, is likely the most pertinent explanation for the lack of observable genotype environment interactions, and the significant effect of low pH 'habitat' on reduced fitness (Hoffmann and Sgrò, 2011). The mean pH in the low pH habitat during the transplant was on average 0.43 pH units lower than the low pH rearing conditions in the laboratory (*in situ* pH range: 6.14 - 7.90; mean ± SD: 7.36 ± 0.35, $pCO_2 > 5000$ μatm, Table 1). Increased CO_2 venting was likely the general cause for the observed low pH, as no pH measurements during the last week of the experiment were at or above the expected pH (Table 1, Fig. 6). Additionally, hourly measurements in the field showed consistent patterns of severe pH fluctuation, where pH decreased each night before increasing the next day (Fig. 6, outliers), the latter likely being the results of seagrass and algae diurnal photosynthetic activity. The field site's pH intensity and frequency (i.e. variability) was not replicated in the laboratory, nor was it expected during the experiment. During the past six years of pH monitoring at the low pH site, the pH was highly variable (Ricevuto et al., 2014). However, pH only reached levels lower than those recorded here in one study (Calosi et al., 2013; pH 7.19), and only for one documented week. While grandparents of the low pH originating population were subjected to previous natural pH fluctuations, the levels of the pH during the experiment may have surpassed a pH threshold (Scheffer et al., 2001; Dupont et al., 2009; Christen et al., 2013), resulting in the overall lowered fitness of both *Simplaria* sp. populations in the low pH site.

The seagrass may also be creating a micro-environment that adds to the magnitude of the low pH fluctuations, potentially causing effects to the spirorbids settled on it (Garrard et al., 2014; Wahl et al., 2016). High levels of photosynthesis are thought to provide a refuge from low pH conditions during the day; seagrass can create a localized change in pH up to 1 unit higher according to Hendriks et al. (2014). An example of this effect was observed in the spirorbid *Spirorbis spirorbis* settled on the algae *Fucus serratus* (Saderne and Wahl, 2013). In more detail, when the spirorbids were exposed to high pCO_2, a reduction in the growth rate was observed, whereas the calcification response measured during irradiation hours was 40 % higher with respect to that recorded during dark hours. Spirorbid presence in low pH vent site could therefore

be attributable to a pH buffering effect from photosynthetic and respiratory processes of the host seagrass on the carbonate system, if there is a positive net effect throughout the diurnal cycle (Saderne and Wahl, 2013). This natural pH variability has not been investigated in the *Posidonia* meadows at a micro-scale < 1 mm, but the dial fluctuations measured in the low pH site support the hypothesis that the spirorbids are naturally subjected to high variability due to photosynthetic processes. This type of variability is thought to drive selection to favor high phenotypic plasticity and/or select for more robust genotypes, as seen in Pansch et al. (2014) where barnacle tolerance to naturally low pH was higher when populations originated from highly variable pH environments, compared to non-fluctuating environments. It is therefore possible that the pH variability in the low pH site may similarly promote plasticity or robust genotypes during 'normal' venting periods, and that high venting periods may degrade any previously developed tolerances (Pansch et al., 2014). The implementation of detailed monitoring of abiotic parameters to future natural evolution experiments will help to resolve the uncertainty regarding how varying scales of temporal pH fluctuations common to coastal systems will influence plasticity and adaptation, an important and overlooked component of constraining evolutionary predictions in the context of global change (Wahl et al., 2016).

Natural pathogens may also have influenced my results (Kawecki and Ebert, 2004). Predation and pathogens in populations subjected to environmental change tend to act continuously on the average phenotype, reducing the mean fitness and impeding diversification by reducing population sizes (Van Valen, 1973; Morgan and Buckling, 2004; Meyer and Kassen, 2007; Bell and Collins, 2008). In this experiment, I controlled for main predators in the field, such as fish and crustaceans through trap protection and the use of mesh caps on these traps without limiting water flow or food. However, pathogens could not be accounted for as easily. In particular, protozoans appeared to be relatively abundant in low pH conditions, but not in ambient pH conditions in the laboratory culture. There is a substantial research body documenting the presence of protozoa on and within spirorbid tubes (reviewed in Kuprianova et al., 2001), however whether the nature of this relationship causes harm remains inconclusive (Knight-Jones et al., 1975). Generally, protozoa are associated with consuming bacteria (Barker and Brown, 1994); therefore, the increase in protozoa may indicate a change in bacterial communities with low pH (Lidbury et al., 2012). Accounts of bacterial/ microbial communities in low pH environments from other venting sites have demonstrated decreased low pH tolerance of certain species with changed microbial communities (Morrow et al., 2015). This highlights the importance of indirect community and host interactions in response to low pH, and the need for experiments specifically testing hypotheses regarding multi-species adaptive interactions (see Morrow et al., 2015).

One of the main difficulties of natural evolution studies using small CO_2 vents is the unattainability of population-level replication for many species among different vents.

This is partly because the populations living in most CO_2 vent systems are not easily comparable, i.e. different 'tolerant' populations and/or species (see Kroeker et al. (2011) and Fabricius et al. (2014), and differences between the Castello and Papua New Guinea CO_2 vent dominating species). Many of these systems are also in different regions with different conditions and stressors, e.g. Italy, Mexico, and Japan (Boatta et al., 2013; Crook et al., 2016). The small spatial scale of these CO_2 vent sites also limits the possibility to replicate populations of adequate sizes in order to acquire F2 individuals, as in the Castello CO_2 vents where population replication was not possible due to the limited quantity of individuals. Due to these caveats, the variability of the results of non-replicated studies must be cautiously assessed. In my results here, high variance in the standard error around the means for each population in most assessed traits occurred (Fig.7, Fig. 8). Despite this, the effect of 'pH habitat' was consistently significant among all traits and the interactive effects in all traits were nowhere near significant, suggesting that my findings are reliable. The variability, therefore, likely reflects either the relatively high plasticity of the studied populations, or variability in the small-scale local conditions that occurred during experimental field exposure. Replicated studies through time and the use of multiple (separated) reference sites might help to overcome these within-site replication limitations, and also help to determine if changes in inter-population variability would result in significant population effects.

Regardless of the considerations discussed above, my results may be an indication of the inability of the *Simplaria* sp. metapopulation within this system to adapt to highly variable low pH conditions. Increased mortality in this species was coupled with decreased reproductive effort and increased growth rates as a result of low pH exposure. Increased mortality is not necessarily detrimental to a population if it leads to selection for adaptive changes in life history traits, such as increased reproductive output (see Stearns 1983; Reznick et al. 1990). However, the high mortality in all populations in the low pH *in situ* habitat are associated with lower reproductive capacity, leading to lower mean fitness and therefore a reduced opportunity for adaptation (Bell and Collins, 2008).

In contrast, increased reproduction and increased mortality in the low pH population was indicative of a trade-off during the laboratory grow-out period, where mortality and recruitment in the low pH was twice that of the ambient pH after F1-generation recruitment. Furthermore, the only individuals able to produce two broods during the field experiment were from the low pH population, despite suffering from high mortality levels in low pH. These high mortality levels alongside increased recruitment levels within the low pH rearing phase align well with the idea that the two populations tested here have two different morphologies (phenotypes). This appears to be a trade-off that could lead to an adaptive phenotypic response within the population if allowed more time, but also resembles a high risk (Stearns, 1989a). An alternative explanation for the grow-out period mortality results may be attributed to natural mortality variability.

Comparisons of post-settlement mortality levels ranged from 79.5 % to over 90 % in populations of similar species of brooding spirorbids (Kupriyanova et al., 2001), indicating that the variance between the mortality levels *Simplaria* populations may be within normal ranges. Further work is necessary to establish this species' natural mortality levels by comparing the responses of other nearby *Simplaria* sp. populations. Replicated transplant experiments through time could also help to determine if increased reproduction is a significant adaptive trait or trade-off (Kawecki and Ebert, 2004).

Interestingly, exposure to low pH conditions prompted increased tube growth rates in all surviving individuals, which was coupled with decreased maturation and lower reproductive output. Tube growth rate was the only trait that increased in low pH, and notably the only trait that was not directly representative of Darwinian fitness. It is thought that tube size is generally correlated with body size, maturation and reproductive output in spirorbids (Kupriyanova et al., 2001), but my findings indicate that there may be a non-adaptive trade-off caused by low pH exposure hampering this relationship. For example, the energetically costly activity of mineralization could be detracting resources/energy away from reproductive efforts under low pH (Knoll, 2003). In a physiological context, these findings support those of Lombardi et al. (2011a), where bryozoans transplanted into low pH vent sites switched resource allocation away from defense to favoring rapid growth. Wood et al. (2008) also showed increases in calcification rates and metabolism in a brittlestar, but at the cost of muscle wastage, which was thought to be unsustainable. Furthermore, similar patterns were also found in four other marine calcifiers, where exposure to low pH implied a shift in the energy budget expenditure away from survival-related processes and into calcification (Findlay et al. 2011). The increased tube growth rates in *Simplaria* sp. exposed to low pH seem to indicate a reallocation of energy away from long-term survival towards calcification investment, which may be compounding the species risk to OA. This finding also highlights the need to measure traits linked to Darwinian fitness, as only measuring size or growth rates can misrepresent actual evolutionary responses to OA.

4.6 Concluding Remarks and Applied Relevance

The reciprocal transplant experiment I carried out provided evidence against the idea that either local adaptation or phenotypic plasticity are evolutionary strategies supporting increased fitness levels in populations of *Simplaria* sp. from the low pH habitat during an abnormally intense venting period at the Castello CO_2 vents. These results indicate that actual adaptive constraints to low pH as a selective driver can exist for this and other calcifying species and/or populations, which may be particularly relevant when the intensity and duration of pH exposure surpasses historically known variation for such populations (Kelly et al., 2012; Parker et al., 2010). This idea aligns well with the general notion that near-future low pH projections will act as a severe threat

to marine calcifiers, lowering their ability to persist through the next century (Dupont and Pörtner 2013).

The temporal scale of OA may also compound this concern. OA may exert a selective force on current marine populations that is too strong for adaptation within the given time frame (Carroll et al., 2007; Reznick and Ghalambor, 2001), despite evidence that certain marine calcifying species are able to rapidly adapt to climate changes (Schluter et al., 2014). Ocean acidification is occurring at a rate ten times faster than any time in the last 55 million years (IPCC, 2014). The pH range in some areas of the Castello CO_2 vent site is similar to that predicted in global oceans by 2100 if high CO_2 emissions continue (RCP 8.5, 50-100 years), but the vents have likely been active for approximately 2,000 years (Lombardi et al., 2011a). This time lag may have provided local species and populations a considerably longer time period to colonize and adapt than future marine populations will have. The lack of adaptive evolutionary responses in the benthic *Simplaria* sp. within the tested populations at this site, however, alludes to the possibility that the rate and magnitude of future OA conditions may not bode well for the maintenance of high levels of biodiversity in marine species through evolutionary responses. Subsequently, we may expect an increase in extinction rates of certain calcifying species under future OA conditions, as already documented through the geological record (Benton and Twitchett, 2003; Veron, 2008). This would likely have important consequences for marine ecosystem functioning (Solan et al., 2004).

This study demonstrates the functionality of using natural pH gradients with F2 generation recruits in an *in situ* reciprocal transplant experiment to test for the presence of local adaptation. This experimental approach is powerful in that it relies on the natural evolutionary pathways that populations have previously experienced – pathways that are impossible to exactly replicate in breeding experiments or experimental evolution (Bell and Collins 2008). Furthermore, this approach is considered to be the best way to assess whether changes are adaptive by separating environmental effects from genetic effects (Nuismer and Gandon 2008; Merilä and Hendry 2014), and as such can directly help us determine the importance of plasticity as a mechanism enabling adaptive evolution (Munday et al. 2013; Sunday et al. 2013). Predictably, the diversity of phenotypic plasticity responses (i.e. non-adaptive or adaptive plasticity) and the time scales in which they are presented (i.e. inter-generational or transgenerational plasticity (TGP)) make it difficult to determine the role plasticity will play in evolutionary change (West-Eberhard, 2003; Ghalambor et al. 2007; Chakraverti et al. 2015; Rodríguez-Romero et al. 2015). Expanding the basic reciprocal transplant approach to include common garden experiments performed under both low and ambient pH 'common' conditions, would help to broaden our understanding of plasticity as a mechanism of rapid adaptation by relating adaptation to TGP (Parker et al. 2015; Rodríguez-Romero et al. 2015; Thor and Dupont 2015; Chakraverti et al. 2016; Ross et al. 2016).

In this study neither local adaptation nor plasticity were found to improve the fitness of a calcifying tubeworm population from low pH vents, yet my results illustrate how realistic natural *in situ* plastic and adaptive responses can be multifaceted. I suggest that the *in situ* reciprocal transplant approach be used as a means to improve our limited knowledge of contemporary evolution of marine species under global change. The approach can also be used as a tool to refine future research with aims that can produce coordinated evolutionary predictions within the context of global change. Improving our understanding of how and why natural populations succeed or fail to adapt to ocean acidification, and global change drivers in general, will help guide resource management and conservation efforts (Palumbi 2001; Ashley et al. 2003; Stockwell et al. 2003, van Oppen et al. 2015).

5 To brood or not to brood: Are marine invertebrates that protect their offspring more resilient to ocean acidification?

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5.1 Abstract

Anthropogenic atmospheric carbon dioxide (CO_2) is being absorbed by seawater resulting in increasingly acidic oceans, a process known as ocean acidification (OA). OA is thought to have largely deleterious effects on marine invertebrates, primarily impacting early life stages and consequently, their recruitment and species' survival. Most research in this field has been limited to short-term, single-species and single-life stage studies, making it difficult to determine which taxa will be evolutionarily successful under OA conditions. Here, these limitations are circumvented by relating the dominance and distributions of the known polychaete worm species living in a naturally low pH seawater vent system to their life-history strategies. These data are coupled with breeding experiments, showing all dominant species in this natural system exhibit parental care. The results here provide evidence supporting the idea that long-term survival of marine species in low pH conditions is related to life-history strategies where eggs are kept in protected maternal environments (brooders) or where larvae have no free-swimming phases (direct developers). These findings are the first to formally validate the hypothesis that species with life-history strategies linked to parental care are more evolutionarily protected in an acidifying ocean compared to their relatives employing broadcast spawning and pelagic larval development.

5.2 Introducion

There is a pressing need to advance our predictive ability on the fate of marine biodiversity considering the rapid changes occurring due to the process of ocean acidification (OA). Most predictions in this field have been based on short-term, single-species and single-life stage studies, making it difficult to determine which taxa will be evolutionarily successful under realistic OA conditions. Observations of the marine organisms persisting in natural low pH/ high CO_2 vent systems provide a unique opportunity to circumvent these limitations and establish general eco-evolutionary

patterns that can support our critical understanding of the fate of marine biodiversity. The aim of this research was to investigate such a system to formulate a framework for identifying marine invertebrate species that are more likely to be able persist under the environmental conditions predicted to occur in the world's future oceans.

The Castello vent ecosystem provides the foundation for this framework. At this site, underwater CO_2 volcanic emissions interact with a sea grass and rocky reef habitat (Section 1.6). CO_2 bubbling from the seafloor drives the seawater pH down to equal to or lower than business-as-usual IPCC projections for 2100 (pH 6.5-7.8; Kroeker et al., 2011), effectively creating a "chemical island" approximately 2,000 years old (Lombardi et al., 2011a). The biological focus is on polychaete worms, as they are the most abundant taxonomic group in the vents after the highly mobile crustaceans (i.e. tanaids, isopods). Additionally, the polychaetes' consistent vent-dominance and the trends seen in their seasonal abundances indicate the possibility of either multi- and/or transgenerational exposure (Calosi et al., 2013; Cigliano et al., 2010; Kroeker et al., 2011; Ricevuto et al., 2014). The group also exhibits highly diverse reproductive and developmental modes (Giangrande, 1997).

In order to develop a predictive framework, I first related the type of early life-history strategies employed by species living in the vents with their known distribution and abundances. I then performed breeding experiments to characterize the developmental modes associated with the only broadcast spawning species, *Platynereis* spp. from, and around, the Ischia CO_2 vents. After which, I determined the distributions of two different *Platynereis* spp. in order to further correlate low pH abundance with life-history strategies using congeneric species. I then expand the pattern between the polychaete species that are comparably dominant in low pH areas of the Castello vents and their life history traits, to propose a framework that can be broadly used to identify other types of marine metazoans that are more likely to be able to adapt to, and survive, under the predicted environmental conditions.

5.3 Methods & Results

5.3.1 Distributions & life history strategies of the Castello vent polychaetes

Four studies have documented detailed marine species differences in abundance and distribution within the vents (Cigliano et al., 2010; Giangrande et al., 2014; Ricevuto et al., 2014; Chapter 2). From the results of these studies, all polychaete species that were identified in both the low pH and extreme low pH north and south sites were considered here, regardless of their abundance. Those species lacking any information on their life history strategies were excluded. The distribution and abundance data for each of these species along the Castello vent pH gradient was then synthesized, with pH sites grouped

into extreme low pH (S3 & N3), low pH (S2 & N2), and all ambient, control sites (SC, NC, or other control equivalent sites). Their life history traits were then categorized.

As a result, twelve of the total thirteen species with known reproductive characteristics colonizing high CO_2 vent areas were found to be brooding direct developers, ten of which had higher abundances in the venting areas than in nearby control CO_2 areas (Table 5.1). The exception was one species that on a morphological basis appeared to be *Platynereis dumerilii* (Audouin & Milne-Edwards, 1834), the only broadcast spawning pelagic developer with higher abundances in the vents (Cigliano et al., 2010; Giangrande et al., 2014; Ricevuto et al., 2014). The observation that brooding polychaete species dominate the CO_2 vent areas, along with evidence for physiological and genetic adaptation in vent-inhabiting *Platynereis dumerilii* (Calosi et al., 2013), prompted further examination of this particular species.

Table 5.1: Early life-history strategies of all polychaete species present in the lowest pH vent site. Percent abundance of each species in the extreme low, low and ambient pH sites are noted, as well as co-dependent brooding traits (interstitial species, small adult size, hermaphroditism). *Polyophthalmus pictus* omitted due to limited reproduction data. Samples with less than two specimens *per* site were considered 'rare' and not included. Calcifying Serpulidae (Spirorbinae) data based on Chapter 2 sampling and classification.

	Species, Family	**Life-history Strategies**	**Abundance in the Castello pH sites (%)**			**Adult Size/ ecology**
			Extreme low pH	Low pH	Ambient pH	
Sibling Species	*Platynereis massiliensis* (Moquin-Tandon, 1869); Nereididae	Brooder; mucus tube egg brooding and direct development (Schneider et al., 1992)	91%	/	9%	15-50 mm, sequential hermaphrodite
	Platynereis dumerilii (Audouin & Milne-Edwards, 1833); Nereididae	Broadcaster; swarming, external fertilization, and Planktotrophic-pelagic larval development (Schneider et al., 1992)	6%	/	94%	15-50 mm
Vent Species (water pH 6.4 -7.8)	*Amphiglena mediterranea* (Leydig, 1851); Sabellidae	Mucus tube egg brooding and direct larval development (Rouse and Gambi, 1998)	21%	55%	23%	5-15 mm
	Spio decoratus Bobretzky, 1970; Spionidae	Brooder; small, transparent membranous sacs hold eggs (clutches) with either benthic or pelagic juvenile development (Giangrande et al., 1992)	17%	17%	67%	10-12 mm
	Simplaria sp. Serpulidae, calcifier	Brooder; modified brood chamber releasing lecithotrophic larvae (non feeding) with ~ < 1 hr. pelagic phase (Kupriyanova et al., 2001)	19%	38%	43%	3 mm, hermaphrodite
	Exogone naidina (Oersted, 1845); Syllidae	Brooder, Direct Dev.; eggs and embryos are individually attached to the ventral side of the mother's body, becoming benthic larvae before detachment (external gestation) (Mastrodonato et al., 2003)	27%	35%	38%	Interstitial
	Exogone (Parexogone) meridionalis Cognetti, 1955; Syllidae	Brooder, Direct Dev.; external gestation (Mastrodonato et al., 2003)	44%	39%	17%	Interstitial
	Parafabricia mazzellae (Giangrande et al., 2014) Fabriciidae	Intra-tubular brooding and direct larvae development (Giangrande et al., 2014)	85%	6%	9%	Interstitial
	Brifacia aragonensis (Giangrande et al., 2014) Fabriciidae	Intra-tubular brooding and direct larvae development (Giangrande et al., 2014)	74%	19%	7%	Interstitial
	Fabricia stellaris stellaris (Muller, 1774); Fabriciidae	Intra-tubular brooding and direct larvae development (Giangrande et al., 2014)	28%	43%	29%	Interstitial
	Novafabricia posidoniae Licciano & Giangrande, 2004; Fabriciidae	Intra-tubular brooding and direct larvae development (Giangrande et al., 2014)	12%	59%	29%	Interstitial
	Rubifabriciola tonerella (Banse, 1959); Fabriciidae	Intra-tubular brooding and direct larvae development (Giangrande et al., 2014)	67%	33%	0%	Interstitial.
	Syllis prolifera Krohn, 1853; Syllidae	Stolonization, where reproductive adults form specialized gamete chambers (sexual satellites) capable of swarming; fertilized eggs sink becoming benthic metatrochophore larvae in less than 24hr. (Franke, 1999)	48%	21%	31%	10-25 mm, Sequential hermaphrodite

5.3.2 Distributions & life history strategies of the Castello vent *Platynereis* spp.

To determine whether the *Platynereis* spp. adaptations proposed by Calosi et al (2013) have led to reproductive isolation, I attempted to crossbreed *Platynereis* individuals collected from within the vent sites (vent populations) with those collected from control sites (control populations) outside the vent area, in the laboratory.

A male from the control population in the initial stages of transforming into a pelagic, swimming reproductive *P. dumerilii* was introduced into a container with an immature adult *Platynereis* sp. from the vent population. Within two hours, the male prompted this vent-originating worm to develop large yellow eggs, likely a pheromone-induced response between the two sexes (Fischer and Dorresteijn, 2004). These eggs filled the female body cavity and were five times larger than the average *P. dumerilii* eggs. The female proceeded to build a complex tube structure consisting of inner microtubes where she deposited large, fertilized eggs that immediately stopped developing (Figure 5.1).

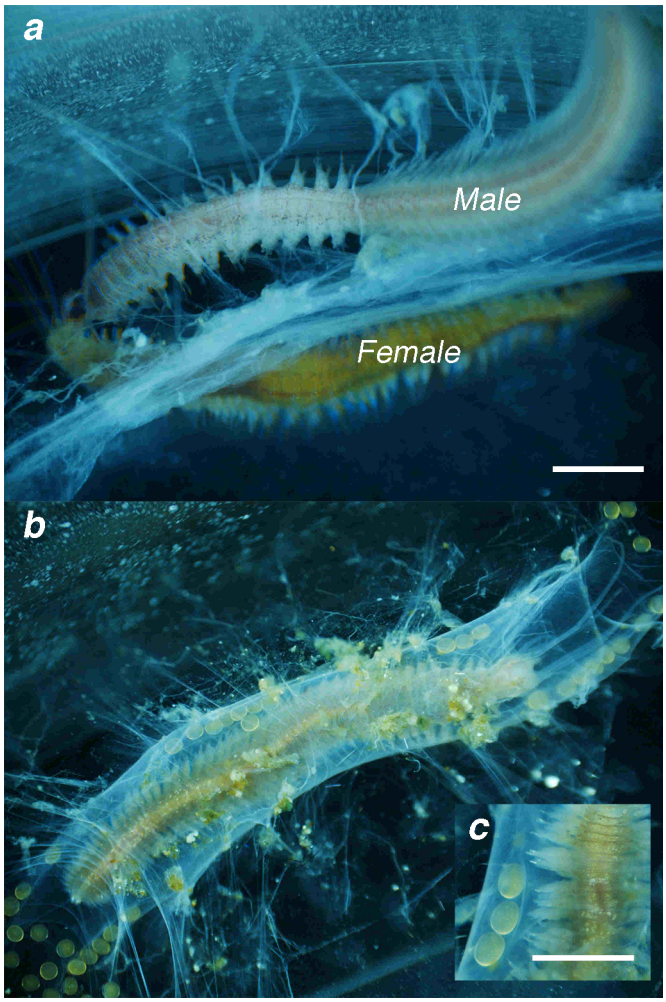

Figure 5.1 a. Initial cross-breeding activity with (top) *Platynereis dumerilii* male transforming into a pelagic, swimming epitoke full of sperm and (below) the *Platynereis massiliensis* female developing large yellow yolky eggs, (250 μm in diameter); b. Female inside tube laying and moving 74 eggs into inner brood tubes after 12 h of pairing with the male; c. Close-up of inner-parental mucus tubes holding large yellow eggs. Scale: 0.5 mm.

To determine if these two individuals represented different species, I matched the reproductive description of the female's brooding behavior to the parent's genetic identities using a CO1 barcoding approach. With this approach, DNA was extracted from two partial specimens with confirmed reproductive modes using the DNEasy Blood and Tissue Kit (Qiagen), following the manufacturer's protocol. A ~600 base pair segment of the mitochondrial cytochrome *c* oxidase subunit I was amplified using universal primers (Folmer et al., 1994) for the brooding species and polychaete-specific PolyLCO/Poly-HCO primers for the broadcasting species (Carr et al., 2011). PCR products were cleaned with Exo-SapIT (Affymetrix). Cycle sequencing was performed using BigDye Terminator v 3.1 (Life Technologies). Sequences were cleaned using Zymo Research DNA Sequencing Clean-up Kit™. Sequences were analyzed in an ABI3130 Genetic

Analyzer (Life Technologies) and edited in Sequencher v. 4.8 (Genecodes). Sequence alignment and calculation of Kimura 2-parameter genetic distances were conducted in MEGA 6 (Kumar et al., 2004).

The CO1 sequence of the pelagic form was only 0.7 % different from the published sequence of *P. dumerilii*, but the brooding form's sequence was 26 % different, indicating that it represents a separate species. Observational results confirm the female found in the vents is actually *Platynereis massiliensis* (Moquin-Tandon, 1869), a sibling species of *P. dumerilii* (Schneider et al., 1992). These two sibling species are morphologically indistinguishable as immature adults but are easily discernible upon maturation, having evolved opposing reproduction modes with morphologically different gametes (Hauenschild, 1951; Schneider et al., 1992). *Platynereis massiliensis* are protandric sequential hermaphrodites that first mature as males and fertilize a female partner's eggs laid inside a brood tube. The female then dies and the male continues ventilating and protecting the embryos inside the tube as they develop into young worms (Schneider et al., 1992). The father then changes sex and the process is repeated in the next reproductive event. *Platynereis dumerilii* have separate sexes and maturation invokes morphological changes allowing the benthic forms to leave their tubes and swarm in a single spawning event in the surface water. Adults swim to the surface, in synchronization with the full moon, in a pheromone-induced search for the opposite sex (Fischer et al., 2010; Schneider et al., 1992). They then release their gametes and die. Fertilization occurs in the seawater and the larvae go through a subsequent six-week pelagic phase (Fischer and Dorresteijn, 2004).

The CO1 analysis provided the first genetic record for *P. massiliensis*, as well as a genetic template to match previously sequenced individuals from both inside and outside the venting areas to their correct species identity. As such, the distribution of both species in and around the vent was found using this genetic template and the published sequence data from Calosi et al. (2013) for *P. dumerilii*. Results suggest that the vent site is dominated by brooding *P. massiliensis* (10:1 with *P. dumerilii*), and the control site is dominated by broadcasting *P. dumerilii* (15:1 with *P. massiliensis*), these differences being significant ($\mathbf{X}^2$: 9.808, $p < 0.005$). Additional observations of several mating pairs successfully producing juveniles inside their maternal tubes from *P. massiliensis* parents collected exclusively from the vent site further validate this distribution pattern, where the brooding species are preferentially located in low pH sites.

5.4 Discussion: Proposing a predictive framework

pH-driven brooding preference in the vents

Existing ecological knowledge suggests that the two *Platynereis* species have comparable sizes, habitats and functions, and as such are overcoming similar mechanical, chemical and physical constraints (Schneider et al., 1992). Furthermore, the known

species ranges appear to overlap on a large spatial scale: ripe females and adult males of *P. massiliensis* have been found in the Gulf of Naples (Italy) (Hauenschild, 1951), Banyuls-Sur-Mer (France) (Schneider et al., 1992), on the Isle of Man coast (British Sea) (Southward, 1923), in a Denmark fjord (Rasmussen, 1973), and Norfolk (UK) (Hamond, 1960). *Platynereis dumerilii* is also found in these localities, however comparing the species' global distributions from current records holds some uncertainty, as observations are limited and not confirmed on a molecular basis (Hartmann-Schröder et al., 1996). It is therefore unknown what prompted speciation; only that it may have been sympatric (occurring in the same habitat) some time ago. Regardless, the distribution of the brooding *P. massiliensis* in the localized venting area of this study clearly shows how this species favors this high CO_2 habitat, whereas the sibling broadcasting *P. dumerilii* species does not. This pattern can be interpreted as a solid example of pH-driven brooding preference (Calosi et al., 2013).

Using the local distribution information of the *Platynereis* spp. congeners, I revisit the synthesis of life history strategies for the complete vent polychaete community and affirm that each dominant species exhibits parental care by a form of brooding (Table 5.1). The most parsimonious mechanism driving this trend appears to be that of the direct physical protection of early life stages from the water conditions (Dupont et al., 2010; Kurihara, 2008; Melzner et al., 2009). Alternatively, or in part, this trend may be attributed to (1) an evolutionarily-based selection for phenotypes tolerant to low pH among brooding species, (2) selection of traits associated with brooding; or (3) selection through some other vent characteristics besides low pH conditions. The possibility that these CO_2-dominating brooding species have selected phenotypes tolerant to low pH is supported by the general ability of polychaetes to rapidly adapt to chronically disturbed habitats (Giangrande, 1997; Wilson, 1991). Additionally, traits commonly associated with brooders such, as short larval dispersal, continuous reproduction, in part through hermaphroditism, and small adult sizes having smaller broods *per* reproductive event support population growth continuously selecting for fitness in a specific habitat (Giangrande, 1997; Strathmann and Strathmann, 1982; Strathmann, 1974). In the most acidified zones, reduced habitat complexity and an increase in algal growth have been documented (Fabricius et al., 2014; Giangrande et al., 2014; Kroeker et al., 2013b). This may cause a loss of brooder predators or competitors not as phenotypically plastic to CO_2 stress, or result in greater availability of sheltered habitat-based type of *refugia* and/or better food resources for brooding interstitial species living in the algae (Porzio et al., 2010; Rouse and Fitzhugh, 1994; Worsaae and Kristensen, 2005). The thirteen polycheate species living in the low pH vent site have many of these traits (Table 5.1), but further investigation of OA-mediated biological and ecological effects on species' long-term OA tolerance is needed to distinguish the exact mechanisms responsible for their low-pH brooding dominance in the vents (i.e. changing feeding preferences or microbial shifts (Connell et al., 2013)).

Beyond polychaetes: expanding the pH-driven brooding preference to OA

While the proposed explanations for the pH-driven brooding preference may not be solely contingent on water chemistry (i.e. pH), the dominant species in this open, 'chemical island' CO_2 vent habitat do appear to be adapted to OA conditions in their reproductive and developmental modes. To broaden and further corroborate the evidence on a relationship between species' life history strategy and tolerance to an important global change driver such as OA, I found examples in the literature from other polychaete worms, starfish, cowries, and oysters, all following parallel adaptive pathways under climate-related stressors (Table 5.2). These species have been found inhabiting areas undergoing rapid environmental alterations and appear to have evolved direct development from broadcasting ancestors to enable them to counteract the detrimental effects of continuous disturbances. Many of these examples show species complexes in which broadcast spawning ancestors retain sensitivity to high CO_2/low pH and other environmental extremes marked by their absence in these disturbed sites, while species showing forms of parental care persist in the disturbed area (Åkesson, 1973; Gray, 1979)

Table 5.2: Review of marine taxa exhibiting climate-related tolerance and greater parental care compared to their congeneric counterparts, respectively. Poecilogonous and species complexes are noted. Comparisons use the best available data.

Marine taxa having evolved brooding and parental care and exhibiting higher stress tolerance; life history strategy	**Congeners having less parental care and lower stress tolerance; life history strategy**	**Presumed environmental factors tied to loss of parental care**	**Reference**
Cowries, Gastropoda, Cypraeidae*:* Seven genera/sub-genera independently evolved direct development with crawl-away juv.s	All genera have representative broadcast-spawning sibling clades	OA *via* high CO_2 upwelling zones, eutrophication, temperature	(Meyer, 2003)
Chilean oyster, *Ostrea chilensis:* veligers brooded in infrabranchial chamber of female and pelagic larval phase is from minutes up to 24 h	Olympia oyster, *Ostrea lurida:* brooding for 10 days in mantle cavity, veliger larvae with 2-3 week long pelagic stage	OA *via* high CO_2 upwelling zones and estuaries with extreme salinity fluctuations	(Chaparro et al., 2002, 2009; Hettinger et al., 2013)
Cushion star, *Cryptasterina hystera:* live bearing direct developers	*Cryptasterina pentagona:* gonochoric broadcast-spawning sibling species	Rapid environmental alteration, temperature based (warming)	(Byrne, 2005; Puritz et al., 2012)
Sea star, *Crossaster papposus:* lecitotrophic larvae, development through non-feeding larvae	Echinoderm species with planktotrophic larvae	OA manipulation experiments	(Dupont et al., 2009; Dupont et al., 2010b)
Slipper limpet, *Crepidula fornicata:* egg capsule brooding	Mollusc larvae from broadcast spawning parents (as morphological variables)	OA manipulation experiments	(Noisette et al., 2014)
Capitella capitata, benthic larvae	Species complex/ Sibling species	Pollution and oil spill colonization	(Grassle and Grassle, 1977, 1976; Gray,1979)
The dorvilleid polychaete, genus *Ophryotrocha*	Species complex/Sibling species	Highly organic (polluted) areas such as harbours	(Åkesson, 1973)
Polydora ciliate, brooder	Species complex/Sibling species	Pollution, red tide, fish pond; long term disturbance	(Gray, 1979)
Strebolospio benedicti, brooder	Both strategies (poecilogony)	Oil spill	(Gray, 1979)
Pygospio elegans, brooder	All can have both strategies (poecilogony); brooding is a relatively rare life-history strategy in non- disturbed habitats	Organic matter, pollution.	(Garaffo et al., 2012; Gray, 1979; Wilson, 1991)
Peloscolex benendeni, direct development, and *Heteromastus filiformis*, lecithrotrophic larvae	Assumed species complex	First colonizers after major disturbances, consistent dominances in highly polluted areas	(Gray, 1979)
Streblospio shrubsolii, brooder	Assumed species complex	Pollution, oil	(Gray, 1979)

Using a multispecies comparative method I further confirm the idea that today's organisms exhibiting brooding or direct development may be more successful in response to future OA than their pelagic broadcast spawning counterparts. One important mechanism in this proposed response hinges on dispersal capacity and extinction of brooders in the future ocean. Brooding dispersal capacity is theoretically limited by low mobility of the early developmental phases, but existing evidence counter-intuitively indicate high dispersal ability in many brooder species (Johannesson, 1988; Levin, 1984). The "Rockall paradox" reviews examples of such situations, where isolated islands are void of any pelagic broadcast spawning invertebrates. In these cases, it is noted that pelagic spawning parents assume a risk that their offspring will find suitable habitats for survival and reproduction. This strategy potentially presents difficulties as pelagic larvae may not be able to find, settle and reproduce in distant places (Johannesson, 1988). The possible link of these isolated islands to the "chemical island" of Ischia's Castello vents may be that pelagic larval settlement and recruitment success in acidified oceans is highly reduced (Cigliano et al., 2010; Connell et al., 2013; Kroeker et al., 2013b; Ricevuto et al., 2014), supporting the hypothesis of direct developer tolerance in our acidifying oceans. On the global scale of OA, pelagic larvae may be searching in vain for a 'less acidified' habitat that can retain a viable population base.

5.5 Conclusion

Current research on evolution and adaptation to OA is primarily focused on quantifying genetic variability of OA tolerant traits as an indicator of adaptive capacity into the expected future oceanic conditions (Sunday et al., 2011; Foo et al. 2012; Kelly et al., 2013). Within this context, brooders may reach extinction far before their pelagic counterparts, as they typically hold lower genetic variability (Strathmann, 1974). However, the evidence presented here points to the opposite pattern. It would be worthwhile to investigate extinction risks of brooding and pelagic-developing species in the context of global OA at different spatial and temporal scales, in an attempt to constrain the effects of both exposure to ongoing global OA and local extreme events. In fact, while brooding-associated traits may be less advantageous under local extreme events (due to dispersal limitation on a short time scale – within a generation), they may actually prove to be more adaptive in a globally disturbed ocean (on a longer time scale – trans- and multiple generations). This polychaete-based analysis, supported by a selection of other invertebrate taxa, provides compelling comparative evolutionary-relevant evidence that direct developers/brooders may do better in the globally acidifying ocean than their relatives employing broadcast spawning and pelagic larval development. The general principle I present here will be useful to inform our capacity to identify which marine taxa will likely be more tolerant to ocean acidification, largely advancing our predictive ability on the fate of marine biodiversity simply based on an aspect of species' life history strategies.

6 Conclusion

6.1 Introduction

Ocean acidification is rapidly changing marine environments, and the reaction of marine metazoans to such changes is presumed to be detrimental (Fabry et al., 2008; Kroeker et al., 2013a; Wittmann and Pörtner, 2013). Our current understanding of this reaction is limited and often overlooks potential evolutionary processes (Munday et al., 2013; Reusch, 2014). Therefore, the main aim of this thesis was to determine how marine metazoans might persist as OA conditions intensify. This was done by studying the evolutionary processes in a polychaete assemblage from a natural high CO_2/ low pH venting system representative of global ocean acidification projections for or before the year 2100. In this conclusion I revisit my three objectives (Section 1.8) and summarize how each chapter has contributed to our knowledge of OA persistence by investigating (a) OA tolerant phenotypes, and (b) plasticity and local adaptation. After which, I reinforce the third objective, (c) of formulating framework to identify persistent marine invertebrate taxa using experimental results in this thesis. I then reflect on possible future research directions.

6.2 Tolerant phenotypes

There are specific traits or phenotypes that appear to allow species to live in low pH habitats. The Castello vent system provided a unique opportunity to investigate such 'tolerant traits' by identifying and comparing the polychaete species which were persisting in higher abundances at the low pH areas to those at the nearby ambient pH areas, and why. In chapter 2 and 5, I characterized the distribution of polychaetes at sites associated with low and ambient pH. First, I used a field survey to characterize the distribution of dominant calcifying polychaetes along the pH gradient. Secondly, I used a combination of recent datasets and genetic fingerprinting (CO1) to characterize the distribution of a dominant non-calcifying polychaete in the same area. Through these investigations I discovered two species new to the low pH vent site, with *Simplaria* sp. as a possible morphotype of *Simplaria pseudomilitaris* (Chiriot-Quiévreux, 1965) having distinct, highly pronounced spines covering the operculum plate, and *Platynereis massiliensis*, likely an overlooked sibling species of *Platynereis dumerilii*. Interestingly,

both of these species were found persisting in low pH sites, and both had closely related taxa with comparatively low abundances in the low pH (termed non-tolerant species).

In order to determine the traits responsible for these species' OA tolerance, I compared their life history traits to those of their closely related non-tolerant counterparts. For the calcifying polychaetes, I did this with a comparative species recruitment experiment using *Simplaria* sp. and the closely related species, *Pileolaria militaris* (Chapter 2). With the non-calcifying *Platynereis* sister species, I did this by describing their known life history traits (Chapter 5). The results from this research indicate that brooding, direct development, and the ability to produce more offspring with faster settlement rates are important traits to persist in low pH.

These findings clearly highlight how early life history modes play an important role in determining OA tolerance within this natural system. Interestingly, early life stages in marine invertebrates have generally been thought to be the most vulnerable life stage with respect to OA conditions (Byrne, 2011a). While these past studies are predominantly focused on species that have pelagic broadcast spawning life histories (Dupont and Pörtner, 2013; Dupont et al., 2009), the findings here agree by highlighting how non-pelagic early life stages and direct developmental modes are associated with less vulnerability (reviewed in Table 5.1 & Table 5.2). Comparative species models investigating the responses of different early life histories strategies may help to elucidate the physiological mechanisms acting to promote persistence (i.e. comparisons between congeneric species with varying developmental periods, varying planktonic stage durations, non-feeding *vs*. feeding larvae) (Somero, 2010).

6.3 Plasticity and adapatation

Identifying tolerant phenotypes by comparing extant taxa from natural systems is a first step towards informing OA persistence (Reznick and Ghalambor, 2001). The subsequent step in informing OA persistence is to determine *how* phenotypic plasticity and adaptation act as mechanisms of low pH tolerance phenotypes (Calosi et al., 2013; Parker et al., 2015; Thor and Dupont, 2015). Yet, the extent of these mechanisms to promote OA persistence are not well understood (Ghalambor et al., 2015; Merilä, 2015), and the majority of work determining their scope is theoretical (Munday et al., 2013).

This paucity originates from the lack of research focused on, and capable of, assessing plasticity, adaptation, and their interaction (Dupont et al., 2013). To address this gap, this thesis assessed plasticity and local adaptation (Chapter 3 & 4) in two same-species (*Simplaria* sp.) populations with differential parental pH backgrounds in the naturally low pH vent system. I first determined if plasticity and/or adaptation were responsible for the differential tolerances to low pH at early and mature life stages (F1) with two laboratory-based reciprocal transplant experiments (Chapter 3). I then used second-generation (F2) offspring from the same two populations to determine if local

adaptation had occurred, and whether plasticity was responsible for the differential tolerances to low pH in the persistent *Simplaria* sp. population using a field-based reciprocal transplant experiment.

Evidence of local adaptation promoting persistence was found. Furthermore, phenotypic plasticity was an important mechanism promoting this adaptation, as indicated by evidence of genetic accommodation in the population of *Simplaria* sp. from the low pH vent sites in the first two transplant experiments (from embryonic development to adulthood; Chapter 3). The OA-specific adaptations observed were the ability to rapidly metamorphose, build initial tubes that were less prone to dissolution, and increase tube growth rates only after persisting through the juvenile phases. *Simplaria* sp. in low pH also had increased fecundity suggesting that increased reproductive output is also an important aspect of OA persistence *via* adaptation, as indicated in other species subjected to long-term pollution and OA condition (i.e. polychaetes: Grassle and Grassle, 1974; copepods: Thor and Dupont, 2015). These results point to the likelihood that a novel *Simplaria* sp. population has colonized and/or is in the process of adapting to the natural low pH system, explaining their abundance in low pH (Kawecki and Ebert, 2004). Until now, no past research has demonstrated such adaptation where plasticity in response to OA conditions has facilitated genetic adaptation in a natural OA system (Browman, 2016; Reusch, 2014; Sunday et al., 2013).

The ability for plasticity and/or adaptation to promote persistence was constrained when tested *in situ* (Chapter 4). Plasticity and adaptation were tested using the same *Simplaria* populations, and the *in situ* responses indicated that neither local adaptation nor plasticity was responsible for the species' natural low pH persistence. These results are likely explained by the intensity and frequency of low pH conditions that occurred during the *in situ* transplant stage (Hoffmann and Sgrò, 2011). The pH during over a third of the transplant was on average 0.36 pH units lower than the populations experienced during the past six years. Evidence that this pH change is the prime factor responsible for the lack of adaptation, as compared to Chapter 3, is supported by examples from other natural systems where environmental changes that are suddenly intensified (in a matter of days) stunt adaptation and result in extirpations (Bell and Collins, 2008). Despite my best efforts to avoid experimental artifacts these results may also be confounded by small sample sizes, age discrepancy in the F2 offspring where the low pH population was significant smaller/younger than those from the control population, or from the lack of population-level replication (Dam, 2013; Nuismer and Gandon, 2008). Repeated local adaptation tests with reciprocal transplants on these populations through time could help to determine the power of plasticity and adaptation to sustain the low pH population (Kawecki and Ebert, 2004).

6.4 Improving the predictive power of evolutionary persistence

The previous two sections overviewed the three means for exploring persistence in the Castello vent site using its polychaete assemblage: possession of tolerant phenotypes, plasticity and adaptation. The prevailing questions regarding future predictions remain: what do these results mean for extant species or population in today's oceans, and which species or populations will be able to survive in future oceans?

The results in this thesis provide evidence that traits associated with OA tolerance may be broadly applied to identify marine organisms that are more likely to be able to adapt to, and survive under the environmental conditions predicted to occur in the world's oceans. The example from Chapter 5 illustrates this clearly, where traits linked to the *Platynereis* sister species and their distributions were expanded to include those of the entire tolerant (dominant) polychaete assemblage from the Castello vent site. By identifying the life history strategies in the polychaete assemblage dominant in the Castello vents, a clear pattern that brooding behavior is an active habitat choice emerged. Brooding behavior seems to be an important strategy for the polychaete communities' persistence in the low pH vent site with all dominant species conforming to the trend. By projecting this pattern to other congeneric marine invertebrates in different systems, including temperature and pollution, the same relationship was found in other polychaete worms, starfish, cowries, and oysters, supported this key finding as a means to identify species with increased performance under climate change drivers. Furthermore, OA tolerant traits (species level) were positively correlated to adaptive traits in the low pH brooding *Simplaria* sp. population (population level), mechanistically corroborating the idea that today's brooding species may be better able to adapt in future oceans.

Spawning life histories can be locally adaptive and evidence of these non-climate related adaptations is found in multiple taxa; the decorator crab, *L. dubia*, pea crab, *P. novaezelandiae*, and the sea slug, *E. viridis*; with a larval duration of several weeks and range of tens of kilometers (Sotka, 2005). Analogously, local adaptation is not always driven by the low dispersal capacity or gene flow associated with brooding (Sanford and Kelly, 2011). The results here indicate that brooding is an adaptive trait due to a functional *mechanism(s)* particular to the environmental change, such as limiting the exposure of sensitive offspring to the low pH seawater environment. This is concerning as most marine invertebrates (approx. 80 %) have pelagic broadcast spawning life histories (Thorson, 1950), and any disturbance in these life history strategies will have far reaching consequences for species persistence and marine communities (Giménez, 2004). Additional population-level investigations of these patterns, associated adaptations and the mechanisms involved should be viewed as a critical next step for OA research.

6.5 Future research directions

Comparative studies of functional traits along natural climate–relevant gradients can help to identify the biological mechanisms enabling specific species to persist in

future climates where others cannot (Law, 2007; Massot et al., 2008; Root et al., 2003; Solan et al., 2004; Williams et al., 2008). There is a multitude of abundance and distribution datasets that are currently available for a broad range of marine species that can be utilized for such analyses (Reusch, 2014). Applying functional traits analyses to these datasets has the potential to quickly generate the needed categorical data for marine taxa tolerances that marine conservationists and managers are seeking now (Ashley et al., 2003; Palumbi, 2001; Stockwell et al., 2003). However, the capacity of these analyses is limited as they can only lead to evolutionary inferences. The more labor and resource intensive experimental evolution and natural evolution studies are more capable of constraining predictive theories of persistence with empirical evidence (Munday et al., 2013).

Proving or disproving evolutionary theories with evidence is likely the best way to improve our predictive capacity (Bell and Collins, 2008). Investigating such evolutionary theories through laboratory natural selection experiments (Bennett et al., 1992; Garland and Rose, 2009; Harshman and Hoffmann, 2000), quantitative genetics based breeding experiments (Falconer and Mackay, 1996), and natural selection studies, as described in depth throughout this thesis, have provided results that have and can continue to reshape our understanding of how marine life will react in the context of climate change. For instance, the concept that natural selection is a slow phenomenon that cannot be observed has now been predominantly discredited in light of all the experimental evolution studies that have indicated that it is possible to see directional evolution on short time scales, with conclusive data into the physiological drivers responding to selection (Garland and Rose, 2009, p. 5). For example, Lohbeck et al. (2012) showed OA tolerance after one year of selection (or 500 generations) in a strain of asexual coccolithophores, a form of calcareous microalgae. However another strain exhibited evolutionary changes of little obvious advantage under the same time and selection regime (Collins and Bell, 2006, 2004; Lohbeck et al., 2012). Furthermore, the adaptations reported in the comparably long-lived *Simplaria* sp. (Chapter 3) have likely occurred within the century, with preliminary results indicating that the vent system has been active for approximately 100 years (Lucey, unpublished data). Extreme decadal venting variation has also been observed throughout the last three decades (Gambi M.C., *pers. obs*.), suggesting that the adaptive responses of *Simplaria* sp. may have occurred within the last 30 years (i.e. rapid adaptation). This further supports the idea that organisms with long generation times have an decreased adaptive potential compared to those that are short-lived (Grant and Grant, 2006; Pespeni et al., 2013b). Together, this evidence substantiates the hypotheses that noticeable evolutionary responses can originate in a variety of marine organisms when exposures to OA occur on realistic time scales, regardless of generation times (Hairston et al., 2005; Hoffmann and Sgrò, 2011; Lindsey et al., 2013).

Another a primary forecast/theory common to current evolutionary research is that all evolutionary changes are based on the amount of standing genetic variation within a population (Sgrò et al., 2011). This genetic variation directly relates to the capacity for adaptation *via* natural selection. If genetic variation in natural populations is sufficient for them to undergo a selective process, the resulting generations may have a higher capacity to adapt to climate drivers (e.g. Pistevos et al. 2011; Sunday et al. 2011a; Chan et al. 2011; Foo et al. 2012; Lohbeck et al. 2012; Fitzer et al. 2012). Full factorial breeding designs quantify and compare additive genetic variation, maternal effects and narrow-sense heritability among species and populations and make assumptions regarding the capacity to adapt (Sunday 2011, Kelly 2013). For example, Sunday et al. (2011) and Kelly et al. (2013) both posit that the sea urchin and/or population with the most genetic variation will be better suited to adapt in the future. However, such experimental measures may not be sensitive to the nuances involved in determining what is *sufficient* for populations to undergo a selective process. The evidence that brooding species (with lower genetic diversity compared to broadcast spawners) are better suited to persist in OA conditions indirectly challenges this idea (Bishop and Pemberton, 2006; Keever et al., 2013; Meyer, 2003; Chapter 5). Additionally, the evidence of adaptation in the *Simplaria* sp. (Chapter 3 & 4) further illustrates the need for caution in the interpretation of adaptation capacity *via* genetic variation along varying timescales. Measures of genetic variation in response to a single stressor, or a particular level of stress, may be potentially insufficient to accurately predict how such variation will result in potential evolutionary rescue. Population genetics may be a way to validate standing genetic variation assumptions by comparing levels in adapted and non-adapted populations along different timescales.

The last theory that I believe is important for future research is that plasticity impedes or is indifferent to the rate of evolution. Growing interest in plasticity, primarily trans-generational plasticity, is demonstrating how plasticity is an important component of future responses (Rodríguez-Romero et al., 2015; Ross et al., 2016; Thor and Dupont, 2015). Yet, there is little research looking at the relationship between plasticity and adaptation in response to climate change. We currently do not know if plasticity can facilitate or even speed up the process of adaptive evolution with respect to the changing environment (West-Eberhard, 2003). It appears from the response of *Simplaria* sp. in Chapter 3, that environmentally induced non-heritable variation such as phenotypic plasticity can result in adaptation. As the rate of OA, and climate change in general, is faster than every before, further investigation into this theory should be seen as an essential goal.

One additional topic important to future research concerns calcifiers' risk to OA impacts on evolutionary timescales. Categorizing the effects of calcifiers has been challenging, and few conclusions have been made to date (Browman, 2016). Geologically,

OA appears to have caused a mass extinction of the deep sea calcifiers, however not all species went extinct and a remarkable radiation event took place (Hönisch et al., 2012). This resulted in many different marine calcifiers, predominately mobile planktonic species, appearing in the record during the PETM (the last time the oceans experienced conditions resembling modern OA) (Hönisch et al., 2012; Knoll, 2003; Ridgwell and Schmidt, 2010). Recent laboratory studies support the general finding that lowered pH will adversely affect calcifiers, but they also provide evidence that OA responses can greatly differ depending on the specific species (Chan et al., 2012; Christen et al., 2013; Lane et al., 2012; Saderne and Wahl, 2013). Likewise, current adaptation to OA conditions appear possible for certain extant calcifying species, but not all (i.e. coccolithophore; Lohbeck et al. (2012), *Simplaria* sp.; Chapter 3).

These differences are thought to occur because their responses are driven by highly controlled physiological processes specific to each species (Wittmann and Pörtner, 2013). A better understanding of how these processes may be affected by long-term OA exposure may provide some clarity to calcifier risk. For example, new research has demonstrated that mussels are able to change their mineralogy so they are better suited for OA conditions, switching from aragonite to the less soluble calcite when exposed for multiple generations (Fitzer et al., 2016). This could be an interesting pattern to investigate with other calcifying species exhibiting adaptive potential, such as the low pH *Simplaria* sp. population identified in Chapter 2, a species that also employs a different calcification process compared to mussels. Additionally, trade-offs in marine calcifiers adapting to OA conditions may dictate the costs associated with their adaptive capacity (Hereford, 2009; Pörtner et al., 2006). One hypothesis is that growth may be compromised by energy invested in calcifying structure/mineralogical composition. This has led to a common assumption that size matters, and if growth rates decrease, fitness decreases (e.g. Parker et al., 2012; Pespeni et al., 2013b; Sunday et al., 2011). In contrast to this supposition, evidence of increased tube growth was consistently associated with stress and mortality (Chapter 3a, 4), and only during maturation did tube growth rates increase (Chapter 3b).

Multiple climate stressors further complicate the issue (Todgham and Stillman, 2013). It is unlikely that evolutionary mechanisms for one climate stressor are the same for another (i.e. temperature and OA), however there has been little investigation on the topic. Avoidance behaviors have been attributed to local adaptation to hypoxia in a population of copepods (Decker et al., 2003). It would be interesting to determine if the tolerant copepods in the Castello vents are also exhibiting such 'avoidance behavior' by migrating into to low pH area during the day for food, and leaving at night when the pH drops due to high plant respiration rates. Migration also appears to be a form of avoidance behavior related to warming for many mobile species (Somero, 2010). Are the planktonic calcifiers that survived during the PETM representative of such a mobility-

driven form of adaptation, and if so does that mean that sessile organisms will be at more risk to the combined effects of temperature, hypoxia and OA?

It is evident that an understanding of how – and how fast – marine populations will be able to change to avoid local and global extinctions is a complex and nuanced undertaking. Whether plasticity and/or adaptation will be able to ameliorate extinction risk appears provisional to specific taxa sensitivities as well as the rate and intensity of the climate change. The uncertainties surrounding these assertions, and those regarding the interactions between ecology, evolution and the multiple climate changes underline the pressing need to test theories regarding plasticity and adaptation with the concerted aim of informing future predictions (Bell and Collins 2008). Integrating natural evolution studies with experimental evolution studies in this context could help to elucidate the interactions that are likely to reshape modern marine populations and communities as they react to the continuous and rapid rate of climate change during these next decades (Gaylord et al., 2014; Lindsey et al., 2013; Visser, 2008).

6.6 Final Conclusion

The main aim of this thesis was to determine how marine metazoans might persist as OA conditions intensify. This was done using a polychaete assemblage from a system representative of global OA projections for or before the year 2100. Initially, OA persistence was found to occur when species had specific traits related to OA tolerance. Such traits allowed some species to live in OA conditions more readily than others, such as short settlement periods, and high fecundity, along with brooding and direct development life strategies (Chapter 2 & 5). Persistence was also attained through genetic adaptation to low pH. Furthermore, phenotypic plasticity was an important mechanism facilitating this rapid adaptation, as indicated by evidence of genetic accommodation (Chapter 3). However, the ability for plasticity and/or adaptation to promote persistence appeared to be restricted by extreme low pH *in situ* conditions (Chapter 4). Despite the *in situ* limitations on adaptation and plasticity, some observed adaptive traits (population level) were positively correlated with tolerant traits (species level), such as ability to brood, rapidly metamorphose or settle. These strategies were also found to be important to the evolutionary persistence of a broader range of marine taxa under multiple types of climate related stress, and may therefore represent a way to identify taxa with less extinction risk due to ongoing climate change.

7 Glossary

Definitions as used in this document

1) **Active and passive plasticity**: plasticity considered active is associated with adaptive plasticity (e.g. plant growing toward sun), but can also be passive when the cause is indirect (e.g. baby's head is flattened going through the birth canal) (West-Eberhard, 2003)

2) **Adaptation:** adaptation refers to both the current state of being adapted to a given environmental condition (i.e. to possess an **adaptive trait** with a current functional role in the life of an organism that is maintained and evolved by means of natural selection), as well as the dynamic evolutionary process that leads to the adaptation of a trait and a organism. Adaptations enhance the fitness of individuals by natural selection in natural populations (Falconer and Mackay, 1996)

3) **Adaptive plasticity**: phenotypic changes that move the phenotype closer to the fitness optimum for genetic selection (Ghalambor et al., 2007)

4) **Canalization**: refers to an evolved reduction in developmental flexibility that buffers development of an adaptive phenotype against environmental or genetic perturbations (Grether, 2005; West-Eberhard, 2003)

5) **Congeneric species**: an organism belonging to the same taxonomic genus as another organism

6) **Darwinian fitness**: a measure of the capacity of a variant type to invade and displace the resident genotype in competition for the available resources (Demetrius and Ziehe, 2007; Garland and Rose, 2009). Evolution selects for highest fitness as a function of lifetime fecundity and survival (Dam, 2013; Roff, 1997)

7) **Developmental plasticity**: occurs when exposure to a novel environment at a specific life stage affects its performance in that environment at a different phase of life, either intra-generationally or trans-generationally (West-Eberhard, 2003)

8) **Fitness**: the capacity to survive and reproduce (Garland and Rose, 2009)

9) **Functional trait:** a trait that strongly influences organismal performance (McGill et al., 2006)

10) **Genetic accommodation** is a modern term used to describe the process of heritable changes that occur in response to a novel induction. It is a mechanism of evolution

wherein a novel phenotype, penetrated either through a mutation or environmental change, is refined into an adaptive phenotype through quantitative genetic changes, and can result in either increased or decreased environmental sensitivity of a plastic phenotype (Crispo, 2007)

11) **Genetic assimilation:** a type of genetic accommodation where the phenotype is favorably selected by the process of directional selection, and is genetically determined and canalized (a loss of plasticity or a flat reaction norm) (Ghalambor et al., 2007)

12) **Genetic compensation:** is when the environmentally induced phenotype is non-adaptive, and selection favors genetic variation that occurs in a different direction than the plastic response, resulting in increased, or decreased plasticity, or no change in the level of plasticity (Grether, 2005)

13) **Genotype**: at the population level, it is the average differences among genotypes, across environments; at the individual level it is the actual set of genes affecting the phenotype and shaping all aspects of the norm of reaction (i.e. both its plasticity and 'height' in an environment-phenotype space) (Pigliucci, 2005)

14) **Local adaptation:** the fine-tuning of a population to their local environment *via* natural selection, which results in resident genotypes that have a higher fitness in their native habitat than genotypes from more distant populations (Reznick and Ghalambor, 2001; Sanford and Kelly, 2011)

15) **Non-adaptive plasticity**: the environmentally induced phenotype in the new environment has on average reduced fitness or is further away from the new adaptive peak compared to the ancestral phenotype (Ghalambor et al., 2007)

16) **Persistence**: the continued or prolonged existence of an organism or taxa. The ability to persist is the first step that is confronted by any organism that faces directional selection (Reznick and Ghalambor, 2001)

17) **Phenotype**: the composite of an organism's observable characteristics or traits, including its morphology, development, biochemical or physiological properties, phenology, behavior, and products of behavior; an expression of both genetic and environmental factors (Chevin et al., 2010)

18) **Phenotypic plasticity**: At the population level, it is the average differences among environments, across genotypes; at the individual level, it is an attribute of the individual reaction norm, indicating that the genotype (through interactions with the environment) generates different phenotypes depending on the external conditions.

The only case of zero plasticity is when the reaction norm is flat and parallel to the environment axis (DeWitt and Scheiner, 2004; Merilä and Hendry, 2014; Pigliucci, 2005; West-Eberhard, 2003); a phenomenon of a genotype producing different phenotypes in response to different environmental conditions; a property of an individual or genotype that may be adaptive, non-adaptive or neutral with regard to an individual's fitness (Ghalambor et al., 2007)

19) **Reaction norms**: reaction norms are used to demonstrate the evolutionary patterns of each population under the driving selective variable (Dam, 2013)

20) **Reversible and irreversible plasticity**: Stearns (1989b, p. 438) uses the word 'plasticity' for a trait change that cannot be undone, or is *irreversible* (e.g. bubble gum); and a trait change that can change back to the original trait as *reversible* (e.g. rubber balloon (West-Eberhard, 2003)

21) **Speciation**: the evolution of complete reproductive isolation- this can be induced in the laboratory, and that this process can feasibly operate in nature (Johannesson et al., 2010)

22) **Synchronic analysis:** concerns itself with the evolution and change that has occurred at a particular moment of time (Reusch, 2014)

23) **Tolerance:** the ability of an organism to endure [low pH] conditions through the composite of an organism's observable traits or phenotypes, including its morphology, development, biochemical or physiological properties, phenology, behavior, and products of behavior (Stearns and Koella, 1986)

24) **Trait**: a well-defined, measurable property of organisms, usually measured at the individual level and used comparatively across species (McGill et al., 2006).

25) **Trans-generational plasticity**: the environment experienced by the parents influences the performance of offspring in the same environment through nutritional, somatic, cytoplasmic, or epigenetic transfer between generations (West-Eberhard, 2003)

26) **Transgenerational plasticity**: the environment experienced by parents influences the performance of offspring in the same environment through nutritional, somatic, cytoplasmic, or epigenetic transfer between generations; may take two generations of environmental acclimation to be expressed (Sunday et al., 2013)

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9 Appendices

9.1 *Simplaria* sp. life cycle assessment

9.1.1 Introduction

Approximately eighty percent of all marine invertebrates exhibit a diverse range of life stages and characteristics appropriate to functioning during various different developmental life stages (Thorson, 1950). Likewise, the tubeworm *Simplaria* sp. has early life stages include embryos that are protected in brood chambers attached to the parent, free-swimming, non-feeding trochophore larvae metamorphs that settle in the benthic environment and juveniles, and which then commence tube formation (Potswald, 1978). Adult worms are sessile filter feeders that build calcareous tubes (Kupriyanova, 2003; Potswald, 1978). Due to the taxonomic uncertainty of *Simplaria* sp., however, very little is known about this species' life history. A preliminary objective in this study was therefore to determine differences in developmental timing and overall survival of both populations under their respective pH environment to categorize, for the first time, the basic life histories of the species.

9.1.2 Materials & Methods: Grow-out period and aquarium system

Two generations of the *Simplaria* sp. populations were reared for Chapter 3 & 4 in a grow-out system consisting of two experimental seawater-culturing aquaria simulating the individuals' respective pH field habitats (N < 500 *per* parent population; see collection details in Chapter 3). The details for this culture system are described in Chapter 3. This grow-out period followed both low pH and ambient, control originating pH populations through two generations, a period which lasted over six months (162 d). During this period, I assessed mortality, development to maturation, and the number of new recruits in each population and generation. Assessments were bimonthly.

9.1.3 Results: *Simplaria* sp. life cycle dynamics

Wild caught parents continuously reproduced during the first month in the laboratory, which resulted in 599 F1 recruits from the control and 1,076 F1 recruits from the low pH parent populations. The first F1 recruit from the control pH habitat was observed 5 d after relocation to the laboratory, whereas the first low pH F1 recruit was observed 7 d after relocation (Figure 9.1). Greatest mortality levels were observed within the first three months of F1 recruitment with 76 % mortality in the low pH, and 55 % in the control population. In the following four months, mortality declined to 45 % and 24 %, in the low pH and control pH populations, respectively. The low pH population's

mortality was comparably higher throughout the grow-out period, however mortality levels for both control and low pH populations were equal at the 4-month mark (Figure 9.2).

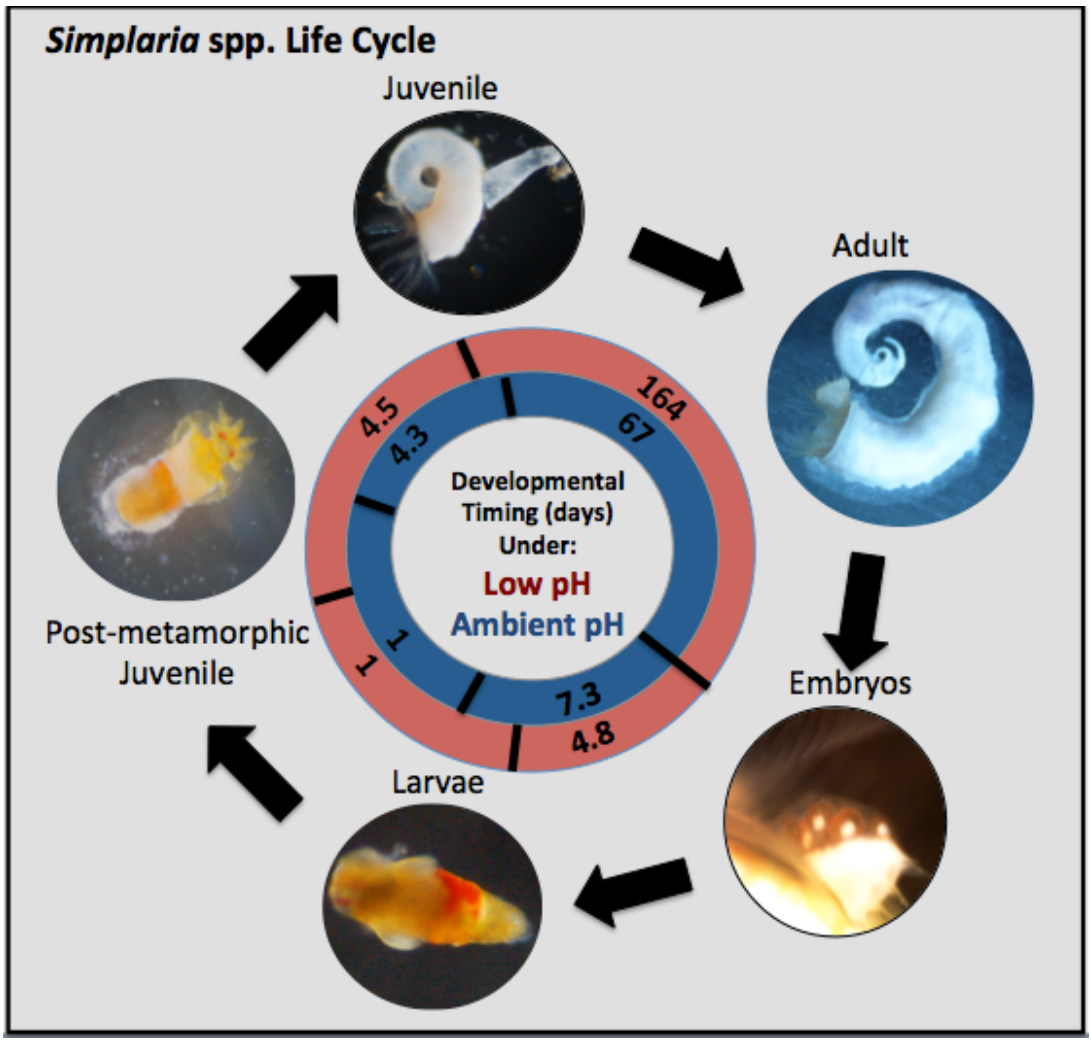


Figure 9.1 Life cycle and corresponding developmental times (in days post-embryo) of each life stage of *Simplaria* sp., with parents acclimated to, and offspring grown under, low (7.7) and ambient (8.1) pH, in the red and blue rings, respectively.

Approximately 34.1% of the initial F1 recruits from the control population survived and became reproductively mature adults. In the low pH population, only 13.3 % of the initial F1 recruits survived to maturity. However, the actual quantities of mature individuals were not dissimilar, with quantities of 143 mature individuals in the low pH and 204 in the control pH. Additionally, the time frame for the first F1 individual

to reach maturation was five months in control population *vs*. six months in low pH population (Figure 9.1).

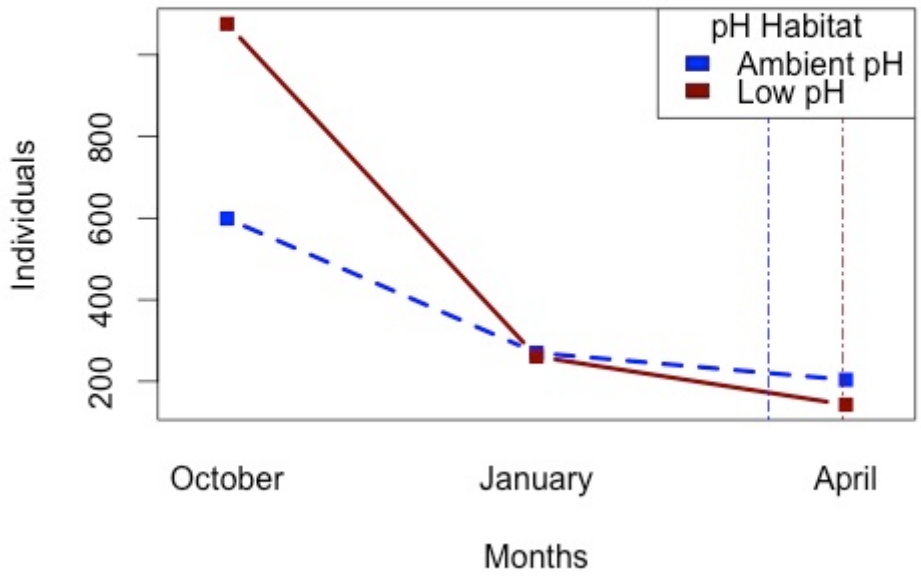


Figure 9.2 F1 Total surviving F1-offspring through time, with red solid lines indicating both field and laboratory acclimation to low pH, and blue dotted lines indicating both field and laboratory acclimation to ambient pH. Vertical dashed lines indicate date of first F2 recruits for each population.

9.1.4 Discussion and Conclusions

Full life-cycle assessments demonstrate that individuals from the low-pH population incurred a 17 % reduction in developmental rate, as well as a 20.1 % decrease in survival when raised in low pH conditions, potentially hindering any positive adaptive effects found in Chapter 3, at a larger, population scale. Conversely, these rates may represent an artifact of natural populations' mortality rate variation (Knight-Jones, 1981).

The development of the new Mediterranean species, *Simplaria* sp., from low pH habitats indicate a potential trade-off between increased reproductive output and slowed development, compared to the control population (Reznick, 1985). These results suggest adverse impacts imposed at the population-level due to low pH conditions. Furthermore, incrementally slow, yet high mortality between one and five months in the low pH population may negate any positive adaptive effects seen in Chapter 3 at a population level (Harvey and Hall-Spencer, 2015). These results allude to the need for exploring population level consequences to fully represent any OA impacts (Bell and Gonzalez, 2009).

9.2 *Simplaria* sp. tube mineralogy assessment

9.2.1 Introduction

The abundance of many marine calcifiers in the Castello vents is dramatically reduced relative to surrounding ambient areas (Kroeker et al., 2011). The assumed cause of this reduction in calcifying taxa has been widely attributed to the low pH and altered carbonate chemistry at the vents, which makes it difficult to produce and maintain calcareous parts (Knoll, 2003). As such, low pH and high pCO_2 conditions present in the Castello vent sites are likely to also cause tube corrosion and dissolution in the calcifying polychaete, *Simplaria* sp. These effects may be more pronounced depending on the mineral composition of the tubes (Knoll and Fischer, 2011). Aragonite and calcite have differing solubilities, with aragonite being more prone to dissolution. Therefore, the physical threat of OA to organisms may be highly dependent on the mineral composition of their calcified parts (Fitzer et al., 2014). Therefore, the aim of this report was to determine the mineralogical composition of adult *Simplaria* sp. tubes.

9.2.2 Materials & Methods

The morphology and mineralogical composition of adult *Simplaria* sp. tubes from the southern gradient were investigated using scanning electron microscopy (SEM) (Quanta FEG 650, SEM, Oregon, USA; magnification ranges of 25x up to 100x) and high resolution Raman spectroscopy (Renishaw InVia, Raman microscope, UK). Tubes were dried at 25 °C for 48 h before both analyses. Before SEM analyses, tubes were cleaned with an ultrasonic bath and fine brush.

9.2.3 Results

SEM revealed signs of corrosion in the center of the tubes from specimens of *Simplaria* sp. collected in the extreme low pH site (S3) (Figure 2.2c and 2.3d). Additionally, visual observations revealed notable morphological differences in the specimens from S3 and N3 compared to the other sites. The S3 and N3 tubes were found with more tube material localized around the tube mouth, or tube collar (Figure 2.2). Many individuals from S3 visibly exhibited some tube corrosion, with varying levels of damage distending from the spiral center. Individuals from the low pH area (S2) had little to no signs of tube corrosion or damage (N2 was not assessed) and tubes from the ambient (SC and NC) had little or no signs of corrosion or damage.

Qualitative mineralogical investigation revealed that tubes of adult *P. militaris/Simplaria* sp from the southern gradient were mainly aragonitic, with calcite peaks present in tubes of some individuals. Aragonite was also found to be the dominant biomineral throughout the tube area, from the tube collar region to the spiral center (Figure 9.3).

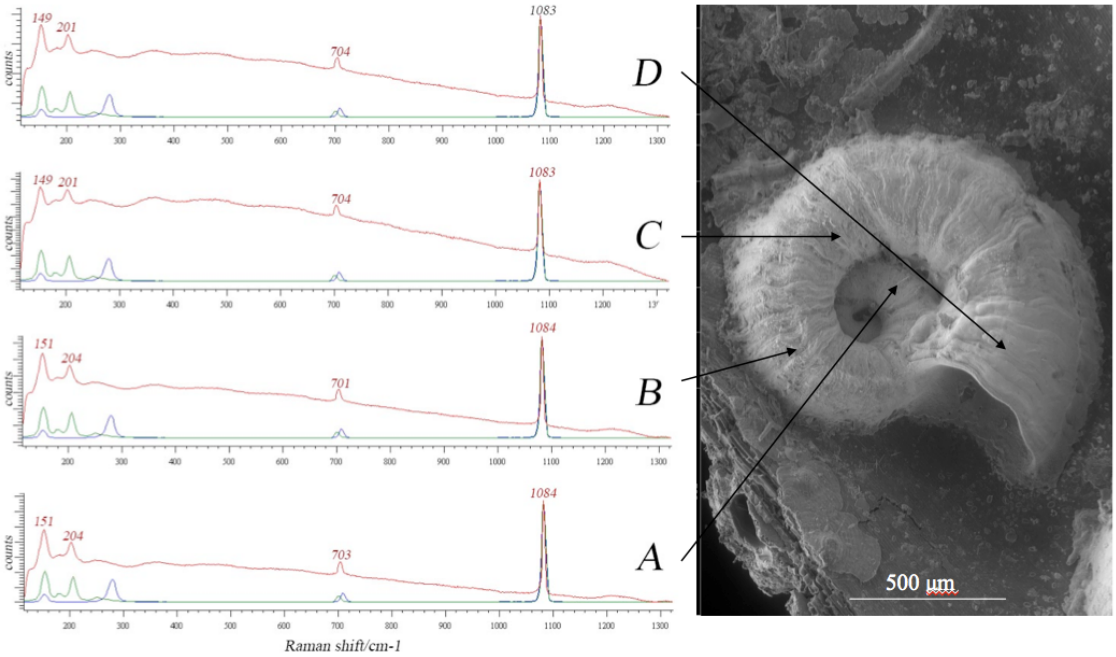


Figure 9.3 One spirorbid mineralogy tube sample from the extreme-low pH site, S3; red line indicates the sample, while the blue and green lines show the calcite and aragonite reference profiles, respectively. A-D represent the positions on the tube where the RAMAN laser point was focused; inset photograph maps these points along the tube.

9.3 Chapter 2: *Simplaria* sp. taxonomic details and count datasets

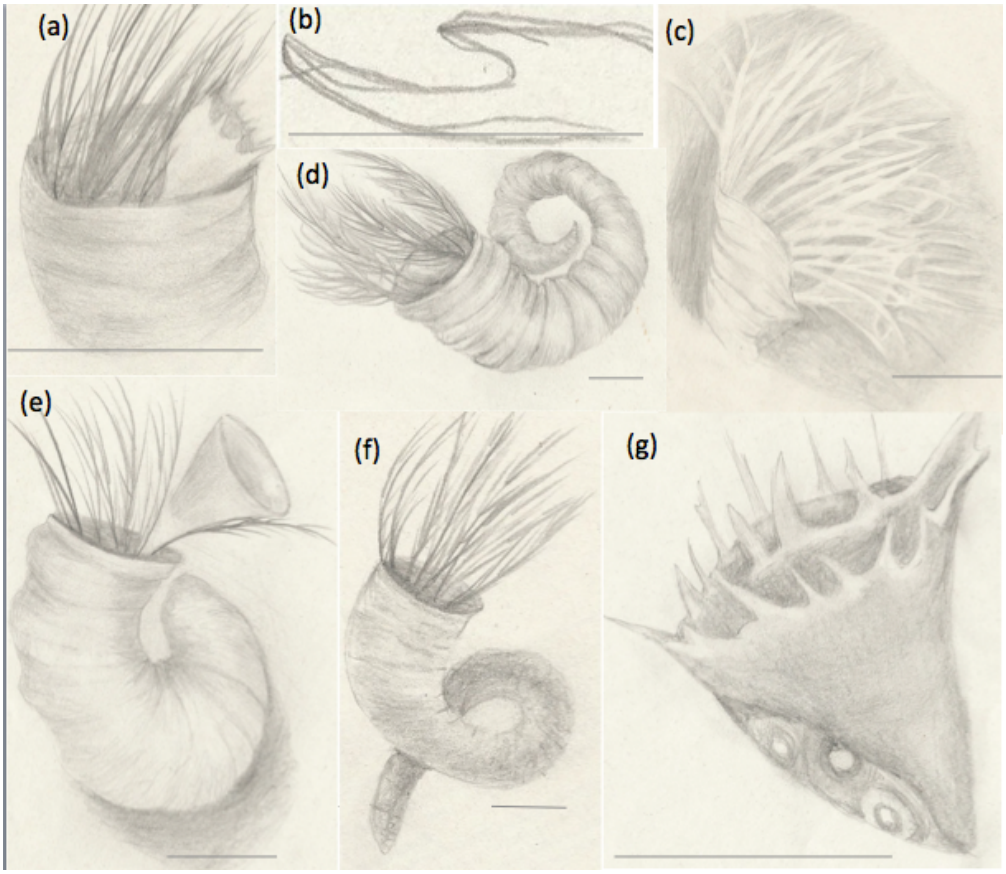


Figure 9.4 *Simplaria* sp. from the vent site S2; (a) Mouth of tube, (b) calcified spine on ventral point of mature operculum, (c) tentacular crown, (d) tube, (e) tube and juvenile operculum, (f) 2-week old tube, (g) mature adult operculum full with visible embryos; Scale bars indicate 0.5 mm. Drawings by C. Lucey

Table 9.1 Dissecting ratios, as the ratio between the number of specimens accurately identified to the total number of specimens found, and interpolated the total number of each species for each replicate: [# of *P. militaris* identified]: [Total spp. found] * [Total spp. found] by replicate.

Site	Replicate	Total spp.	*P. militaris*	*Simplaria* sp.	Other spp.	Identified	Ratio Identified	Percent *Simplaria* sp.
NC	*A*	146	62	48	13	123	0.84	39%
	B	178	68	54	12	134	0.75	40%
	C	161	26	32	12	59	0.37	54%
	D	243	78	29	5	112	0.46	26%
N2	*A*	50	12	16	0	28	0.56	57%
	B	80	19	33	2	54	0.68	61%
	C	54	25	35	1	61	1.13	57%
	D	94	20	54	1	75	0.8	72%
N3	*A-D*	46	5	5	0	10	0.22	50%
SC	*A*	113	4	28	3	35	0.31	80%
	B	71	1	25	0	25	0.35	100%
	C	132	0	57	2	59	0.45	97%
	D	180	5	78	5	83	0.46	94%
S2	*A*	75	2	39	1	42	0.56	93%
	B	106	9	42	3	54	0.51	78%
	C	234	1	99	3	103	0.44	96%
	D	158	0	41	1	42	0.27	98%
S3	*A-D*	47	0	13	0	13	0.28	100%

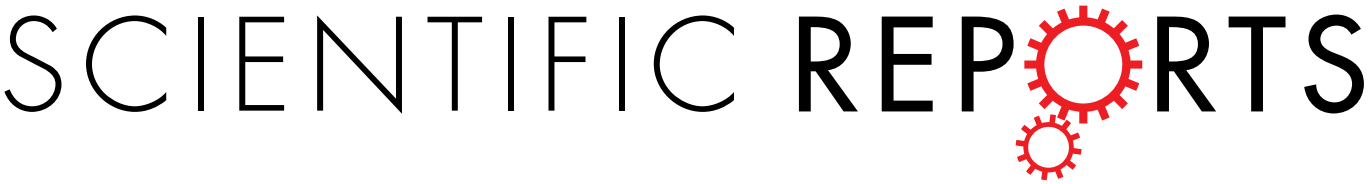
9.4 Chapter 3: Additional data

Table 9.2 Mean values ± standard error for all traits measured in the marine polychaete *Simplaria* sp. in both reciprocal transplant experiments. The number of replicates for each trait is provided in parentheses.

		Reciprocal-Transplant Experiments			
	Transgenerational acclimation pH	Control	Low	Control	Low
	Exposure	Control pH		Low pH	
Exp. 1	Survival (%)	100 ± 0.000 (3)	100 ± 0.000 (6)	58.67 ± 23.98 (5)	96.60 ± 1.80 (6)
	Larval release rate (days)	7.333 ± 1.528 (3)	6.571 ± 4.237 (7)	5.000 ± 4.291 (4)	4.750 ± 1.815 (12)
	Settlement rate (days)	1.000 ± 0.00 (3)	1.000 ± 0.00 (6)	1.187 ± 0.491 (5)	1.064 ± 0.057 (6)
	Post-metamorphic Stage (days)	1.000 ± 0.00 (3)	1.000 ± 0.00 (6)	0.707 ± 0.491 (5)	1.186 ± 0.057 (6)
	Tube surface area, day 7 (mm^2)	0.145 (1)	0.140 ± 0.010 (4)	0.154 ± 0.034 (2)	0.116 ± 0.005 (4)
	Operculum Diameter (mm)	0.177 (1)	0.174 ± 0.010 (4)	0.193 ± 0.020 (2)	0.160 ± 0.004 (4)
	Tube Dissolution (%)	0 (3)	0 (4)	38.88 + 23.23 (4)	13.58 + 7.43 (6)
Exp. 2	Survival (%)	76.2 ± 4.10 (3)	0.795 ± 0.068 (3)	0.786 ± 0.021 (3)	0.869 ± 0.025 (3)
	Net Tube Growth (mm^2)	0.085 ± 0.043 (3)	0.159 ± 0.030 (3)	0.143 ± 0.058 (3)	0.295 ± 0.011 (3)
	Net Operculum Change (mm)	-0.013 ± 0.001 (3)	0.010 ± 0.013 (3)	-0.034 ±0.023 (3)	0.021 ± 0.017 (3)
	Peripheral flange area (mm^2)	-0.044 ± 0.028 (3)	0.008 ± 0.009 (3)	-0.021 ±0.010 (3)	0.020 ± 0.022 (3)

9.5 Published manuscripts (Chapter 4 & 5)

See following pages.



OPEN

To brood or not to brood: Are marine invertebrates that protect their offspring more resilient to ocean acidification?

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Anthropogenic atmospheric carbon dioxide (CO_2) is being absorbed by seawater resulting in increasingly acidic oceans, a process known as ocean acidification (OA). OA is thought to have largely deleterious effects on marine invertebrates, primarily impacting early life stages and consequently, their recruitment and species' survival. Most research in this field has been limited to short-term, single-species and single-life stage studies, making it difficult to determine which taxa will be evolutionarily successful under OA conditions. We circumvent these limitations by relating the dominance and distribution of the known polychaete worm species living in a naturally acidic seawater vent system to their life history strategies. These data are coupled with breeding experiments, showing all dominant species in this natural system exhibit parental care. Our results provide evidence supporting the idea that long-term survival of marine species in acidic conditions is related to life history strategies where eggs are kept in protected maternal environments (brooders) or where larvae have no free swimming phases (direct developers). Our findings are the first to formally validate the hypothesis that species with life history strategies linked to parental care are more protected in an acidifying ocean compared to their relatives employing broadcast spawning and pelagic larval development.

We focused on the unique coastal vent ecosystem of Ischia island (Italy), where underwater CO_2 volcanic emissions interact with a seagrass and rocky reef habitat[1]. CO_2 bubbling from the seafloor drives the seawater pH down to equal to or lower than business-as-usual IPCC projections for 2100 (pH 6.5–7.8[1,2]), effectively creating a "chemical island" approximately 2,000 years old[3]. Our biological focus is on polychaete worms, as they are an abundant taxonomic group in the vents[1]. Their consistent vent-dominance and the trends seen in their seasonal abundances indicate the possibility of either multi- and/or transgenerational exposure[4–7]. Furthermore, the group exhibits highly diverse reproductive and developmental modes[8].

We related the type of early life history strategies employed by species living in the vents with their known distribution and abundances[1,5,6]. We found twelve of the total thirteen species with known

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			Abundance in the Castello pH sites (%)[1,5,7,9, personal data*]			
	Species, Family	Life-history Strategies	Extreme low pH	Low pH	Ambient pH	Co-dependent brooding traits
Sibling Species	*Platynereis massiliensis* (Moquin-Tandon, 1869); Nereididae	Brooder; mucus tube egg brooding and direct development[11]	91%	/	9%	15–50 mm, sequential hermaphrodite
	Platynereis dumerilii (Audouin & Milne-Edwards, 1834); Nereididae	Broadcaster; swarming, external fertilization, and Planktotrophic-pelagic larval development[11]	6%	/	94%	15–50 mm
Vent Species (water pH 6.4–7.8)	*Amphiglena mediterranea* (Leydig, 1851); Sabellidae	Mucus tube egg brooding and direct larval development[44]	21%	55%	23%	5–15 mm
	Spio decoratus Bobretzky, 1970; Spionidae	Brooder; small, transparent membranous sacs hold eggs (clutches) with either benthic or pelagic juvenile development[45]	17%	17%	67%	10–12 mm
	Pileolaria spp. Serpulidae, calcifier*	Brooder; modified brood chamber releasing lecithotrophic larvae (non feeding) with ~4 hr. pelagic phase[46]	19%	38%	43%	3 mm, hermaphrodite
	Exogone naidina (Oersted, 1845); Syllidae	Brooder, Direct Dev.; eggs and embryos are individually attached to the ventral side of the mother's body, becoming benthic larvae before detachment (external gestation)[47]	27%	35%	38%	Interstitial
	Exogone (Parexogone) meridionalis Cognetti, 1955; Syllidae	Brooder, Direct Dev.; external gestation[47]	44%	39%	17%	Interstitial
	Parafabricia mazzellae (Giangrande *et al.*, 2014) Fabriciidae	Intra-tubular brooding and direct larvae development[9]	85%	6%	9%	Interstitial
	Brifacia aragonensis (Giangrande *et al.*, 2014) Fabriciidae	Intra-tubular brooding and direct larvae development[9]	74%	19%	7%	Interstitial
	Fabricia stellaris stellaris (Muller, 1774); Fabriciidae	Intra-tubular brooding and direct larvae development[9]	28%	43%	29%	Interstitial
	Novafabricia posidoniae Licciano & Giangrande, 2004; Fabriciidae	Intra-tubular brooding and direct larvae development[9]	12%	59%	29%	Interstitial
	Rubifabriciola tonerella (Banse, 1959); Fabriciidae	Intra-tubular brooding and direct larvae development[9]	67%	33%	0%	Interstitial
	Syllis prolifera Krohn, 1853; Syllidae	Stolonization, where reproductive adults form specialized gamete chambers (sexual satellites) capable of swarming; fertilized eggs sink becoming benthic metatrochophore larvae in less than 24 hr.[48]	48%	21%	31%	10–25 mm

Table 1. Early life-history strategies of all polychaete species present in the lowest pH vent site. Percent abundance of each species in the extreme low, low and ambient pH sites are noted, as well as co-dependent brooding traits (interstitial species, small adult size, hermaphroditism). *Polyophthalmus pictus* omitted due to limited reproduction data. Samples with less than two specimens *per* site were considered 'rare' and not included. Calcifying Serpulidae (Spirorbinae) data based on unpublished sampling and classification.

reproductive characteristics colonizing high CO_2 vent areas to be brooding or direct developers (eggs kept in protected maternal environment/no free-swimming larval phases). Ten had higher abundances in the venting areas than in nearby ambient CO_2 areas (Table 1). The exception was one species, morphologically appearing to be *Platynereis dumerilii* (Audouin & Milne-Edwards, 1834), the only broadcast spawning pelagic developer with higher abundances in the vents[5,7,9].

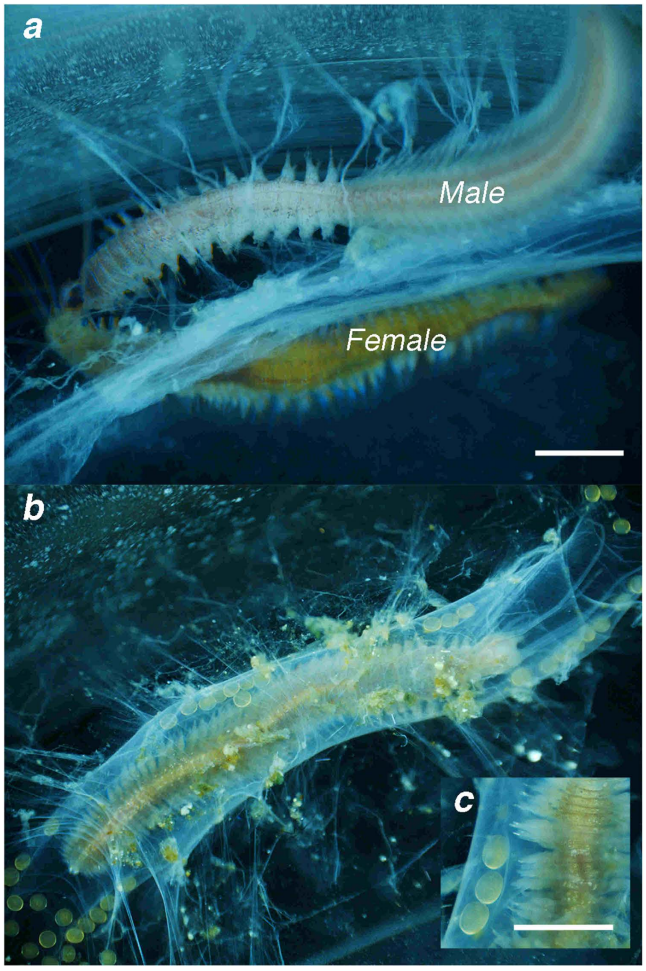

Figure 1. **a**. Initial cross-breeding activity with (top) *Platynereis dumerilii* male transforming into a pelagic, swimming epitoke full of sperm and (below) the *Platynereis massiliensis* female developing large yellow yolky eggs, (250 μm in diameter); **b**. Female inside tube laying and moving 74 eggs into inner brood tubes after 12 h of pairing with the male; **c**. Close-up of inner-parental mucus tubes holding large yellow eggs. Scale: 0.5 mm.

The observation that brooding polychaete species dominate the CO_2 vent areas, along with evidence for physiological and genetic adaptation in vent-inhabiting *Platynereis dumerilii*[6], prompted further examination of this particular species. To determine whether these adaptations have led to reproductive isolation, we attempted to crossbreed *Platynereis* individuals collected from within the vent sites with those collected from control sites outside the vent sites, in the laboratory.

A male from the control population in the initial stages of transforming into a pelagic, swimming reproductive *P. dumerilii* was introduced into a container with an immature adult *Platynereis* sp. from the vent population. Within two hours, the male prompted this vent-originating worm to develop large yellow eggs, likely a pheromone-induced response between the two sexes[10]. These eggs filled the female body cavity and were five times larger than the average *P. dumerilii* eggs. The female proceeded to build a complex tube structure consisting of inner microtubes where she deposited large, fertilized eggs that immediately stopped developing (Fig. 1).

We matched the reproductive description of the female's brooding behaviour to the parent's genetic identities using a COI barcoding approach (Supplementary Methods). While the COI sequence of the pelagic form was only 0.7% different from the published sequence of *P. dumerilii*, the brooding form's sequence was 26% different, indicating that it represents a separate species. Observational results confirm that the female found in the vents is actually *Platynereis massiliensis* (Moquin-Tandon, 1869), a sibling species of *P. dumerilii*[11]. These two sibling species are morphologically indistinguishable as immature adults but are easily discernible upon maturation, having evolved opposing reproduction modes with morphologically different gametes[11,12]. *Platynereis massiliensis* are protandric sequential hermaphrodites that first mature as males and fertilize a female partner's eggs laid inside a brood tube. The female then dies and the male continues ventilating and protecting the developing embryos inside the tube as they develop into young worms[11], after which the father changes sex and the process is repeated in the

next reproductive event. *Platynereis dumerilii* have separate sexes and maturation invokes morphological changes allowing the benthic forms to leave their tubes and swarm in a single spawning event in the surface water. Adults swim to the surface, in synchronization with the full moon, in a pheromone-induced search for the opposite sex[11,13]. They then release their gametes and die. Fertilization occurs in the sea water and the larvae go through a subsequent six-week pelagic phase[10].

Our COI analysis provides the first genetic record for *P. massiliensis*, as well as a genetic template to match previously sequenced individuals from both inside and outside the venting areas to their correct species identity. We did this using published sequence data from Calosi *et al.* (2013) for *P. dumerilii*. Results suggest that the vent site is dominated by brooding *P. massiliensis* (10:1 with *P. dumerilii*), and the control site is dominated by broadcasting *P. dumerilii* (15:1 with *P. massiliensis*), these differences being significant ($\mathbf{X}^2$: 9.808, $p < 0.005$). Additionally, we observed several mating pairs successfully producing juveniles inside their maternal tubes from *P. massiliensis* parents collected exclusively from the vent site.

It is not known what prompted speciation in these two species[11]. Existing ecological knowledge suggests that they have comparable sizes, habitats and functions, and as such are overcoming similar mechanical, chemical and physical constraints[11]. Additionally, the known species ranges appear to overlap on a large spatial scale: ripe females and adult males of *P. massiliensis* have been found in the Gulf of Naples (Italy)[12], Banyuls-Sur-Mer (France)[11], on the Isle of Man coast (British Sea)[14], in a Denmark fjord[15], and in Norfolk (UK)[16]. *Platynereis dumerilii* is also found in these localities, however we are cautious to compare the species' global distributions from current records, as observations are limited and not confirmed on a molecular basis[17]. Speciation may have been sympatric in the past (occurring in the same habitat), but the distribution of the brooding *P. massiliensis* in the localized venting area of this study clearly shows how this species favours this high CO_2 habitat, whereas the sibling broadcasting *P. dumerilii* species does not. This pattern can be interpreted as a solid example of pH-driven brooding preference[18].

Using the local distribution information of these congeners, we revisit the synthesis of life history strategies for the complete vent polychaete community and affirm that each dominant species exhibits parental care by a form of brooding or direct development (Table 1). The most parsimonious mechanism driving this trend appears to be that of the direct physical protection of early life stages from the water conditions[19–21]. Alternatively, or in part, this trend may be attributed to (1) an evolutionarily based selection for phenotypes tolerant to low pH among brooding species, (2) selection of traits associated with brooding; or (3) selection through some other vent characteristics besides low pH conditions. The possibility that these CO_2-dominating brooding species have selected phenotypes tolerant to low pH is supported by the general ability of polychaetes to rapidly adapt to chronically disturbed habitats[8,22]. Furthermore, the traits commonly associated with brooders, such as short larval dispersal, continuous reproduction, in part through hermaphroditism, and small adult sizes having smaller broods *per* reproductive event, support respective population's survival by continuously selecting for fitness to a specific habitat[8,23,24]. Low pH habitat-based changes may be indirect factors influencing brooding preference as well[4,9,25]. For instance, habitat complexity and increased algal growth may cause a loss of brooder predators or competitors not as phenotypically plastic to CO_2 stress, such as microbial shifts deterring pelagic larval recruitment[26] Alternatively, a greater availability of sheltered habitat-based types of *refugia* and/or better food resources for brooding interstitial species living in the algae may occur[27–30]. The thirteen polycheate species in this study live in the low pH vent habitat and have many of these traits (Table 1), but further investigation of OA-mediated biological and ecological effects on species' long-term OA tolerance is needed to distinguish the exact mechanisms responsible for low pH brooding dominance[31,32].

These possibilities show that brooding and/or direct development may not be solely contingent on water chemistry, however the dominant species in this open 'chemical island' CO_2 vent habitat do appear to be adapted to OA conditions in their reproductive and developmental modes. To broaden and further corroborate our evidence on a relationship between species life history strategy and tolerance to an important global change driver such as OA, we found examples in the literature from other polychaete worms, starfish, cowries, and oysters, all following parallel adaptive pathways under climate and environmental-related stressors (Table 2). These species have been found inhabiting areas undergoing rapid environmental alterations and appear to have evolved direct development from broadcasting ancestors to enable them to counteract the detrimental effects of continuous disturbances. Many of these examples show species complexes in which broadcast spawning ancestors retain sensitivity to high CO_2/low pH and other environmental extremes marked by their absence in disturbed sites, while species showing forms of parental care persist in the disturbed area[33,34].

This multispecies comparative method substantiates the idea that today's organisms exhibiting brooding or direct development may be more successful in responding to future OA than their pelagic broadcast spawning counterparts. One important consideration in this proposed response hinges on dispersal capacity and extinction of brooders in the future ocean. Brooding dispersal capacity is theoretically limited by low mobility of the early developmental phases, but existing evidence counter-intuitively indicate high dispersal ability in many brooder species[35,36]. The "Rockall paradox" reviews examples of such situations, where isolated islands are void of any pelagic broadcast spawning invertebrates. In these cases, it is noted that pelagic spawning parents assume a risk that their offspring will find suitable habitats for survival and reproduction. This strategy potentially presents difficulties, as pelagic larvae may not be able to find, settle and reproduce in distant places[35]. The possible link of these isolated islands to the

Marine taxa having evolved brooding and parental care and exhibiting higher stress tolerance; life history strategy	Congeners having less parental care and lower stress tolerance; life history strategy	Presumed environmental factors tied to loss of parental care	Reference
Cowries, Gastropoda, Cypraeidae: Seven genera/sub-genera independently evolved direct development with crawl-away juveniles	All genera have representative broadcast-spawning sibling clades	OA *via* high CO_2 upwelling zones, eutrophication, temperature	49
Chilean oyster, *Ostrea chilensis*: veligers brooded in infrabranchial chamber of female and pelagic larval phase is from minutes up to 24 h	Olympia oyster, *Ostrea lurida*: brooding for 10 days in mantle cavity, veliger larvae with 2-3 week long pelagic stage	OA *via* high CO_2 upwelling zones and estuaries with extreme salinity fluctuations	50–52
Cushion star, *Cryptasterina hystera*: live bearing direct developers	*Cryptasterina pentagona*: gonochoric broadcast-spawning sibling species	Rapid environmental alteration, temperature based (warming)	53,54
Sea star, *Crossaster papposus*: lecitotrophic larvae, development through non-feeding larvae	Echinoderm species with planktotrophic larvae	OA manipulation experiments	19,31
Slipper limpet, *Crepidula fornicata*: egg capsule brooding	Mollusk larvae from broadcast spawning parents (as morphological variables)	OA manipulation experiments	55
Capitella capitata, benthic larvae	Species complex/ Sibling species	Pollution and oil spill colonization	33,56,57
The dorvilleid polychaete, genus *Ophryotrocha*	Species complex/Sibling species	Highly organic (polluted) areas such as harbours	34
Polydora ciliata, brooder	Species complex/Sibling species	Pollution, red tide, fish pond; long term disturbance	33
Streblospio benedicti, brooder	Both strategies (poecilogony)	Oil spill	33
Pygospio elegans, brooder	All can have both strategies (poecilogony); brooding is a relatively rare life-history strategy in non-disturbed habitats	Organic matter, pollution.	22,33,58
Peloscolex benendeni, direct development, and *Heteromastus filiformis*, lecithrotrophic larvae	Assumed species complex	First colonizers after major disturbances, consistent dominances in highly polluted areas	33
Streblospio shrubsolii, brooder	Assumed species complex	Pollution, oil	33

Table 2. Review of marine taxa exhibiting climate-related tolerance and greater parental care compared to their congeneric counterparts, respectively. Poecilogonous and species complexes are noted. Comparisons use the best available data.

"chemical island" of Ischia's vents may be that pelagic larval settlement and recruitment success in acidified oceans is highly reduced[4,5,7,26], supporting the hypothesis of direct developer pH tolerance. On the global scale of OA, pelagic larvae may be searching in vain for a 'less acidified' habitat that can retain a viable population base.

Current research on evolution and adaptation to OA is primarily focused on quantifying genetic variability of OA tolerant traits as an indicator of adaptive capacity into the expected future oceanic conditions[37–40]. Within this context, brooders may reach extinction far before their pelagic counterparts, as they typically hold lower genetic variability[24]. However, our evidence points to the opposite pattern. It would be worthwhile to investigate extinction risks of brooding and pelagic-developing species in the context of global OA at different spatial and temporal scales, in an attempt to constrain the effects of both exposure to ongoing global OA and local extreme events. In fact, while brooding-associated traits may be less advantageous under local extreme events, due to dispersal limitation on a short time scale – within a generation, they may actually prove to be more adaptive in a globally disturbed ocean (on a longer time scale: across multiple generations). Our polychaete-based analysis, supported by a selection of other invertebrate taxa, provides compelling comparative evolutionary-relevant evidence that direct developers/brooders may do better in the globally acidifying ocean than their relatives employing broadcast spawning and pelagic larval development. The general principle we present here will be useful to inform our capacity to identify which marine taxa will likely be more tolerant to ocean acidification, largely advancing our predictive ability on the fate of marine biodiversity simply based on an aspect of species' life history strategies.

Methods for the sequencing procedure

DNA was extracted from two partial specimens of confirmed reproductive modes using the DNEasy Blood and Tissue Kit (Qiagen), following the manufacturer's protocol. A ~600 base pair segment of the mitochondrial cytochrome *c* oxidase subunit I was amplified using universal primers[41] for *Platynereis massiliensis* and polychaete-specific PolyLCO/Poly-HCO primers for *P. dumerilii*[42]. PCR products were cleaned with Exo-SapIT (Affymetrix). Cycle sequencing was performed using BigDye Terminator v 3.1 (Life Technologies). Sequences were cleaned using Zymo Research DNA Sequencing Clean-up Kit™.

Sequences were analyzed in an ABI3130 Genetic Analyzer (Life Technologies) and edited in Sequencher v. 4.8 (Genecodes). Sequence alignment and calculation of Kimura 2-parameter genetic distances were conducted in MEGA 6[43]. The sequences have been deposited in GenBank under accession numbers KP127953 (*P. massiliensis*) and KP127954 (*P. dumerilii*).

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Author Contributions

1) N.M.L. conceived the idea; 2) All co-authors planned the project/methods; 3) N.M.L., L.D. and A.S. carried out the experiment/sequencing; 4) N.M.L. interpreted the results with support from M.C.G., A.S. and P.C.; 5) N.M.L. wrote the first draft of this MS, and all co-authors contributed to the writing of the finalised version.

Additional Information

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