Potential Effects of Ocean Acidification on Cold-Water Marine Invertebrates

by

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Abstract

Anthropogenic emissions of carbon dioxide are causing an overall decrease of ocean pH, now termed ocean acidification (OA). This change in water chemistry has potentially dire implications for organisms living in marine environments, especially at high latitudes where carbonate saturation states are already low. OA has been shown to affect processes such as calcification, physiology, reproduction and development, with species-specific differences being observed. The goal of the present study was to better understand the effects of OA on weakly calcifying, cold-water species with lecitotrophic larvae, using the sea cucumber *Cucumaria frondosa*. In addition, it aimed to gain information on how OA might affect deep-sea organisms, using the hermaphroditic polychaete *Ophryotrocha* sp. Focal species were exposed to a ~0.4 unit decrease in pH for 19-26 weeks using a realistic flow-through system. I then investigated the reproductive output of each species, as well as its behaviour, spawning, larval development and calcification. Lipid and fatty acid profiles were also examined in *C. frondosa*. Findings varied between the two species. Gamete synthesis was disrupted by low pH in *C. frondosa*. Consequently, mature oocytes exhibited morphological discrepancies and negative buoyancy, leading to high embryonic mortality. Skeletal ossicles were affected in terms of abundance, microstructural appearance and chemical composition. In comparison, *Ophryotrocha* sp. exhibited a decrease in spermatozoa production but an increase in the number and size of oocytes under low pH. There was a trend towards a lower effective fecundity and development appeared to be slower under OA conditions, however these observations will require further investigation. The present analysis contributes to a growing understanding of the potential impacts of predicted near future OA gathered through long-term transgenerational exposure. It will assist in filling

gaps in OA research pertaining to weakly calcifying species from cold-water and deep-sea environments that rely on lecithotrophic (maternally-provisioned) development. Together, the results suggest that these species may undergo significant challenges under future OA conditions, with cascading effects on the environments of which they are vital components.

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Co-Authorship Statement

The research described herein, including data collection and analysis, and all written work, was performed by Katie Verkaik under the supervision and guidance of Annie Mercier, with technical and intellectual input from Jean-Francois Hamel. The authorship of journal contributions arising from the thesis chapters will therefore be:

Chapter 2 and Chapter 3: K. Verkaik, J.-F. Hamel, A. Mercier.

Chapter 1: General Introduction

1.1 Ocean Acidification

Anthropogenic emissions of carbon dioxide (CO_2) are hypothesized to be leading to the acidification of the world's oceans. Human activities such as the burning of fossil fuels generate an increase in the partial pressure of atmospheric CO_2 (ρCO_2), part of which is absorbed by oceans that are acting as a sink for excess CO_2. When CO_2 dissolves in water, it forms carbonic acid which dissociates, forming an equilibrium between hydrogen ions, bicarbonate ions and carbonate; the concentration of hydrogen ions in seawater increases, resulting in a decrease in pH, now termed ocean acidification (OA) (Caldeira and Wickett 2003). This concentration change causes an equilibrium shift resulting in the depletion of available carbonate and dissolution of deposited forms (Gattuso and Lavigne 2009). The calcium carbonate saturation state is also lowered, which then increases the rate of dissolution (Orr et al. 2005).

Reports predict an overall 0.4 unit decrease in pH by 2100, which could have widespread impacts on marine life (Caldeira and Wickett 2003; Raven 2005). Some locations are already particularly at risk as they are exhibiting measurable seasonal changes in seawater pH, which will only be compounded by OA. The melting of ice, in combination with OA conditions is altering the carbon chemistry of sub-polar and polar waters, such as the Canadian Basin (Yamamoto-Kawai et al. 2009). Additionally, regions with seasonal coastal upwelling, such as the coasts of Northern California and Oregon, are experiencing natural decreases in pH, with OA appearing to be extending the area affected by these phenomena (Feely et al. 2008). Not only are these locations of high concern under threat from predicted future OA scenarios, but they provide us with a glimpse of the effects on marine life we might observe in the years to come.

1.2 Methodological Considerations in the Study of OA

When discussing OA, researchers not only examine pH, but also other associated water chemistry parameters. Firstly, there are two pH scales: pH_{NIST} and pH_{TOTAL}. pH_{NIST} was devised by the National Institute of Standards and Technology and provides standardized buffer solutions to calibrate probes and measure pH. pH_{TOTAL} can be measured via the *m*-cresol purple indicator method or using TRIS buffers (Feely et al. 2008) and is typically 0.1 pH units higher than $pH_{NIST.}$ In recent publications, pH is measured daily (using one or both of the above scales). Researchers also measure total alkalinity (once a day to once every week), typically through titrations methods (Feely et al. 2008). Then, pH and total alkalinity values, in combination with temperature and salinity, are fed to computer software programs (CO_2 calculations system, e.g. CO_2SYS) to determine partial pressure of CO_2 (ρCO_2), saturations states of calcite (Ωca) and aragonite (Ωara) and dissolved inorganic carbon (Feely et al. 2008). Alterations to any of these parameters in the wild may have effects on marine organisms, in varying degrees depending on the biological and physiological processes of different species. Therefore, it is necessary to consider the full range of water chemistry dynamics to determine which component will lead to greater impacts in each species. For echinoderms, it is hypothesized that as changes in pH/ρCO_2 will be more important than mineral saturation states, as they transport bicarbonate for calcification and use CO_2 as their main source of inorganic carbon (Byrne et al. 2013). It should be noted that while I discuss the results in terms of changes in pH, it is impossible to determine if the main driver for an observed change is low pH or high $\rho CO_{2.}$

1.3 Potential Impacts of OA on Marine Invertebrates

For organism with calcified components, calcification is suggested to be the principal process to be effected by OA, as it is dependent on the amount of available carbonate. Marine

organisms that possess calcium carbonate shells and skeletons are readily susceptible to decreased calcification rates (Orr et al. 2005). The rate of dissolution is correlated to the crystalline form (aragonite, high-magnesium or low-magnesium calcite) being secreted, with the first two types being the most soluble (Morse et al. 2007). The fact that the predominance of each varies across taxa will make organisms more or less vulnerable to impacts on the formation and maintenance of skeletal components under OA conditions (Morse et al. 2007).

Reports show that in marine invertebrates exposed to a decrease in pH, calcification rates decline, altering the growth of taxa such as algae, corals and molluscs (Guinotte and Fabry 2008). For example, coccolithophores (calcifying eukaryotic phytoplankton) exhibited thinner shells and morphological deformities when exposed to an increase in CO_2 levels (Riebesell et al. 2000). Corals are able to survive and reproduce under acidified conditions but have difficulties maintaining their skeletal structure (Fine and Tchernov 2007; Leclercq et al. 2000; Ries et al. 2009). In addition, the shell growth of molluscs such as scallops, oysters, clams and whelks can be negatively impacted at elevated ρO_2 (McClintock et al. 2009; Ries et al. 2009). Most echinoderms utilize high-magnesium calcite to form skeletal ossicles, and thus are hypothesised to be significantly impacted by OA as well (McClintock et al. 2011; Weber 1969). Studies have also examined the potential effects of OA on their calcifying larvae, with evidence that they may be even more susceptible than adults (Byrne 2011; Dupont et al. 2008; Kurihara 2008).

In recent years, OA research has been shifting from calcification towards other processes such as physiology, reproduction and larval development, with echinoderms becoming a focal group (Dupont et al. 2010a). Many echinoderms are important as keystone species, ecosystem engineers, bioturbators and key components of grazing communities (Bowmer and Keegan 1983;

Micael et al. 2009; Sköld and Rosenberg 1996). Research thus far suggests that the impact of OA on this group is species specific and dependent on the life-history stage being examined.

Hypercapnia (CO_2 accumulation in the internal fluids) and acidosis have emerged as two of the main physiological impacts associated with OA (Collard et al. 2013). In several species of sea urchin, OA conditions have caused changes in the pH of the coelomic fluid (Dupont and Thorndyke et al. 2012; Calosi et al. 2013; Holtmann et al. 2013; Moulin et al. 2014; 2015; Spicer et al. 2011; Stumpp et al. 2012; Uthicke et al. 2014), which can interfere with processes such as calcification and metabolism.

Studies on reproduction and larval development have found mixed results. For example, studies have looked at the impact of OA on the fertilization of four different sea urchin species, of which only one suffered a significant reduction in percent fertilized eggs and cleavage rate due to decreases in pH (Byrne et al. 2009; Carr et al. 2006; Havenhand et al. 2008; Kurihara and Shirayama 2004). The larvae of six sea urchins (temperate, polar and tropical species) were studied, revealing a decrease in growth and calcification but no apparent impact on survival to the 4-arm pluteus stage (Byrne et al. 2013; Clark et al. 2009; Kurihara and Shirayama 2004). Similarly, when the larvae of the sub-arctic sea urchin *Arbacia dufresnei* were exposed to a decreased pH (7.4), a delay in development was observed (at the pluteus and prism stages) but no increase in percent abnormal larvae was recorded (Catarino et al. 2012). In comparison, exposure to a 0.2 unit decrease in pH resulted in 100% mortality of the eastern Atlantic brittle star *Ophiothrix fragilis* before the 8-arm pluteus stage (Dupont et al. 2008).

Although it is generally hypothesized that adult echinoderms are robust to OA, significant effects may only be observed in the long term or when examined across various life stages (Dupont et al. 2010a). Long-term studies remain rare but can be quite informative. The sea

urchin *Hemicentrotus pulcherrimus*, collected from rocky intertidal habitats in Japan, was exposed to increased ρCO_2 (1000 atm) over 10 months and showed a delay in gonad development and spawning period, but no increased mortality (Kurihara 2013). In the sea urchin *Strongylocentrotus droebachiensis* sampled from waters off the coast of Norway and Denmark, fecundity decreased in females and fewer offspring reached the juvenile stage after adults were exposed to elevated ρCO_2 (1217 µatm) over four months. However, when adult females were exposed for 16 months, there was no effects on fecundity or differences in larval survival observed relative to adults exposed to control conditions (Dupont et al. 2013). The results of this latter study support the hypothesis that adult sea urchins can acclimatize to changes in ρCO_2, and that negative carry-over effects may decrease or be lost over the long term. If acclimation is not possible there could be massive consequences (i.e. decreased population numbers, changes in food web dynamics, or changes in interactions between species) at the community and ecosystem levels (see review by Dupont and Pörtner 2013).

1.4 Research Gaps

A large number of studies investigating OA, especially the early ones, are laboratory-based, single-species, perturbation experiments, typically focusing on short-term exposure of one life-history stage, under static conditions (see review by Dupont and Pörtner 2013). Although these experiments are providing valuable information, they do not allow us to fully understand what might happen under realistic conditions and/or at the ecosystem level (e.g. in open systems where interactions among species and other biotic parameters are at play). Ideally, long-term gradual changes need to be examined as these organisms will not just be exposed to extreme scenarios overnight. Research also needs to include more organisms that have no or weak calcium carbonate structures and species from neglected biota, e.g. those from subpolar/polar

regions (Dupont et al. 2010a; see reviews by Dupont and Pörtner 2013 and Garrard et al. 2013) and deep-sea environments.

Another important aspect that has been virtually overlooked in OA research is the potential difference between lecithotrophs (species producing non-feeding larvae that rely on yolk reserves from maternal investment) and planktotrophs (species with feeding larvae). The few OA studies on lecithotrophs have focused on early life stages and shown varying results. When exposed to low pH (7.7), larvae of the sea star *Crossaster papposus* exhibited faster growth rates than under normal conditions (Dupont et al. 2010b). In the sea star *Meridiastra calcar* embryonic cleavage was not affected but the number of embryos reaching the hatched gastrula stage decreased (Nguyen et al. 2012). Contrasting results were observed with the lecithotrophic sea urchin *Heliocidaris erythrogramma*; one study reported a decrease in fertilization success (Havenhand et al. 2008) and another found fertilization and early development to be unaffected (Byrne et al. 2009). Overall, it is hypothesized that lecithotrophs may be more robust to climate change because they do not rely on planktonic food sources for growth (Havenhand et al. 2008; Byrne et al. 2009; Dupont et al. 2010a; review by Ross et al. 2011; Nguyen et al. 2012); however, the fact that oocyte provisioning by the mother may be impacted has not been considered. To date, transgenerational studies have only focused on species producing planktotrophic larvae (Dupont et al. 2010b; see review by Ross et al. 2011).

In addition, climes and environments are not equally well studied when it comes to OA. For instance, although it forms the largest ecosystem on Earth, the deep sea has not been given much consideration in OA research (Guinotte et al. 2006; Wernberg et al. 2012). Unique water parameters associated with depths >200 m (such as temperature, salinity, light, carbonate saturations states and pressure) may modulate the effects of OA. Through circulation and vertical

mixing, anthropogenic CO_2 will reach the deep sea, with some reports suggesting a 0.2-0.4 unit decrease in pH to depths of 1000 m by the end of the 21st century (Gruber and Sarmiento 2002; Sabine et al. 2004; Ilyina et al. 2010). Moreover, organisms in the deep sea typically experience much more stable conditions than their counterparts in shallow waters and thus could respond more drastically to small changes in pH (Guinotte et al. 2006; Wernberg et al. 2012). Based on the effects observed on shallow-water species, it has been hypothesized that, due to the water chemistry associated with the deep sea, calcification of cold-water deep-sea corals will be negatively impacted, with the risk of destroying these understudied ecosystems (Ramirez-Llodra et al. 2011; Turley et al. 2007). A recent study has been performed at depth using experimental release of liquid CO_2, revealing mixed responses in a variety of deep-sea species and phyla; some survived and thrived under low pH conditions (e.g. microbes, gastropods, fishes) and others experienced massive mortalities (e.g. echinoids, holothuoroids, amphipods; Barry et al. 2013).

1.5 Study Organisms

Although sea cucumbers are found in all habitat types across shallow and deep waters, only a few studies have focused on the potential impacts of OA on this taxonomic group. When exposed to a decrease in pH, the warm-water sea cucumber *Holothuria* sp. showed a decrease in percent motile spermatozoa (Morita et al. 2010). Collard et al. (2014) observed that short-term exposure (6-12 days) to low pH caused extracellular acidosis in the coelomic fluid of the sea cucumbers *Holothuria scabra* and *H. parva*. The sea cucumber, *Apostichopus japonicus* was exposed to a 0.62 unit decrease in pH, which led to an observed 6% decrease in post-fertilization success, as well as prolonged duration of the early-auricularia stage and decreased duration of the mid-auricularia (Yuan et al. 2015). While affected by OA, sea cucumbers may also have the ability to buffer the effects of changing pH. In the Great Barrier Reef, studies on the species

Stichopus herrmanni and *Holothuria leucospilota* revealed that they dissolved $CaCO_3$ through digestion, thus aiding in the natural turnover of this compound, and that they excreted ammonia, which can increase seawater alkalinity and buffer pH changes (Schneider et al. 2011).

The sea cucumber *Cucumaria frondosa* (Figure 1-1) is an annually-spawning and slow-growing species (Hamel and Mercier 1996a; 1996b) that has been commercially exploited in the North Atlantic for over 30 years and has a growing commercial market in Asia (Nelson et al. 2012). It is one of the most extensively studied echinoderms; its life cycle, gametogenesis, spawning, embryonic and larval development, settlement, growth, feeding habits, diet, physiology and population structures have been covered in the literature (Hamel and Mercier 1995, 1996a, 1996b, 1996c, 1998, 1999, 2008a, 2008b; Singh et al. 1998, 1999, 2001; So et al. 2010, 2011). Considering that populations of *C. frondosa* can be extensive, with biomasses reaching millions of tons in some locations of eastern Canada, any decline due to OA conditions could interfere with fundamental ecosystem functioning (Hamel and Mercier 1998) and economic benefits (Nelson et al. 2012; Therkildsen and Petersen 2006).

Polychaetes are another important component of marine ecosystems, acting as bioturbators, ecosystem engineers and prey items in all habitats (Lewis 2013). Although they are understudied with respect to OA, Lane et al. (2013) demonstrated that the embryonic and larval development of the shallow-water, tube-dwelling polycheate, *Hydroides elegans* was not affected when exposed to low pH (7.7, 7.5); however, the ability of juveniles to calcify and produce tubes was negatively impacted. Lewis at al. (2013) showed that elevated seawater ρCO_2 had a greater effect on the survival of larvae of the coastal polychaete *Pomatoceros lamarckii* than on spermatozoa motility or fertilization success. In addition, Cigliano et al. (2010) studied settlement success of various benthic invertebrates along pH gradients associated with a shallow

water CO_2 vent habitat and found that serpulid polychaetes had lower recruitment at decreased pH levels. The same study showed that syllid polychaetes exhibited higher abundances at the most acidified sites (Cigliano et al. 2010). The intertidal polychaete *Arenicola marina* from Northern Europe exhibited a decrease in spermatozoa motility and lower fertilization success after exposure to low pH (7.7, 7.47) (Campbell et al. 2014). In one of the only studies with a deep-water focus, Barry et al. (2013) found that in a deep-sea polychaete community, species richness and density decreased following pH changes of $\geq$0.2 units.

The polychaete genus *Ophryotrocha* is present in both coastal and deep-sea environments (Thornhill et al. 2012; Wiklund et al. 2012). The species, *Ophryotrocha* sp. (Figure 1-2) used here was collected at bathyal depths (500-1500 m); it had been thriving and reproducing normally for several years in the laboratory prior to this study, offering a rare opportunity to examine the potential effects of OA on a deep-sea species. Adults are simultaneous hermaphrodites, and are characterized by seasonal feeding and annual reproduction (Mercier et al. 2014).

1.6 Goals of the Research and Chapter Structure

The purpose of the present study was to explore the effects of decreased pH on a weakly calcifying, subpolar lecithotrophic sea cucumber (*C. frondosa*) and a hermaphroditic polychaete from the deep sea (*Ophryotrocha* sp.). Both of these species have exhibited the ability to acclimatize to changes in environmental conditions (e.g. drastic temperature changes in shallow waters, salinity changes in estuaries, food limitations in the deep sea, surviving pressure changes). One of the main objectives was to examine these effects in a realistic way, using a flow-through system that maintained a 0.4 unit decrease in pH under seasonally varying

conditions (of pH, temperature, light, salinity, and nutrient levels). This thesis contains four main chapters.

In chapter 2, I studied the effects of exposure to a 0.4 unit decrease in pH for 19 weeks on gametogenesis, spawning and development in *C. frondosa* using gonad tubule content analysis, histology and microscopy. In addition, I studied the effects on processes that may compete with reproductive effort such as skeletogenesis (ossicle formation and structure), and lipid synthesis.

In Chapter 3, I studied the effects of the same 0.4 decrease in pH for 26 weeks on *Ophryotrocha* sp. using histology and microscopy. In addition, effects on the microstructure of the body wall and chaetae as well as the elemental composition of chaetae were assessed.

The results obtained with both species are summarized in Chapter 4 where I discuss the potential impact of OA in a more general context, including its overall significance for populations of sea cucumbers and deep-sea worms and the how these effects could alter the communities of which they are vital parts. I conclude with suggestions for future research in the field of OA.

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1.8 Figures

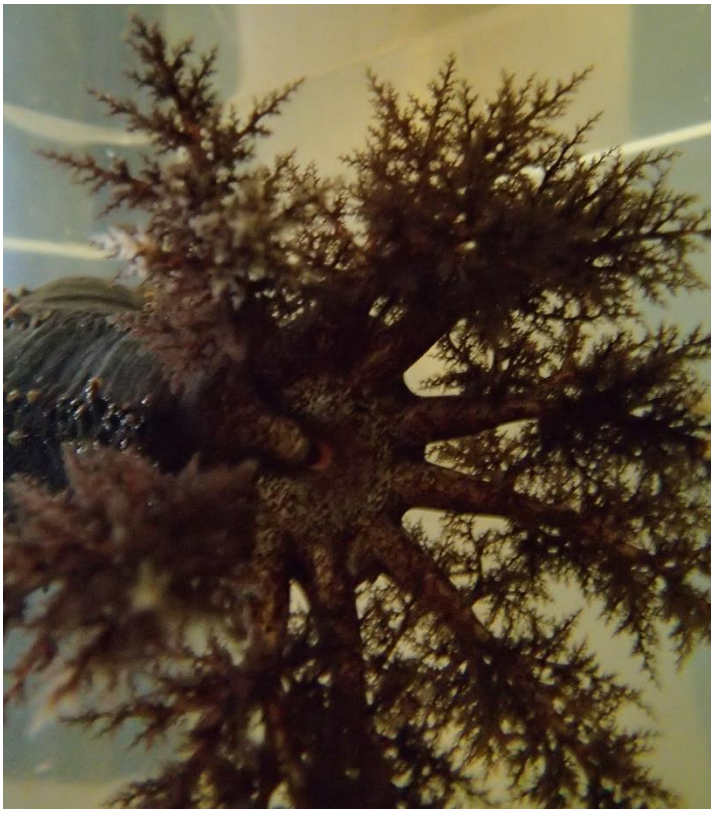

Figure 1-1: Focal species *Cucumaria frondosa* (adult size: 10 to 14 cm in length)

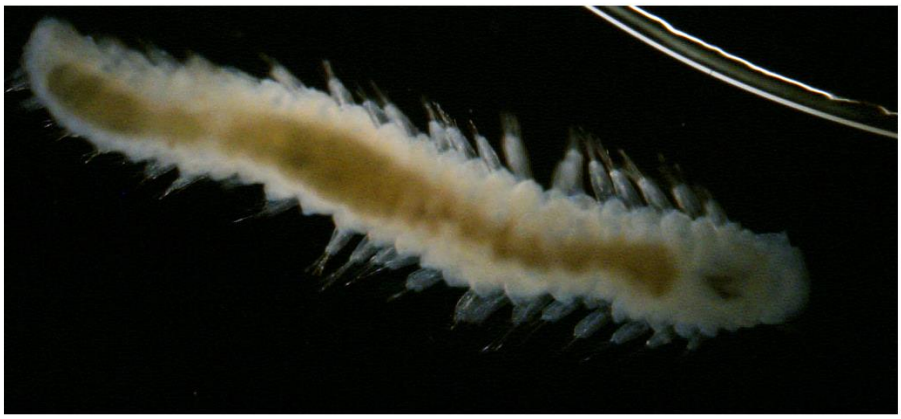

Figure 1-2: Focal species *Ophryotrocha* sp. (adult size: 10-15 mm in length)

Chapter 2: Carry-over effects of ocean acidification in the cold-water lecithotrophic holothuroid *Cucumaria frondosa*

2.1 Abstract

Ocean acidification (OA), defined as a decrease in seawater pH due to anthropogenic CO_2 emissions, is predicted to affect the world's oceans in the near future. Echinoderms, which are widely distributed across most marine biota, have been identified as a primary group of interest relative to the impact of OA. However, to date studies remain limited on species that are weakly calcified, produce lecithotrophic larvae and live in polar/subpolar environments. The cold-water sea cucumber *Cucumaria frondosa* is one of the most abundant echinoderms in the world. It is annually-spawning, slow-growing and has been commercially exploited in the North Atlantic for over 35 years. The purpose of the present study was to examine the effects of predicted near-future OA scenarios on gametogenesis, spawning and embryonic development of *C. frondosa*, as well as on processes that may compete or coincide with reproductive effort such as ossicle formation and lipid synthesis. Sea cucumbers were exposed to a 0.4 unit pH decrease over 19 weeks using a realistic flow-through design allowing for natural fluctuations in pH, light, temperature, salinity and nutrient levels. There were two treatment groups, ambient pH and low pH (0.4 units lower than ambient). Results suggest that low pH/high ρCO_2 interferes with gamete synthesis, leading to discrepancies in oocyte/embryo buoyancy and morphology as well as developmental tempo, translating into 100% mortality before the blastula stage. Differences in the abundance, microstructural appearance and chemical composition of ossicles, as well as in the lipid contents of muscles, gonads and spawned oocytes were observed. Such findings draw attention to previously understudied impacts of OA, including transgenerational effects in cold-water species with maternally provisioned eggs, and the resulting implications for temperate, subpolar and polar ecosystems.

2.2 Introduction

Anthropogenic emissions of carbon dioxide (CO_2) are being absorbed by the world's oceans, causing an increase in the concentration of hydrogen ions in seawater and a consequent decrease in pH, commonly termed ocean acidification (OA) (Caldeira and Wickett 2003). The overall effect of this equilibrium shift is the depletion of available carbonate and the dissolution of deposited forms of carbonate (Gattuso and Lavigne 2009). Reports have predicted a ~0.4 unit decrease in pH by 2100 which could have widespread impacts on marine life (Caldeira and Wickett 2003; Raven 2005).

Echinoderms are important components in marine ecosystems, filling roles as keystone species, ecosystem engineers, bioturbators, important links in food webs and key components of grazing communities (Bowmer and Keegan 1983; Micael et al. 2009; Sköld and Rosenberg 1996). Most echinoderms utilize high-magnesium calcite to form skeletal ossicles, and thus are hypothesised to be severely impact by OA (McClintock et al. 2011; Weber 1969). However, research thus far indicates that the impact of OA on echinoderms is species and life-stage specific. While studies have suggested that the early life stages may be more susceptible to OA than adults (Dupont et al. 2008; Kurihara 2004; see review by Byrne et al. 2011), the available evidence remains ambiguous (Guinotte and Fabry 2008; Orr et al. 2005).

Most early studies investigating OA have relied on laboratory-based, single-species, perturbation experiments, typically focusing on short-term exposure of one life stage, under static conditions (reviewed by Dupont and Pörtner 2013). Although these experiments provided valuable insight, they do not necessarily reflect realistic scenarios since these organisms will not

be exposed to the associated OA pH changes overnight. More realistic studies (e.g. in flow-through systems, under seasonally/daily changing pH, light, temperature and salinity) are needed to accurately determine or better understand impacts, including any carry-over effects between consecutive life stages. If negative effects are found at any one stage, or species do not exhibit the ability to acclimate after long-term exposure, there could be dire consequences (i.e. decreased population numbers, changes in food web dynamics, or changes in interactions among species) observed at the community and ecosystem levels (e.g. in open systems where species interactions and other biotic parameters are at play) (see review by Dupont and Pörtner 2013). As pH in the natural environment will not be the only fluctuating parameter, the combination of a reduced pH with changes in salinity, temperature and seasonal composition of seawater can reveal unexpected trends.

Apart from acknowledging the need for more realistic experimental studies, research on OA has begun to explore how biological processes other than calcification, such as reproduction and basic physiology, may be impacted in echinoderms (e.g. Catarino et al. 2012; Dupont et al. 2012; Kurihara et al. 2013; Moulin et al. 2014, 2015; Siikavuopio et al. 2007; Stumpp et al. 2012; Uthicke et al. 2014). One of the main issue associated with OA is hypercapnia (CO_2 accumulation in the internal fluids) and acidosis (Collard et al. 2013). Hypercapnia/acidosis can interfere with calcification, nutrition and metabolism (Pörtner 2008; Melzner et al. 2009). To avoid negative impacts on these processes, organisms strive to maintain homeostasis through respiration, circulation, and acid-base regulation (Collard et al. 2013). The OA research focus is also shifting towards organisms that have no or weak calcium carbonate structures and inhabit a wider diversity of habitats (Dupont et al. 2010b; see reviews by Dupont and Pörtner 2013 and Garrard et al. 2013). Species with different life-history strategies must also be investigated. For

instance, although 68% of echinoderms produce non-feeding lecithotrophic larvae (Uthicke et al. 2009), only a handful of studies have examined the effects of OA on species with this development mode. And these only measured the response of eggs and early life stages, with the authors generally suggesting that lecithotrophic larvae may be more robust to OA than planktotrophic larvae (Havenhand et al. 2008; Byrne et al. 2009; Dupont et al. 2010a; review by Ross et al. 2011; Nguyen et al. 2012). To our knowledge, the present study is the first to examine transgenerational effects of OA in a lecithotrophic species, providing a more realistic evaluation of their putative response.

Holothuroids (sea cucumbers) have only been the subject of a few OA studies so far. In one study, the sea cucumber *Holothuria* sp. showed a decrease in percent motile spermatozoa with decreasing pH (Morita et al. 2010). Moreover, acid-base physiology in the sea cucumbers *Holothuria scabra* and *H. parva* was negatively impacted during short-term exposure (6-12 days) to low pH, causing extracellular acidosis of the coelomic fluid (Collard et al. 2014). In the sea cucumber *Apostichopus japonicus*, a 0.62 unit decrease in pH elicited a 6% decrease in post-fertilization success, in addition to increasing duration at the early-auricularia stage and decreasing duration at the mid-auricularia stage (Yuan et al. 2015). At another level, it has been shown that holothuroids could potentially help offset ocean acidification. Studies on the species *Stichopus herrmanni* and *Holothuria leucospilota* in the Great Barrier Reef, showed that when calcium carbonate ($CaCO_3$) is dissolved through digestion, sea cucumbers have the ability to aid in the its natural turnover, and that they could increase seawater alkalinity via excretion products, which could ultimately buffer changes in pH (Schneider et al. 2011).

The sea cucumber *Cucumaria frondosa* is one of the most abundant echinoderms in the world. It is a cold-temperate species, present along the Arctic and Atlantic coasts of North

America and northern Europe, that exhibits annual spawning and slow growth (Hamel and Mercier 1996a; 1996b). It has been commercially exploited in the North Atlantic for over 35 years and has a growing commercial market (Hamel and Mercier 2008a; Nelson et al. 2012). Its life cycle, gametogenesis, spawning, embryonic and larval development, settlement, growth as well as its feeding habit and diet, physiology and population structure/genetics have all been studied extensively (Hamel and Mercier 1995, 1996a, 1996b, 1996c, 1998, 1999, 2008a, 2008b; Singh et al. 1998, 1999, 2001; So et al. 2010, 2011). Because its abundance may reach millions of tons in some locations of eastern Canada (Hamel and Mercier 2008a), population declines due to shifting environmental conditions, including OA, could have cascading impacts on fundamental ecosystem functions (Hamel and Mercier 1998) and economic benefits (Nelson et al. 2012; Therkildsen and Petersen 2006).

The purpose of the present study was to examine the effects of predicted near-future ocean acidification, namely a decrease in pH of 0.4-0.5 units relative to ambient values, on various processes in *C. frondosa*, including: (1) gametogenesis; (2) spawning and fertilization success; and (3) embryonic and larval development. In addition, the effects on (i) overall health, (ii) lipid synthesis and (iii) the abundance, microstructural composition and shape of ossicles were analysed. The study explored potential transgenerational effects (across multiple life stages of a species) under naturally fluctuating temperature, light, phytoplankton abundance, salinity and pH, true to nearby oceanic conditions where extensive populations of *C. frondosa* are found. Determining these potential effects will hopefully aid in locating areas of high conservation concern that may incur biodiversity loss under near future ocean conditions.

2.3 Materials and Methods

2.3.1 Specimen Collection

Adult *C. frondosa* were collected in December 2013 by scuba divers in Admirals Cove (47.0969° N: 52.9092° W), south-eastern Newfoundland, from a depth of 15-20 m (water temperature 3-4 °C). Sea cucumbers were kept in the laboratory for less than a month before being used. Only healthy individuals (attached firmly to the substrate, feeding, exhibiting proper coloration and undamaged body wall) were selected.

2.3.2 Experimental Setup

Sea cucumbers were experimentally tested in the laboratory using a flow-through system of unfiltered seawater pumped directly from the ocean for a period of 19 weeks, from December 26, 2013 to May 7, 2014. The beginning of the trial immediately preceded the initiation of gametogenesis in *C. frondosa* (Hamel and Mercier 1996b) and the following time frame encompassed the periods of gamete synthesis and spawning (Hamel and Mercier 1996b; Mercier and Hamel 2010). Two treatment groups were used, ambient pH (control) and low pH (0.4 unit decrease from ambient conditions). The experimental design included 8 tanks (16 L) per treatment group, each containing three individuals, for a total of 24 sea cucumbers per treatment (ambient vs low pH). At the onset of the experiment, the immersed weight and contracted length (from mouth to anus) of each sea cucumber were recorded. Average total wet weight in ambient pH was 148.2 g ± 30.8 and 150.8 g ± 38.1 in low pH and length from 10 to 14 cm in both treatments. All individuals were initially acclimated to the tank system at ambient pH for one week. Seawater with automatically adjusted low pH (-0.4 units relative to ambient; see method

below) was then slowly flowed into the experimental tanks, allowing the animals to acclimate to the decreasing pH over a period of 12 hours.

The pH was maintained by electronically-controlled injection of CO_2, via a CO_2 regulator (Milwaukee Instruments MA957) and reactor (AquaMedic CO_2 Reactor 500) into the header tank that fed the multiple experimental tanks. Both ambient and acidified seawater supplies were continuously distributed to the tanks at a flow rate of ~7.2 L h^{-1}. Water temperature followed local conditions (in Logy Bay, Newfoundland); flow rates kept temperature below 10 °C for the duration of the experiment, mimicking conditions observed in the natural habitat of *C. frondosa*. Photoperiod corresponded to natural sunrise/sunset hours with maximum light intensity set at ~150 Lux (spring conditions). The setup allowed for natural fluctuations in both ambient and experimental pH levels, following any daily, tidal and seasonal changes. Other parameters such as salinity, temperature, dissolved oxygen and plankton abundance also fluctuated naturally throughout the experiment, in parallel to changes in the field. The sea cucumbers were given a supplement (to ensure proportional food availability between treatments) of phytoplankton cells (Phyto-Feast® Live, Reef Nutrition) composed of *Pavlova, Isochrysis, Thalassiosira weissflogii, Tetraselmis, Nannochloropsis,* and *Synechococcus* (~25,000 cells ml^{-1}) three mornings a week for the duration of the trial, as phytoplankton is known to dominate the diet of *C. frondosa* in nature (Hamel and Mercier 1998). The experimental setup was designed to incorporate the conditions necessary for normal reproduction to occur, including inter-individual communication and environmental fluctuations that act as gametogenic and spawning cues (Mercier and Hamel 2009).

2.3.3 Water Chemistry Monitoring

The physical and chemical parameters of the water (pH, temperature, salinity, and DO) were monitored in each tank twice daily (morning and evening) using a YSI 556 MPS multi-probe. Total alkalinity (TA) was measured twice a week in four randomly chosen tanks (two from ambient and two from low pH conditions) using a total alkalinity test kit (Orion; accuracy ± 5 ppm). Temperature, pH_{NIST}, and TA were used to estimate ρCO_2, saturation state of aragonite (Ωarag) and saturation state of calcite (Ωca) using CO2SYS software (Lewis and Wallace 1998) with constants of Mehrbach et al. (1973) as refitted by Dickson & Millero (1987). Data loggers (HOBO Pendant®, Onset Computer Corp.) were used to measure the temperature and light intensity every two hours in one randomly selected tank per treatment.

2.3.4 Behavioural Monitoring

The overall health, behaviour and level of stress of the sea cucumbers were recorded on a weekly basis for 15 weeks. Active feeding, based on deployment and repeated insertion of tentacles into the oral cavity was documented, together with tegument coloration (normal coloration being described as dark brown and uniform). In addition, tentacle reaction time was monitored after gently brushing the tips of two tentacles with a sterile pipette. The retraction and eventual re-extension of the tentacles post-contact was recorded for 2 minutes using an Olympus Stylus TG-2 digital camera. Videos were later analysed and compared between the two groups (n=4-6 individuals per treatment per week). To assess stress levels, cloacal opening rates (through which water circulation occurs, leading to oxygenation) were measured once a week in mid-morning (Doyle and McNiell 1964). As with many other species of holothuroids, cloacal opening (respiration) rates will increase as an indicator of stress (Gianasi et al. 2015; Yang et al. 2006). Rates were determined by counting the number of cloacal openings within a 2 min period

in all individuals where the cloaca were visible without disturbing them (n=7-18 individuals per treatment per week).

2.3.5 Sample Collection

Three sampling periods were established during the experiment. At the onset of the trial (T0), a subsample (n=22) of the initial sea cucumber population was analyzed to determine their baseline status. Subsequently, 4 out of 8 tanks per treatment were randomly sampled after 10 weeks, during the pre-spawning period (T10; March 11 and 12, 2014; n=11-12 individuals per treatment), and the remainder were sampled at the end of the trial, after 19 weeks during the post spawning period (T19; May 6 and 7, 2014; n=11-12 individuals). At T10 and T19, sea cucumbers were photographed and their wet weight and length (mouth to anus) were measured. Then the whole gonad was isolated, blotted repeatedly to remove excess liquid and weighed. The muscle bands (longitudinal and circular) were surgically removed from their attachment to the body wall, blotted and weighed separately. All body walls were vacuum sealed and frozen for later ossicle analysis (see method below). For all individuals at all sampling periods, two sets of three randomly collected gonad tubules were removed from the whole gonad. Gonads of *C. frondosa* in the study area are composed of uniformly developing tubules (Hamel and Mercier 1996a). One set of three tubules was preserved in 4% formaldehyde for a histological study of gametogenesis (at T19 only) and the other group was used to determine fecundity at both time points. Additionally, at T10 and T19, three gonad tubules and the muscle bands (longitudinal and circular) from each individual (n=11-12 per tissue type; n=3 individuals per sex per treatment) were wrapped in aluminum foil (previously burnt at 450 ºC for 5 hours), and kept at -80 °C for a maximum of three months before lipid analysis (see method below).

2.3.6 Sample Analysis

2.3.6.1 Gametogenesis

Only individuals of similar length and weight were used for this analysis to minimize size effects. The average mean wet body wall weight of individuals (n=23) was 24.7 ± 5.4 g in ambient pH and 26.9 ± 12.4 g in low pH. To compare the development of gametes in the two treatments, several complementary methods were used. (1) Gonad index (GI) was calculated as the percent wet weight of the whole gonad to the wet weight of the body wall, without muscle bands, which were shown to fluctuate with level of gamete development (Hamel and Mercier 1996a). (2) Oocyte abundance and size (maximum, or Feret, diameter), as well as the ratio of healthy versus phagocytized oocytes, were established in histological sections. For this, gonad tubules from 6 females (n=3 per treatment) sampled at T19 were used. Gonads were processed using standard methods (Havenhand and Schlegel 2009), i.e. dehydrated using a series of ethanol baths, followed by xylene to remove the alcohol. Samples were then embedded in methacrylate, sectioned (7 µm), and stained with hematoxylin and eosin. Photographs were taken using a Nikon Eclipse 80i microscope coupled with a Nikon DXM1200F digital camera. Cells that were entirely within the field of view were then analysed with the software ImageJ. (3) Histological sections were also used to determine a qualitative stage of oogenesis for each female (Hamel and Mercier 1996a) based on size of oocytes and number of phagocytized cells. (4) Finally, a quantitative maturity stage index (MSI) was ascribed to each sample (Doyle et al. 2012). Briefly, the MSI uses oocyte and gonad metrics to obtain a quantitative measure of oogenesis on a continuous scale (as gonad weight or oocyte density alone do not fully capture gamete maturity). MSI was calculated using the following formula:

$$(\text{Oocyte density}) \times (\text{oocyte diameter}) \times CV \times 0.001$$

where oocyte density is the number of all oocytes present in a 1 cm segment of tubule, oocyte diameter is the mean Feret diameter of these oocytes and the CV (coefficient of variation) is the SD of oocyte diameter × $mean^{-1}$ × 100.

2.3.6.2 Fecundity

To assess differences in gamete abundances (in males and females) between the two treatment groups, three gonad tubules from each individual (n=11-12 individual per treatment for each sampling period) were weighed and their length was measured (see fecundity metrics describe below). A 1-cm segment of each tubule was then isolated from the tip of the tubule and its gamete contents were collected by gently squeezing down the length of the tubule to extract all gametes (tubules were thoroughly examined for residual gametes). The weight of the empty tubule and its contents (male or female gametes) were recorded separately. The removed contents were preserved in 20% ethanol in filtered seawater (low concentration to avoid dehydration causing changes to shape/size). For female samples, the total number of oocytes was calculated as well as their size (Feret diameter). For males, a 10 µL aliquot per tubule (n=3 tubules per individual) was placed in a disposable hematocytometer to determine the number of spermatozoa. All measurements and photos were taken using a Nikon Eclipse 80i microscope coupled with a Nikon DXM1200F digital camera and Simple PCI imaging software. Relative fecundity was measured as the total number of gametes per cm of gonad tubule (number cm^{-1}) using counts of gametes extracted from the tubule segment immediately before spawning (T10). Total fecundity was then extrapolated for the whole gonad.

2.3.6.3 Spawning and Development

Cucumaria frondosa is a broadcast-spawning species that releases large bright-orange lecithotrophic oocytes (Hamel and Mercier 1996b). For the duration of the spawning period, the

intensity of any gamete release were monitored several times a day. Upon initial sighting of oocytes, a sample was taken to determine if they had been fertilized (through elevated fertilization envelop or cleavage), and when broadcast was completed (no more oocytes being released) three water samples of 10 ml were taken to estimate the size of oocytes released. Thirty to 50 embryos were used to determine size (as previously described) and stage of development over time; to determine percent survival and mortality, the number of healthy (round in shape, uniform in colour and showing normal cleavage) or abnormal oocytes (irregular in shape, mottled surface texture and discrepancies in cleavage) respectively, were counted and ratios determined over the total number of oocytes per subsample. This procedure was repeated every two hours for the first 24 h to determine developmental kinetics. Embryos/larvae were then assessed twice a day. Development occurred in the tanks where spawning occurred, thus under the respective pH conditions. A new stage was scored when ~50% of the propagules were at that stage (Hamel and Mercier 1996b). Development was assessed and pictures were taken using the previously described equipment. Subsamples of oocytes (100-300 depending on the total released) from spawning events (n= 2 in each treatment) were preserved for lipid analysis using the aforementioned methods.

2.3.6.4 Lipid Analysis

There is a parallel relationship between feeding, reproduction and the synthesis of novel lipids which can be dependent on the species and their reproductive mode (Hudson et al. 2004). To determine if lipid profiles were impacted during the study, total lipid classes and fatty acids were compared between treatment groups (as per sampling described above). A modified Folch procedure was used to extract lipids (Folch et al. 1957; Parrish 1999). Briefly, samples were homogenized in a 2:1 chloroform:methanol solution using a Polytron PCU-2-100 homogenizer

(Brinkmann Instruments, Rexdale, Ontario, Canada). Chloroform extracted water was used to bring the sample to a methanol:chloroform:water ratio of 1:2:1. Next, samples were sonicated for 5 minutes in an ice bath and centrifuged at 3000 rpm for two minutes. The bottom organic layer was then removed using a double pipetting technique to avoid disturbing the aqueous top layer. Chloroform was then added back into the sample and the procedure was repeated a total of three times. All organic layers were pooled and concentrated using a flash-evaporator (Buchler Instruments, Fort Lee, New Jersey, USA). Final samples were blown down to volume using nitrogen, sealed with Teflon tape and stored at -20°C until measurements of fatty acids and total lipid classes were taken. For fatty acid analysis, 100-200 µL (depending on concentration of original sample) of lipid extract were transferred to lipid clean vials. Fatty acids were extracted using a FAME derivatization; lipid extracts were trans-esterified using Hilditch reagent ($1.5H_2SO_4$:98.5 anhydrous MeOH) for 1 h at 100 °C. FAMEs were analysed on a HP 6890 Series GC system and run for 30 minutes. Chromatographs were compared to a prepared standard and analyzed using Varian Galaxie Chromatography Data System, version 1.93.2 (Agilent Technologies, Colorado, USA).

Total lipid classes, were determined using a series of developing and conditioning sequences routinely used for the separation of aquatic lipid classes on Chromarods (quartz rod covered in silica) (Parrish 1987). Briefly, samples were spotted on Chromarods (x µl-amount will be dependent on original samples) and focused in acetone, then developed twice in hexane:diethyl ether:formic acid (98.95:1:0.05). After drying for five minutes in constant humidity, the rods were scanned using an Iatroscan MK-6 to classify non-polar lipids. The Iatroscan burns samples in a hydrogen flame at a temperature of 800°C; ions are burned off and collected to form peaks for identification. Next, to remove polar lipids two development

sequences were used. First, samples were developed in hexane:diethyl ether:formic acid (79.9:20:0.1), dried and scanned. For the last sequence, samples were developed twice in 100% acetone, then developed twice in chloroform:methanol:chloroform-extracted-water (5:4:1) and scanned a final time. Chromatographs were compared to a prepared standard and analyzed using PeakSimple Chromatography Software, version 2.38 (SRI Instruments, California, USA).

2.3.6.5 Ossicles

To determine whether calcification of *C. frondosa* was affected by low pH, frozen body wall samples were thawed and cut into three regions (anterior, middle and posterior), then weighed and measured. Each region was placed in 150 ml of 5.25% sodium hypochlorite (bleach) for 48 h to digest the tissue, with an additional 50 ml of bleach added to each sample after 24 h. For each body region, the total number of ossicles was assessed under a stereomicroscope (Nikon SMZ1500). For comparison between treatments, total ossicle abundance was extrapolated for each individual (n=5 per treatment group) as the number of ossicles from all regions divided by total wet body wall weight. Three subsamples of ossicles from each region were rinsed three times in distilled water and dried at 60°C for 24 h. The microscopic morphology and elemental composition of these ossicles were assessed via scanning electron microscopy (Phenom ProX SEM). Elements chosen for analysis were those hypothesized to be most affected by OA, primarily calcified components. To compare fine morphology and surface structure, photos of 9 ossicles collected from 3 individuals in each treatment were taken at a high magnification (860x-20,000x). Elemental composition was determined in subsamples of ossicles from each treatment using energy-dispersive X-ray spectroscopy (EDS). Three points of analysis were routinely taken per ossicle (near the outer edge, near an inner opening and near the center of the whole ossicle); any area of the ossicle that

presented obvious differences was also analyzed. Elements present were determined using the Phenom ProSuite elemental identification (EID) software.

2.3.7 Statistical Analysis

The effects of time (T10, T19) and pH treatment (ambient, low) on the various reproductive variables (GI, MSI, fecundity) were tested using 2-way analysis of variance (ANOVA). When interactions were detected, univariate analyses were conducted using t-test or its non-parametric counterpart, Mann-Whitney U test, when the assumption of equal variance was violated. The latter tests were also used to compare the proportions of phagocytized and vitellogenic oocytes between the two pH treatments at T19, the frequency of cloacal movement between treatments at various time points and the total number and elemental composition of ossicles between pH treatments. These analyses were performed in Sigmaplot software (v. 11.0; Systat, Inc.). In addition to t-tests (or Mann-Whitney U test), lipid data were also compared using multivariate statistics; testing for dissimilarity using SIMPER and multidimensional scaling (MDS); with criteria for inclusion of various lipids and fatty acids determined by a correlation set at 0.8-0.9 in the software Primer 6 (v. 6.1.16; Primer-E, Ltd).

2.4 Results

2.4.1 Water Chemistry

Average daily pH measurements showed that a 0.42 ± 0.12 unit decrease relative to ambient pH was maintained for the duration of the experiment irrespective of natural (daily and seasonal) pH fluctuations recorded during the study (Figure 2-1A, B). Overall, pH increased throughout the study, from winter to spring (Figure 2-1A). Daily measures showed that pH was typically lower in the morning and higher in the afternoon (Figure 2-1B). Temperature and salinity in both treatments also increased towards the end of the study period, whereas dissolved

oxygen stayed relatively consistent (Figure 2-1C). Calculated water parameters associated with differences in pH showed that ρCO_2 was approximately 3 times higher in low pH (average ambient ρCO_2 464.9 ± 65.8 and low pH associated ρCO_2 1552.2 ± 231) that saturation states of calcite and aragonite were higher in ambient pH (Table 2-1).

2.4.2 Gonad indices, gametogenesis and fecundity

There was a significant interaction between time and pH on the male GI ($F_{1,16}$=4.613, p=0.047; Figure 2-2A). Based on independent t-tests, there was a significant decrease in GI from T10 to T19 in ambient pH conditions (U=0, df=9, p=0.004); but not in low pH (t_7=1.054, p=0.327). In females, the GI (Figure 2-2B) was not significantly affected by time ($F_{1,21}$=0.855, p=0.366) or pH ($F_{1,21}$=0.0014, p=0.970).

There was a significant decrease in spermatozoa density over time (Figure 2-3A) in males from both ambient and low pH ($F_{1,17}$=61.525, p<0.001) and no interaction between time and pH ($F_{1,17}$= 2.622, p=0.124). Similarly, there was no significant interaction between time and pH on oocyte density in females ($F_{1, 21}$=0.637, p=0.434) but there was a significant decrease in oocyte density ($F_{1,21}$=75.433, p<0.001) over time (Figure 2-3B). Of note is the fact that the decrease in gamete density was visually more pronounced in individuals from ambient pH in both sexes (Figure 2-3A, B) and was associated with more individuals (gonads) showing signs of spawning.

Gonad histology at T19 further revealed that the density of phagocytized oocytes, an indicator of the gonad recovery stage (Figure 2-4A, B), was significantly higher in ambient than in low pH (Figure 2-2C; t_4=5.072, p=0.007). Oocyte size frequencies based on freshly extracted material at T10 and T19, and on histological material at T19, did not show any clear pattern apart from the persistence of larger size classes under low pH (Figure 2-5). Histological sections from T19 also showed that ~65% of gonads in ambient pH and ~59% of gonads in low pH were in the

advanced growth stage. However, while ~35% of individuals were in the recovery stage in ambient conditions, this stage was ~1% under low pH conditions. In contrast, ~40% of gonads in low pH were mature, while no mature gonad was found under ambient pH at T19 (Figure 2-6).

There was no significant interaction between the effects of time and pH on the MSI ($F_{1, 21}$=1.463, p=0.240) but there was a significant effect of time ($F_{1,21}$=4.592, p=0.044). Graphically, the effect appeared stronger in the low pH group. In independent t-tests, there was a significant difference in MSI at T10 compared to T19 in ambient pH (t_{10}=2.277, p=0.046) but no significant difference was detected between sampling times in low pH (t_{11}=0.687, p=0.506; Figure 2-7A, B).

2.4.3 Spawning and Development

There were more spawning events observed in sea cucumbers exposed to ambient pH (n=7) than low pH (n=5). Under ambient conditions, 100% of oocytes were found floating on the surface of the water immediately after their release (Figure 2-8A). However, nearly all the oocytes (~98%) released under low pH conditions were negatively buoyant and sank to the bottom (Figure 2-8B, C). Kinetics of development at ambient pH showed that on average zygotes reached the 128-cell stage within 30 h and the early blastula after 48 h. In comparison, under low pH, zygotes were determined to be at the 64-cell stage after 24 h (observed only in a few embryos). Survival after the first day was 78% in ambient pH and 5% in low pH. After 48 h, survival in ambient pH was 5% compared to 0% in low pH (all oocytes were found decaying on the bottom). Percent survival in ambient pH is likely underestimated due to tank set up which did not retain floating propagules efficiently. Therefore, a portion of embryos from ambient pH were presumably lost through the drain; whereas negative buoyancy insured retention of oocytes in low pH. Eggs or embryos in ambient pH had a smooth, round, uniform orange color and shape (Figure 2-9A, B, C), whereas those released in low pH were similarly colored but irregular in

shape (Figure 2-9D, E, F, G, H). Additionally, some of the latter eggs appeared to have a rough, uneven surface and dimpled cytoplasm (Figure 2-9I).

2.4.4 Behaviour

Rates of cloaca opening were higher in low pH for the first four weeks but stabilized for the remainder of the experiment, with significant differences between treatments restricted to week 1 (t_{12}=-4.625, p<0.001) and week 4 (t_{19}=-2.468, p=0.023). The average tentacle reaction time across the entire 19 week period in ambient and low pH (56.3 ± 8.1 s and 57.5 ± 10.5 s, respectively) was not significantly different between treatments (t_6=-0.189, p=0.856) and neither was the average number of individuals actively feeding at a given time (ambient pH 4.2 ± 2.4 individuals; low pH 4.8 ± 2.9 individuals; t_{22}=-0.544, p=0.592).

2.4.5 Lipids

Globally, SIMPER analysis showed that for both males and females, during both sampling periods, muscle tissues were 25-33% dissimilar, and gonad tissues were 25-34% dissimilar between ambient and low pH (Figure 2-10A, B, C and D). Different lipid classes and fatty acids contributed to this dissimilarity between treatments. Values were reported when contribution was greater than 5% in at least one treatment group (Table 2-2). For male muscle samples from the T10 sampling, the proportion of phospholipids and of free fatty acids 20:5ω3 and 20:1ω9 was higher in low pH, while the proportion of 22:4ω6 and 20:1ω11 was higher in ambient pH (Table 2-2). Similarly, female muscle samples showed higher proportions of 22:4ω6 and 20:1ω11 in ambient pH, while percent of 20:5ω3 and 20:1ω9 was higher in low pH; proportions of phospholipids were similar between treatments (Table 2-2). For male gonad samples, although not found to be statistically significant, there was a trend towards the proportion of phospholipids and sterols being higher in low pH while the proportion of

tritriacylglycerol (TAG) was higher in ambient pH; the essential fatty acid 20:5ω3 was similar between treatments (Table 2-2). In comparison, TAG and ai15:0 were higher in female gonad samples from ambient pH while 20:5ω3 was higher in low pH; phospholipids were similar between treatments (Table 2-2). Muscle samples from the end of trial sampling period (T19) showed the same trends for both sexes. Proportions of phospholipids, 22:4ω6 and 20:1ω11 were higher in ambient pH, while proportions of 20:5ω3 and 20:1ω9 were higher in low pH (Table 2-2). In male gonad samples, percent sterols and 20:5ω3 were higher in ambient pH and TAG, ai15:0 and percent 16:1ω7 were higher in low pH (Table 2-2). Percent phospholipids were similar between treatments at T10. Female gonad samples showed slightly different trends, with proportions of phospholipids (T10 only) and ai15:0 being higher in ambient pH, and proportions of sterols and 20:5ω3 higher in low pH (Table 2-2). Percent TAG was similar between treatments. It should be noted that some of these differences between ambient and low pH were only slightly apparent while others were statistically significant (see Table 2-2); non-significant results were still reported since little is known on the lipid profile in this species and as such these potential trends could guide future research.

Comparison of lipid profiles between tissue types within treatments revealed a large difference, ranging from 47-66% dissimilarity for both sampling periods and both sexes (Figure 2-10A, B, C, and D). Major contributors were the same for each sampling period: proportions of phospholipids, 20:5ω3, 22:4ω6, 20:4ω6, 20:1ω9 and 20:1ω11 were slightly higher in muscle tissue whereas TAG, sterols, ai15:0 and 16:1ω7 were higher in gonad tissue (Figure 2-10A, B, C, and D).

Lipid profiles of naturally-spawned oocytes were 39% dissimilar between treatments. The major contributors included a higher proportion of 16:1ω7 in ambient pH samples and

slightly higher proportions of sterols, 16:1ω5, ai15:0, and 20:5ω3 in low pH treatments, although none were significantly different in t-test analyses (Table 2-2).

2.4.6 Ossicles

There was no significant interaction between body wall region and treatment on the number of ossicles ($F_{2, 22}$=0.0461, p=0.955). In addition, there was no significant difference between treatments for the total number of ossicles (Figure 2-11; t_8=1.596, p=0.149). However, there was a difference in their appearance. Ossicles extracted from individuals under ambient pH showed a smooth and uniform surface (Figure 2-12 A to E), whereas ossicles sampled from low pH (Figure 2-12 F to J) had a rougher surface (Figure 2-12 G, H), with the occasional occurrence of larger calcified protuberances (Figure 2-12 I) and/or tinted areas (Figure 2-12J). Based on elemental analysis, there was no significant difference in content of calcium between ambient (18.3 ± 4.6%) and low pH (17.8 ± 2.3%; t_4=0.148, p=0.889) or magnesium in ambient (3.6 ± 3.1%) and low pH (2.8 ± 2.7; U=0, df=4, p=0.100).

2.5 Discussion

The sea cucumber *Cucumaria frondosa* showed both resilience and vulnerability to predicted near future OA conditions. Several indicators, including rates of respiration, tentacle deployment and feeding, and timing of spawning activity, suggest that *C. frondosa* was able to cope, at least superficially, with a ~0.4 decrease in pH. Importantly, the experimental setup was demonstrated to satisfy the conditions necessary for successful reproduction to occur. However, finer investigations revealed major susceptibilities affecting gametogenesis, oocyte morphology, embryo development and survival, as well as ossicle structure in individuals exposed to low pH for 4 months. Together these findings highlight transgenerational effects, providing evidence that this species may be vulnerable to OA.

The combined analysis of GI, gamete abundance (including relative fecundity) and MSI in *C. frondosa* indicated that a decrease in pH (increase in ρCO_2) subtly hindered gamete production and release in both sexes. Chiefly, there was a less defined reproductive cycle under low pH, with fewer indicators of maturity at T10 (pre spawning) and weaker indicators of recent gamete release at T19. Exposure to elevated ρCO_2 has been shown to cause a similar decreased gonad growth in the sea urchin *Strongylocentrotus droebachiensis* (Siikavuopio et al. 2007; Stumpp et al. 2012; Dupont et al. 2013) and a 1-month delay in gonad maturation and spawning in *Hemicentrotus pulcherrimus* (Kurihara et al. 2013). Interestingly, *C. frondosa* individuals collected below their normal depth of occurrence (at 1200-1450 m) were also shown to exhibit impaired oocyte maturation (Ross et al. 2013), indicating that natural environmental stress can reduce reproductive output in this species. Accordingly, Hamel and Mercier (1996d) indicated that when *C. frondosa* was exposed to adverse environmental conditions and low food availability for an extended period of time, it used its body wall as an energy reserve to sustain gametogenesis. A similar process was reported in the sea urchin *S. droebachiensis* where gonad and body wall were used for energy storage to mitigate environmental challenges such as low food availability (Russell 1998). The present study, however, showed that while pH impacted gametogenesis, it did not interfere with the perception of environmental cues inducing gamete release in *C. frondosa*. The timing of spawning remained consistent across treatments and was also coincident with spawning in other laboratory tanks and in the field.

The fact that gamete development was affected by exposure to low pH/high ρCO_2 may have to do with the coelomic fluid, which is suspected to play a key role in nutrient and hormone translocation/function in echinoderms (Ferguson 1964; Mercier and Hamel 2009). OA conditions have been shown to elicit changes in the pH of the coelomic fluid in several species of sea urchin

(Dupont and Thorndyke et al. 2012; Calosi et al. 2013; Holtmann et al. 2013; Moulin et al. 2014; 2015; Spicer et al. 2011; Stumpp et al. 2012; Uthicke et al. 2014) and sea cucumber (Collard et al. 2014), some of which compensated for acidosis be eliminating protons (e.g. H^+), increasing dietary intake of calcium carbonate or creating a buffer (e.g. bicarbonate). In turn, compensation can be energetically costly, leading to a decrease in somatic and gonad growth and to calcification problems (Collard et al. 2013; Moulin et al. 2014, 2015; Stumpp et al. 2012). In contrast, other species of sea urchin were unable to compensate for coelomic fluid acidosis (e.g. Catarino et al. 2012; Kurihara et al. 2013; Miles et al. 2007; Spicer et al. 2011). Collard et al. (2014) showed that exposure of two holothuroid species to low pH resulted in an increase in coelomic fluid pH, which was not overcome over the short term (6-12 days). Changes in the coelomic fluid pH can by hypothesized to have occurred in *C. frondosa* during exposure to OA conditions in the present study. Whether directly or indirectly (through compensation mechanisms), acidosis could have interfered with normal hormonal pathways function and/or translocation of nutrients necessary for gamete synthesis.

A change in coelomic fluid pH may also explain the ossicle calcification anomalies detected here in *C. frondosa*. To compensate for extracellular acidosis, some species have been shown to dissolve and reabsorb calcium carbonate structures to create a bicarbonate ion buffer (Calosi et al. 2013; Holtmann et al. 2013; Moulin et al. 2014; Stumpp et al. 2012). Walker et al. (2013) showed that exposure to elevated CO_2 led to the presence of deep holes and etching on the surface of the ossicles of the brittle star *Ophionotus victoriae* and Bray et al. (2014) provided evidence non-uniform ossicle pore size, as well as signs of surface degradation in the sea urchin *Arbacia lixula*. Similar structural changes were noted in the current study, evoking ossicle dissolution. However, in adults of *C. frondosa* the majority of ossicles are covered by a layer of

connective tissue, suggesting the involvement of compensation mechanisms rather than dissolution from direct contact with acidified seawater. Furthermore, some ossicles harbored large bumps, reminiscent of hyper-calcification. Increased rates of calcification were documented during arm regeneration of the brittle star *Amphiura filiformis* exposed to low pH (Wood et al. 2008). In addition, a test of the sea urchin *Paracentrotus lividus* showed higher concentrations of Ca^{2+} and Mg^{2+} under low pH/elevated ρCO_2, indicative of increased calcification rates (Calosi et al. 2013). This phenomenon can also be seen from the study of fossil records in deep-sea foraminifers showing a response to rapid changes in CO_2 (Foster et al. 2013). Thus, hyper-calcification may have been the initial response of *C. frondosa* to OA conditions, followed by calcification anomalies, as seen in ossicle formation, elicited by mechanisms deployed to buffer coelomic fluid acidosis over the longer term.

Minor differences in lipid composition of muscles and gonads between ambient and low pH conditions were also detected in *C. frondosa*. Higher levels of the essential fatty acid 20:5ω3 were found in the muscle bands of both sexes under low pH/high ρCO_2 in the pre-spawning period, suggesting that individuals exposed to low pH experienced a delay in the translocation of important fatty acids towards reproduction, as suggested for other marine invertebrate species (see review by Glencross 2009). Furthermore, gonad samples from both sexes displayed trends towards potentially higher proportions of TAG under ambient pH in the pre-spawning period. Common lipid classes known to be used for energy storage in echinoderms are TAG and diacylglycerol ether (DAGE) (Allen 1968; Hayashi and Kishimura 1997; Karnovsky and Brumm 1955; Oudejans and van der Sluis 1979). For species with lecithotrophic (yolk-sustained) larvae, like *C. frondosa*, DAGE is typically dominant over TAG (Villinski et al. 2002, Falkner et al. 2006 and Prowse et al. 2009). The predominance of TAG measured here in the gonads of *C.*

frondosa may be due to incomplete separation of TAG and DAGE (Prowse et al. 2009) leading to an underestimation of DAGE. Together, these results suggest a delay in the conversion of storage lipids towards gonad development under low pH conditions. Moreover, the proportion of TAG had declined in ovaries of *C. frondosa* sampled post spawning under ambient pH, but not under low pH, consistent with the presence of unspawned mature oocytes in the latter.

A combination of poorly provisioned oocytes (due to impaired energy and lipid storage/use) and less successful fertilization (due to lower spermatozoa quality and water pH) could explain the observed carry-over effects of exposure to low pH/high ρCO_2 in *C. frondosa*. Low-pH oocytes were characterized by morphological anomalies/deformities such as uneven shapes and non-uniform cytoplasms. Disruption of vitellogenesis has been reported in the brittle star *Ophuria ophuria* (planktotrophic species) exposed to low pH [according to Lowe et al. (unpublished data) cited in Wood et al. 2008]. Here, oocytes were apparently fertilized normally but embryos died at the early blastula stage, unlike those developing under ambient pH. Similarly, larvae of the crab *Hyas araneus* displayed mortality and delays in development when exposed to elevated ρCO_2 prior to hatching (Schiffer et al. 2014). When adults of the sea urchin *Echinometra* sp. A were exposed to elevated ρCO_2, their spawning and the size of their oocytes were not affected; however, the number of spermatozoa released, percent normal larvae and larval size decreased, independently of adult pre-acclimation (Uthicke et al. 2013). In fertilization trials using gametes of the sea cucumber *Apostichopus japonicus* post-fertilization success (calculated as the percent eggs starting cell division 6 h post fertilization) decreased by 6% following a decrease in pH by 0.6 units (Yuan et al. 2015). In the same study, duration of the early and mid-auricularia stages increased with decreasing pH, but no effect was observed at the late-auricularia stage (Yuan et al. 2015). The embryonic development of *C. frondosa* therefore

appears to be more susceptible to low pH than that of the temperate species *A. japonicus*, noting that the present study included transgenerational effects not examined by Yuan et al. (2015). Due to the variability in responses to OA, major impacts or potential for acclimation may only be detected over relatively long periods of exposure and/or the study successive life stages (Dupont et al. 2010b; Dupont et al. 2013; Uthicke et al. 2014).

Normal fully-mature oocytes of *C. frondosa* are positively buoyant and develop at the water surface until metamorphosis (Hamel and Mercier 1996b). In the present study, nearly all oocytes and embryos developing under low pH/high ρCO_2 were negatively buoyant, remaining demersal until they died. Loss of buoyancy under OA conditions may derive from a higher percentage of sterols in the cell membrane of oocytes; sterols mediate membrane fluidity in some eukaryotes (Parrish 2013). Similarly, higher proportions of the essential fatty acid 20:5ω3 were measured in the pre-spawn ovaries and similar potential trends were noted in spawned oocytes sampled under low than ambient pH. Such essential fatty acids are required for growth, reproduction (Phleger 1998; Santos et al. 2002) and buoyancy control (Pond and Tarling 2011) in marine animals such as fish and copepods, but this remains to be verified in echinoderms (see review by Glencross 2009). At high concentrations, wax esters and 20:5ω3 can reduce buoyancy in copepods at depth (Pond and Tarling 2011). If similar relationships occur in echinoderms, changes in the proportions of sterols and/or 20:5ω3 could alter membrane function in *C. frondosa*, with possible impacts on oocyte buoyancy. Alternatively, lipid organization rather than composition may play a greater role in lecithotrophic holothuroids, which is an aspect that requires further research under OA conditions. Moreover, lipids were the primary component in gonad tissue from *C. frondosa* from the Bay of Fundy, but protein and glycogen were seasonally abundant (David and MacDonald 2002). Therefore, oocyte buoyancy and shape could be

additionally affected if the respective ratios of glycogen, protein and lipids were altered by OA. Irrespective of the underlying cause, negative buoyancy and/or developmental issues likely drove the differences in percent survival of embryos, as a large proportion of propagules in the low pH treatment suffocated in the sediments. If not overcome via acclimation, the impact of OA on oocyte buoyancy in *C. frondosa* would have major consequences on its ability to reproduce successfully.

An important highlight of the present study is that *C. frondosa* produces lecithotrophic oocytes that rely on yolk reserves deposited by the female during gametogenesis to develop. Previous studies of the effects of OA that included a transgenerational investigation of gamete synthesis and subsequent embryonic development were conducted on species that produce planktotrophic (feeding) larvae (Dupont et al. 2010a; see review by Ross et al. 2011). Studies on lecithotrophic species to date had only focused on fertilization or embryonic/larval development. Larvae of the sea star *Crossaster papposus* exposed to low pH exhibited faster growth rates (Dupont et al. 2010a). In the sea star *Meridiastra calcar*, a 0.6 unit decrease in pH did not have an effect on embryonic cleavages, but decreased the number of embryos reaching the hatched gastrula stage (Nguyen et al. 2012). Havenhand et al. (2008) showed that a 0.4 unit decrease in pH negatively impacted fertilization success in the sea urchin *Heliocidaris erythrogramma*. Contrastingly, in the same species, Byrne et al. (2009) found that fertilization and early development were unaffected by low pH (0.3-0.6 unit decrease from ambient). In general, species with lecithotrophic development are suggested to be more resilient to climate change because their larvae do not depend on planktonic food sources (Havenhand et al. 2008; Byrne et al. 2009; Dupont et al. 2010a; review by Ross et al. 2011; Nguyen et al. 2012). However, this assumption is made on the basis that oocyte provisioning by the mothers is not affected. The

present study is apparently the first to examine transgenerational effects in a lecithotrophic species, showing that marine organisms with this development mode may not be more robust to OA than those with planktotrophic species, as initially predicted from the study of their larvae.

A brief word should be said on the benefits of conducting transgenerational studies under realistic conditions during meaningful life-history events, which implies a good basic knowledge of the focal species. While not perfect, the experimental setup used during the present study strove to incorporate the environmental factors (and fluctuations thereof) that mediate gametogenesis and spawning, which would not have been the case under static conditions, over a shorter period, or using a more artificial design. Furthermore, due to the nature of the annual reproductive cycle, significant differences in the number of gametes could only be detected immediately before spawning (a measure of true potential fecundity). Had samplings only occurred after the full 19 weeks, some of the finer negative impacts would have gone unnoticed. Finally, as anticipated, the MSI provided a more sensitive approach than the GI, allowing for significant differences in oogenic development over time to be evidenced in ambient pH. As research on OA moves forward to assess realistic impacts, as close to real-life scenarios as possible, attention to experimental designs and choice of bioindicators will likely become instrumental.

As for the probable impact of OA on the focal species, *Cucumaria frondosa* is a major component of temperate, subarctic and arctic benthic communities; hence, a decrease in its populations under OA conditions could generate unexpected cascade effects. As a primary consumer, it mediates nutrient cycling and provides food to many predators including marine mammals, fishes and predatory invertebrates. Also, it is the target of a growing fishery in the North Atlantic (Hamel and Mercier 2008b). Being ubiquitously distributed in the North Atlantic

and Arctic oceans, including areas affected by freshwater runoff and drastic temperature changes, *C. frondosa* exhibits an apparent adaptability to environmental changes (Hamel and Mercier 2008a; Hamel and Mercier 2008b). Despite this, the current study reveals that population decline is a real concern for this species should the predicted change in pH occur over the course of this century.

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2.8 Tables and Figures

Table 2-1: Experimental conditions. Average values of pH_{NIST}, temperature and salinity over the 19-week experimental period in the two treatments (±SD, n=200). Mean (±SD) daily differences between treatments are also shown since these factors were measured twice daily. Total alkalinity values provided as mean (±SD) based on weekly measurements from two tanks per treatment (n=34). ρCO_2 and saturation states of calcite (Ωca) and aragonite (Ωar) were calculated in CO2SYS using pH, total alkalinity, salinity and temperature.

Parameters	**Treatments**		**Mean Daily Difference**
	Ambient pH	**Low pH**	
pH	8.07 ± 0 .04	7.67 ± 0.06	0.42 ± 0.12
Temperature (°C)	4.8 ± 5.4	4.5 ± 1.3	0.2 ± 2.2
Alkalinity (µmol kg^{-1})	2735 ± 185	2831 ± 178	-
Salinity	36.6 ± 0.22	36.6 ± 0.19	0.0032 ± 0.31
ρCO_2 (ppm)	464.9 ± 65.8	1552.2 ± 231	-
Ωca	5.04 ± 2.4	1.76 ± 0.76	-
Ωar	3.17 ± 1.5	1.10 ± 0.48	-

Table 2-2: Comparison of fatty acid composition (% of total fatty acids) and lipid classes (% of total lipids) from pre-spawn and end of trial gonad and muscle samples (n=3 per sex per treatment). Asterisks (*) identify significantly higher proportion between treatments. P= phospholipids. S= sterols. TAG= Triacylglycerol. Weak contributors (-) were not included. For gonad and muscle samples df=4 and for oocyte samples df=2. For oocyte samples, Mann-Whitney test used, therefore medians reported.

			Female					**Male**				
Time Point	**Sample**	**Fatty Acid/Lipid Class**	**Ambient pH**	**Sd**	**Low pH**	**Sd**	**P**	**Ambient pH**	**Sd**	**Low pH**	**Sd**	**P**
Pre	Muscle	P	66.34	7.9	68.66	4.2	0.678	58.08	16.2	61.57	13.4	0.788
		20:5w3	33.48	2.7	47.88*	7.6	0.036	29.18	10.9	47.62*	2.7	0.047
		22:4w6	12.36*	0.57	0	0	<0.001	12.12	11.7	0	0	0.147
		20:1w11	6.05*	0.21	0	0	<0.001	6.84*	0.65	0	0	<0.001
		20:1w9	0	0	6.69*	0.54	<0.001	1.13	2.0	8.80*	1.0	0.004
End	Muscle	P	62.07	9.9	40.48	31.6	0.322	68.68	19.8	53.84	7.1	0.289
		20:5w3	34.67*	2.1	46.01	1.8	0.002	35.16	6.2	44.06	7.9	0.200
		22:4w6	11.84*	1.572	0	0	<0.001	8.53	7.4	0	0	0.117
		20:1w11	6.74*	0.40	0	0	<0.001	6.79*	0.31	0	0	<0.001
		20:1w9	0	0	8.71*	0.72	<0.001	0	0	8.45	0.70	<0.001
Pre	Gonad	P	11.16	9.1	12.68	1.9	0.792	17.06	10.6	45.30	33.5	0.236
		S	-	-	-	-	-	11.69	6.5	16.86	10.3	0.503
		TAG	66.12	14.9	40.36	13.7	0.092	14.21	6.9	6.61	6.0	0.225
		20:5w3	15.03	1.2	18.49*	1.5	0.035	30.24	6.8	33.03	7.5	0.658
		ai15:0	31.85	4.6	25.01	3.4	0.109	-	-	-	-	-
End	Gonad	S	3.13	0.47	7.37	4.9	0.207	11.98	9.4	7.20	6.2	0.504
		TAG	44.12	5.9	42.61	12.9	0.862	6.10	3.5	21.96	18.3	0.383
		20:5w3	15.60	2.6	18.64	4.5	0.035	24.08	6.2	18.14	3.4	0.218
		ai15:0	31.19	3.1	26.11	9.5	0.429	11.40	2.0	25.96	9.9	0.081
		16:1w7	-	-	-	-	-	19.48	1.1	22.89*	0.23	0.006
N/A	Ooctyes	S	9.73		43.11		0.245					
		20:5w3	19.5		23.48		0.245					
		ai15:0	34.36		40.69		0.245					
		16:1w7	11.96		0		0.245					
		16:1w5	24.59		33.11		0.245					

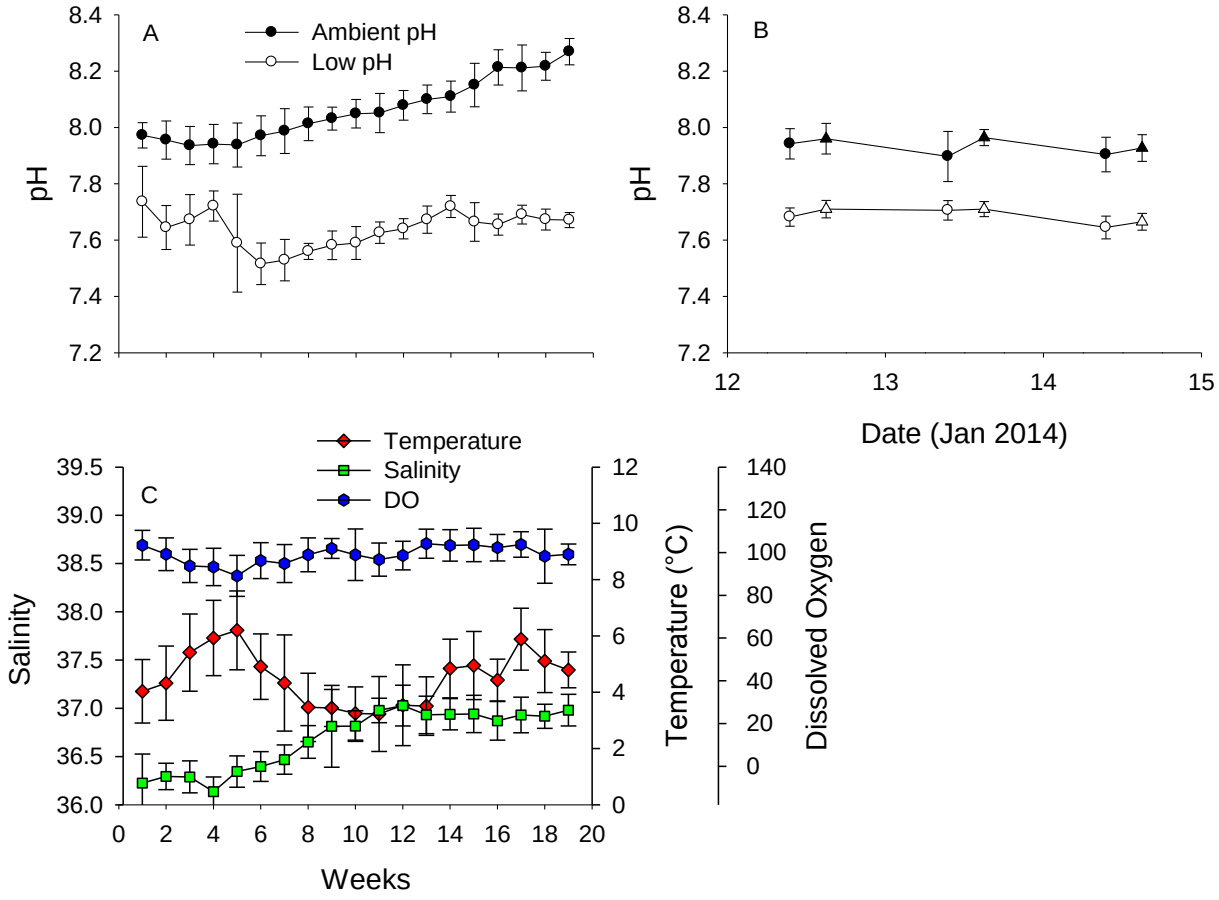

Figure 2-1: Water chemistry over the experimental period (Dec 26, 2013- May 7, 2014). A) pH B) Daily fluctuations shown over 3 days within in the same month (circles show morning values and triangles show afternoon values). C) Temperature, salinity and dissolved oxygen. Data in A, B and C shown as weekly/daily mean ± SD (n=4-8).

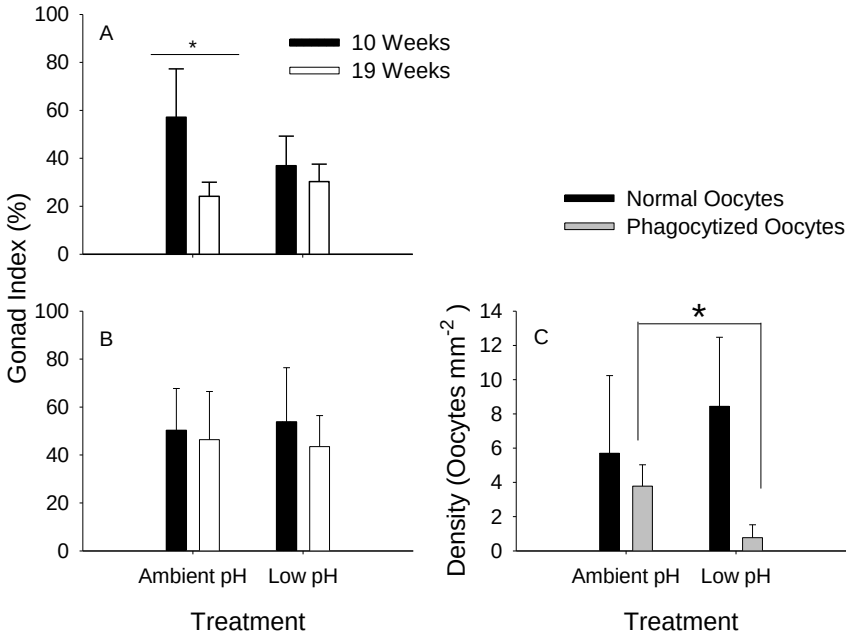

Figure 2-2: Gonad index in A) males and B) females of *C. frondosa* over time under ambient and low pH. C) Number of normal and phagocytized oocytes at T19 in both treatments. Data in A and B shown as mean ± standard deviation (n=5-7). Data in C shown as mean ± SD (n= 3). The asterisk (*) in (A) identifies statistically significant differences between time points for that treatment; refer to text for statistical results. The asterisk in (B) shows statistically significant differences between treatments in the number of phagocytized oocytes; refer to text for statistical results.

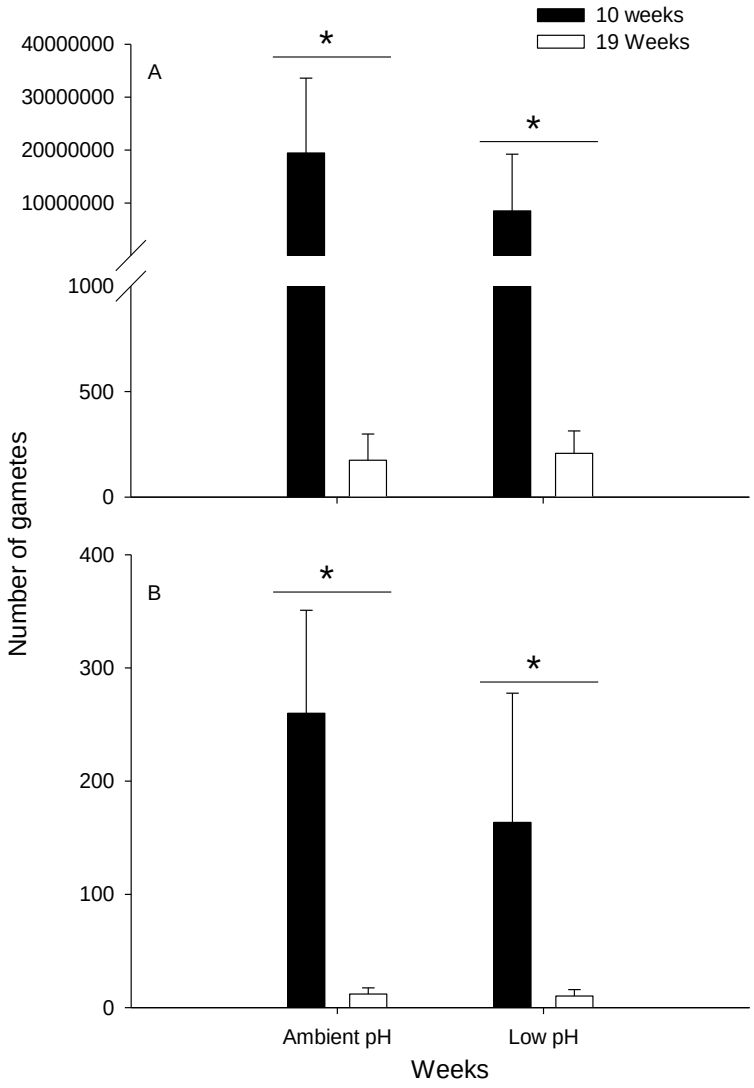

Figure 2-3: Gamete density over time for A) males and B) females. Data shown as mean ± SD (n=5-7). Asterisks (*) identify statistically significant differences between time points; refer to text for statistical results.

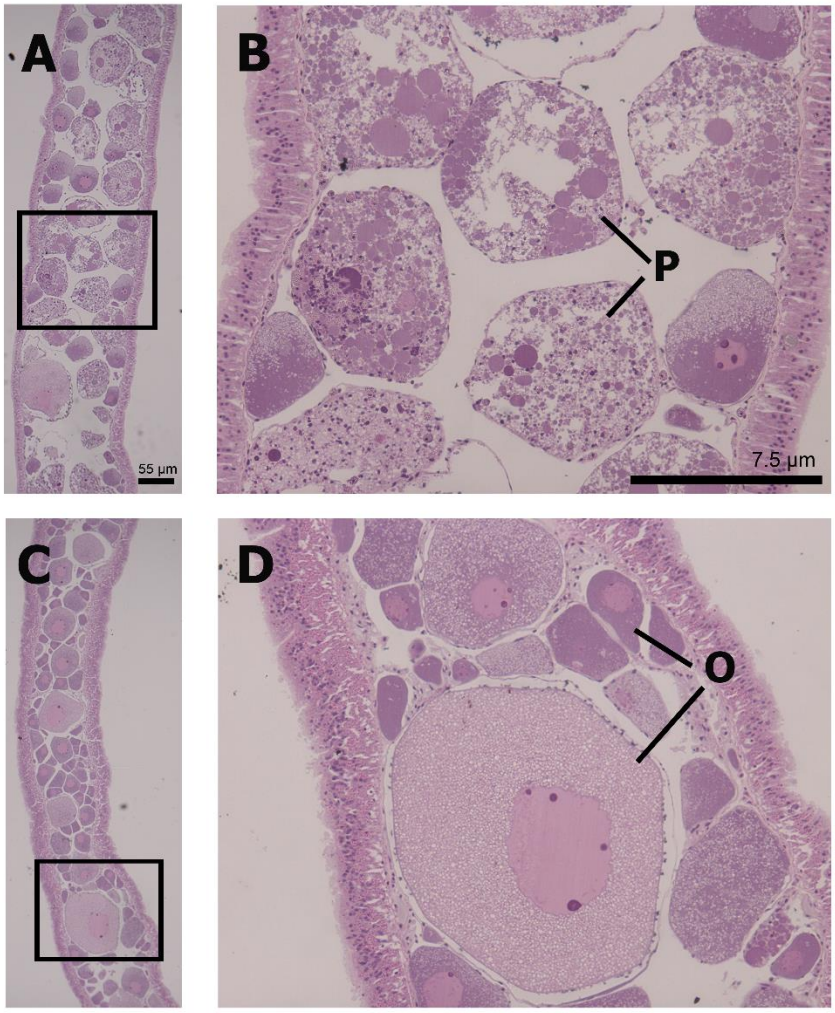

Figure 2-4: Histology of gonad tubules of *C. frondosa* (longitudinal sections) at T19 (May 7, 2014) showing difference between treatments. A and B (close up) from ambient pH; C and D (close up) from low pH. P: phagocytized oocytes; O: oocyte.

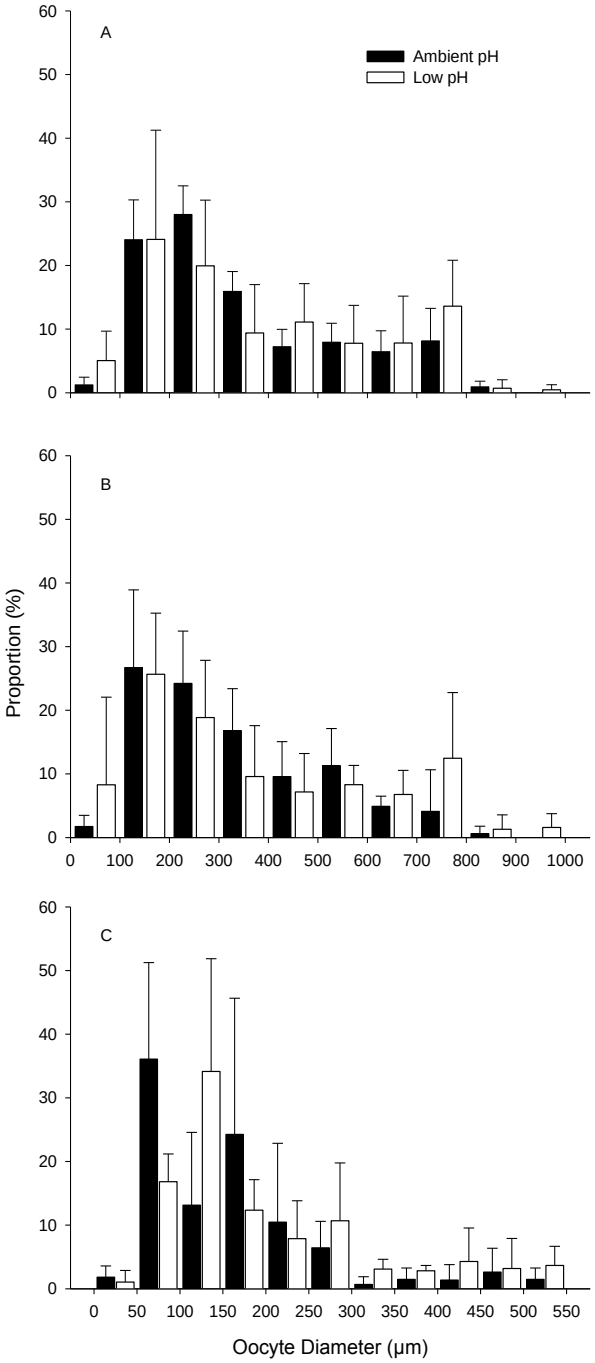

Figure 2-5: Oocyte size frequency distributions determined from A) tubule contents at T10 (March 12, 2014), B) tubule contents at T19 (May 7, 2014) and C) histological examination at T19 (May 7, 2014). Data in A and B shown as mean ± SD (n=5-7). Data in C shown as mean ± SD (n=3).

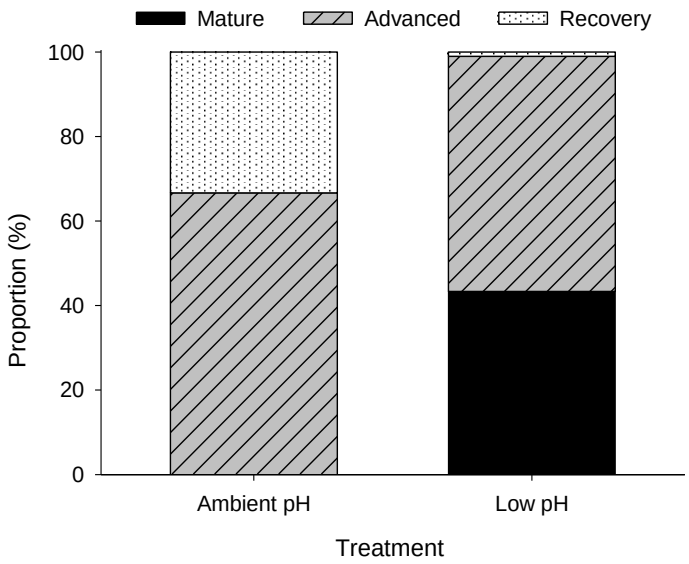

Figure 2-6: Percent gonad tubules in each gametogenic stages in each treatment (n=3) at T19 (May 7, 2014).

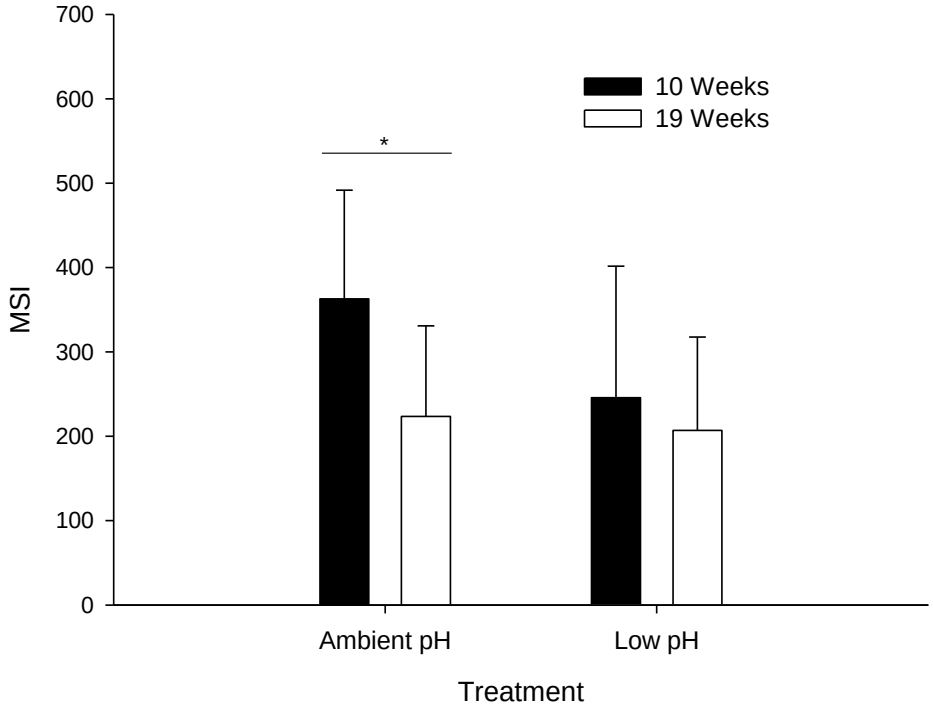

Figure 2-7: Maturity stage index (MSI) at A) T10 (March 12, 2014), and at B) T19 (May 7, 2014). Data shown as mean ± SD (n=5-7). The asterisk (*) identifies statistically significant differences between time points for that treatment; refer to text for statistical results.

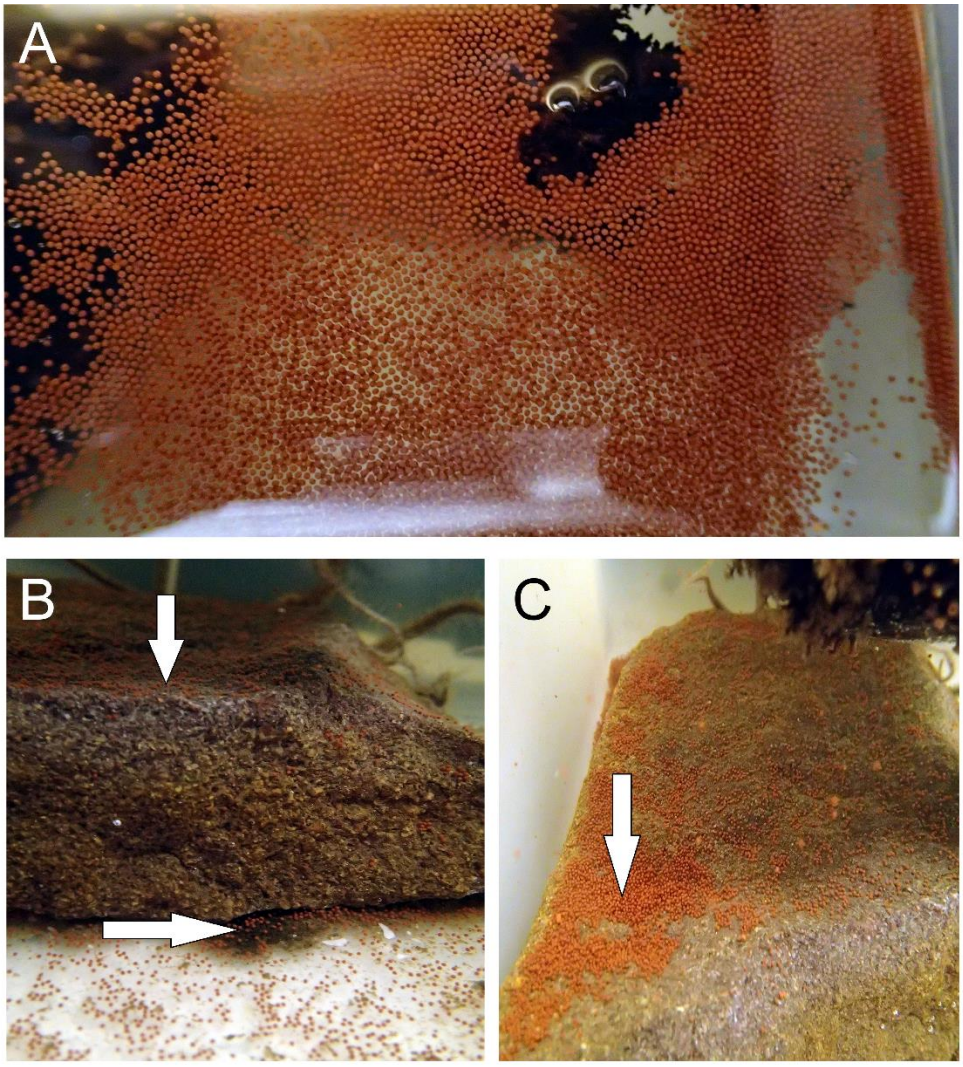

Figure 2-8: Spawning of *C. frondosa* during the study. A) Positively buoyant oocytes released under ambient pH floated to the water surface. B-C) Negatively-buoyant oocytes sank to the bottom of the tanks under low pH conditions. Arrows point to oocytes (small red-orange dots) that measure between 500-600 µm.

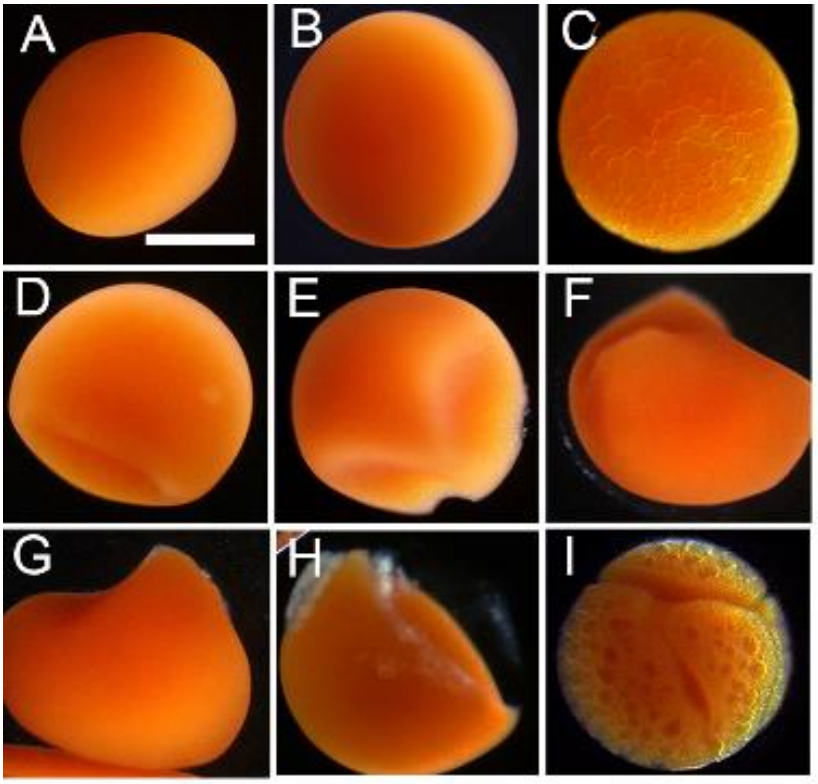

Figure 2-9: Comparison of egg development under ambient pH (A-C) and low pH (D-I). A) Unfertilized egg; B) newly fertilized eggs; C) late blastula. D-H) Irregular shaped oocytes; I) dividing oocyte showing irregular dimpled surface. Scale bar in A represents 30 µm and applies to all panels.

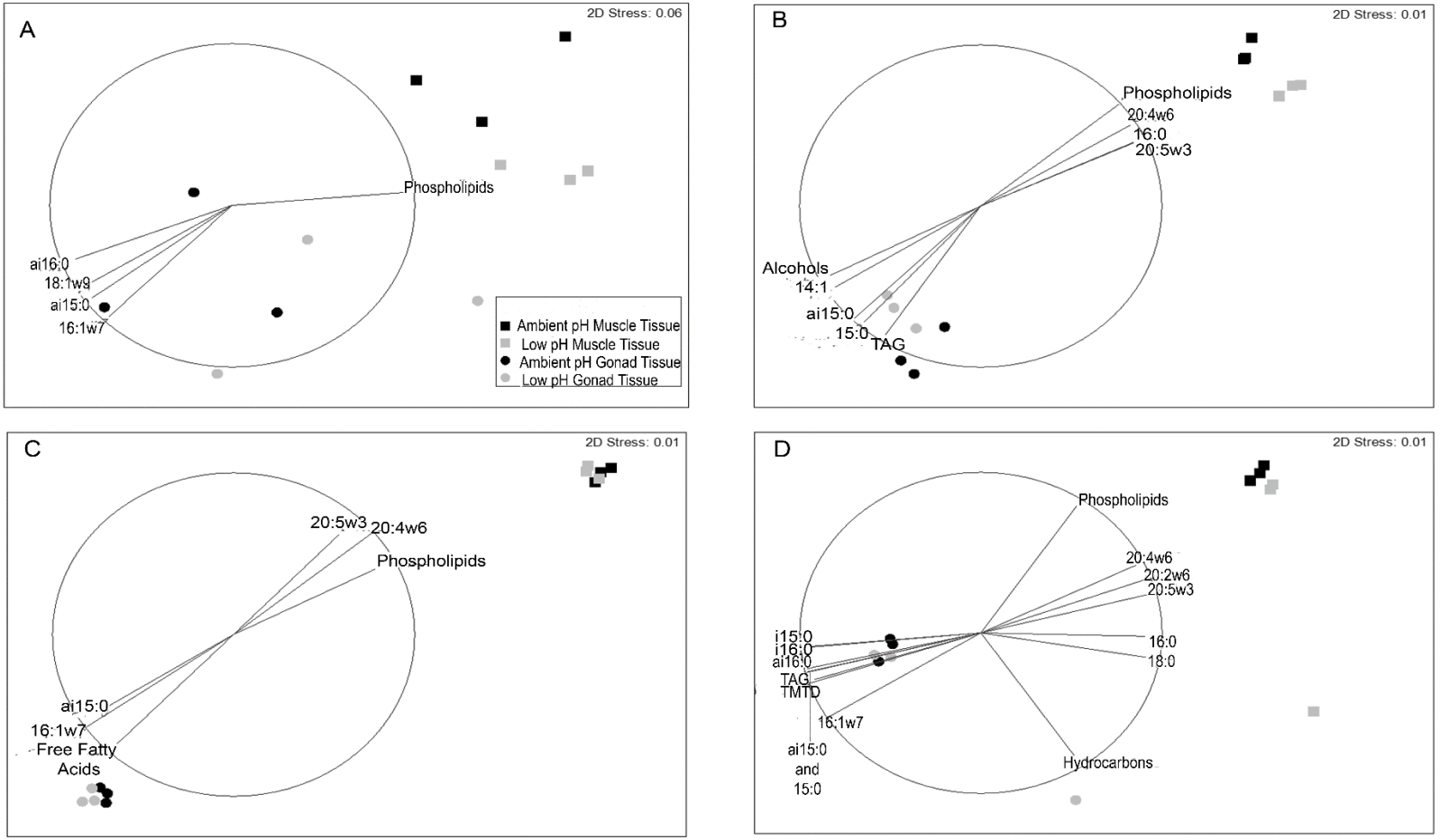

Figure 2-10: MDS comparison of lipid classes and fatty acid composition in muscle and gonad tissue from male and female *C. frondosa* (n=3). A) Male gonad and muscle tissues at T10, correlation set at 0.9. B) Female gonad and muscle tissues at T10, correlation set at set at 0.9. C) Male gonad and muscle tissues at T19, correlation set at 0.8. D) Female gonad and muscle tissues at T19, correlation set at 0.9.

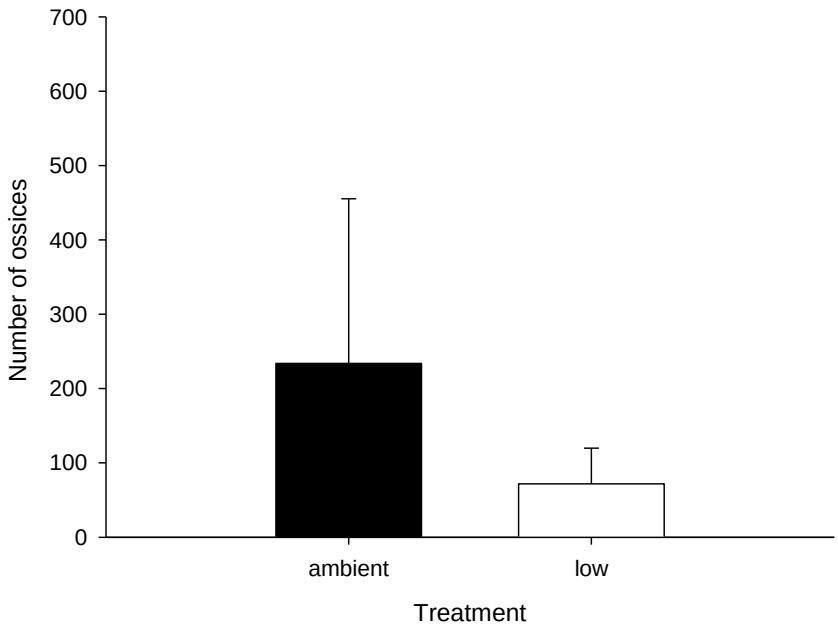

Figure 2-11: Comparison of the number of ossicles between treatments for entire sea cucumber. Data shown as mean ± SD (n=5).

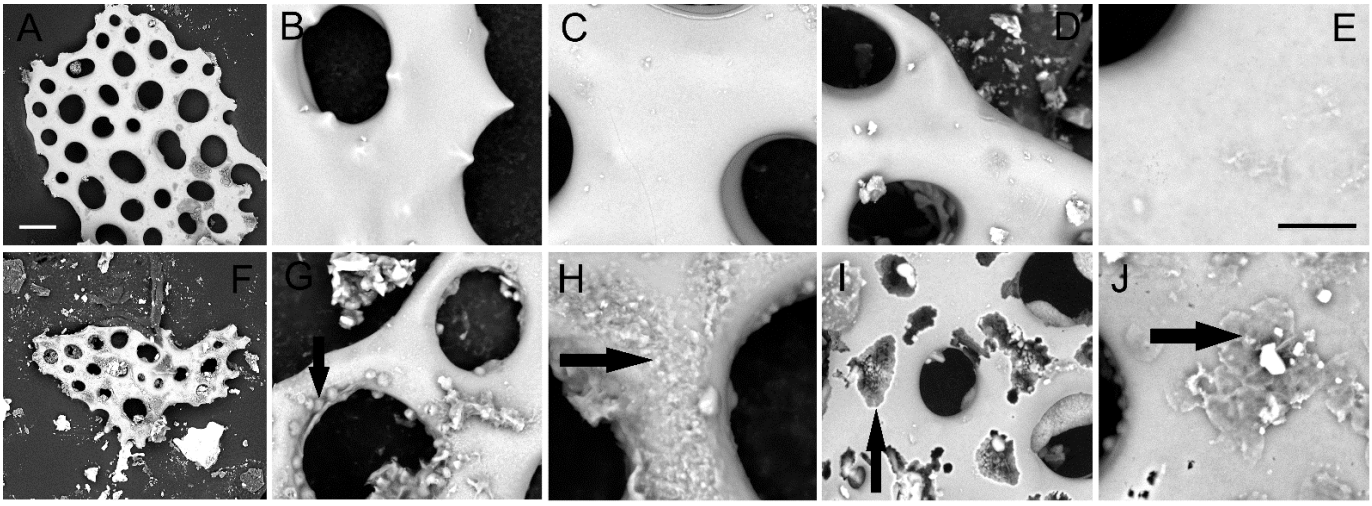

Figure 2-12: Ossicles of C. frondosa from ambient pH (A-D), and low pH conditions (E-H). Arrows highlight abnormalities in low pH: G) shows large calcified bumps and thinning centers, H) shows etched, texturized surface, I) shows erosion of ossicles surface and J) shows texturized surface. Scale bar in A represents 40 µm and applies to A and F; arrows highlight abnormalities. Scale bar in E represents 5 µm and applies to B-E.

Chapter 3: Impact of ocean acidification on reproductive output in the deep-sea annelid *Ophryotrocha* sp. (Polychaeta: Dorvelleidae)

3.1 Abstract

As increasing anthropogenic CO_2 emissions are absorbed by the oceans, a decrease in seawater pH is expected to occur, causing what is now termed ocean acidification (OA). Deep-sea species have been greatly understudied with respect to OA, even though their response may differ from those evidenced so far in shallow-water taxa, due to adaptations that may reflect generally more stable environments. The polychaete worm *Ophryotrocha* sp. collected at bathyal depth was held and reproduced for several years, offering a rare opportunity to study environmental effects in a member of a deep-sea community. This hermaphroditic species exhibits well defined seasonality in feeding and reproduction and its development and growth have been characterized. The purpose of the present study was to explore the effects of OA on gametogenesis following exposure to a 0.4 unit pH decrease under realistic conditions over 26 weeks. Opportunistic assessments of spawning and development were also conducted. A flow-through design allowing for natural fluctuations in pH, temperature, salinity, and phytoplankton was used. Individuals exposed to low pH/high ρCO_2 produced larger and more abundant oocytes but fewer spermatozoa, compared to individuals in ambient conditions. However, lower fecundity (number of eggs released) was ultimately recorded under low pH conditions, together with slower development of the embryos and larvae. Microstructure of the body wall, and appearance and elemental composition of chaeta were not affected. Despite its ability to live and reproduce normally for years in the laboratory, a realistic decrease of pH in the environment of *Ophryotrocha* led to reproductive disruption, highlighting its potential vulnerability to OA. Due to their basal position in food webs, alteration of worm communities in the deep sea could have cascading effects on biodiversity.

3.2 Introduction

Anthropogenic emissions of carbon dioxide (CO_2), leading to an increase in the concentration of hydrogen ions in seawater, are expected to cause a decrease in pH, commonly termed ocean acidification or OA (Caldeira and Wickett 2003). Reports predict an overall decrease of 0.4 unit in pH by 2100, which could have widespread impacts on marine life (Caldeira and Wickett 2003; Raven 2005). Over the past decade, the potential impacts of OA on various species have been studied abundantly (see reviews by Byrne 2011; Dupont and Pörtner 2013; Guinotte and Fabry 2008; Orr et al. 2005).

Despite the fact that it makes up the largest portion of the world's oceans and contains a diverse network of unique organisms, the deep sea has been comparatively neglected with respect to OA studies (Guinotte et al. 2006; see review Wernberg et al. 2012). Through thermohaline circulation and vertical mixing, anthropogenic CO_2 will reach the deep sea, with some reports suggesting a 0.2-0.4 unit decrease in pH to depths of 1000 m by the end of the 21st century (Gruber and Sarmiento 2002; Sabine et al. 2004; Ilyina et al. 2010). Effects of OA at depth may differ from those observed in species from shallow coastal habitats, due to the different physical and chemical parameters associated with the deep sea, such as temperature, light, carbonate minerals solubility and pressure. In general, deep-water environments are typically viewed as being more environmentally stable (Orr et al. 2005). Some studies have used fossil records to gain insight into adverse effects or adaptations in deep-sea species exposed to past OA events. For instance, Foster et al. (2013) used x-ray microscopy to compare the effects of past rapid increases in CO_2 levels on the calcification and diversity of deep-sea foraminifers. They discovered that after an extreme event ~55.5 Ma, the Paleocene Eocene Thermal Maximum or PETM, foraminifers experienced a mass extinction and that surviving species showed an

increased rate of calcification. The same authors examined the effects of the Eocene Thermal Maximum 2 (~ 53.2 Ma), an event that was half the magnitude of the PETM, and found only a small loss in diversity, concluding that there may be a response threshold for calcification and extinction associated with increases in CO_2 (Foster et al. 2013). In addition, based on what is known about the effects of OA scenarios on shallow-water corals, Guinotte et al. (2006), Turley et al. (2007) and Ramirez-Llodra et al. (2011) hypothesized that due to the water chemistry associated with the deep sea, negative impacts on calcification of cold-water deep-sea corals could be expected, with the risk of damaging these valuable ecosystems.

To date, few experimental studies have examined the effects of OA on deep-sea species. Investigations involving the release of liquid CO_2 within experimental setups at depth showed mixed responses to low pH on a variety of taxa (Barry et al. 2005; 2013): some survived and even thrived in low pH conditions (e.g. microbes, gastropods, fishes) while others experienced massive mortalities (e.g. nematodes, flagellates, amoeba, sea urchins, sea cucumbers, amphipods). In laboratory experiments conducted at ambient pressure, the deep-sea urchin *Strongylocentrotus fragilis,* collected from depths of 500-1000 m, exhibited a limited ability to compensate for respiratory acidosis; effects on feeding, growth and gonadosomatic index occurred only under the most extreme scenarios (Taylor et al. 2014). In comparison, the deep-sea hermit crab *Pagurus tanneri,* collected from a depth of 884 m, was shown to exhibit impaired antennular flicking and prey detection when exposed to decreased pH in the laboratory (Kim et al. 2015).

Polychaetes are important members of marine ecosystems, acting as bioturbators, ecosystem engineers, and important prey items in numerous habitats (Lewis 2013). A number of OA studies have focused on shallow-water polychaetes, with variable results. For example,

Widdiecomb and Needham (2007) determined that a reduction in seawater pH had no impact on the size or structure of burrows made by the ragworm *Alitta* (=*Nereis*) *virens*. In contrast, the production and quality of tubes formed by juveniles and larvae of *Hydroides elegans* were negatively impacted by exposure to low pH (Chan et al. 2012; Lane et al. 2013). Many species of polychaetes associated with a shallow volcanic CO_2 vent were found to be more abundant in the most acidified areas, providing evidence for their adaptive abilities (Calosi et al.2013; Ricevuto et al.2014) . Taxon-dependent disparities have also been reported, such as a decreased recruitment of serpulid polychaetes compared to a greater abundance of syllid polychaetes effected by volcanic CO_2 vents (Cigliano et al. 2010). In studies on various intertidal polychaete species, low pH (7.4-7.7) elicited detrimental impacts on sperm motility, fertilization success, larval survival (Lewis at al.2013; Campbell et al. 2014), and on regenerative abilities (Pires et al. 2015). In the only study with a deep-water focus, Barry et al. (2013) mentioned the potential effects of OA on a deep-sea polychaete community using CO_2 release experiments at depth, showing that species richness and density at 3300-3600 m decreased following exposure to pH changes of $\geq$0.2 units.

The polychaete genus *Ophryotrocha* is reported from many coastal and deep-sea environments (Thornhill et al. 2012; Wiklund et al. 2012). The species *Ophryotrocha* sp. used in the present study was collected at bathyal depths (500-1500 m) and has been kept for several years in the laboratory, producing several generations and offering a rare opportunity to study anthropogenic effects in a member of a deep-sea community. Adults exhibit simultaneous hermaphroditism, seasonal feeding and annual reproduction (Mercier et al. 2014). The goal of the present study was to develop our understanding of the impact of OA on deep-sea worm communities, by assessing the effects of decreased pH (~0.4 pH unit decrease, in a flow-through

system) on gametogenesis and reproductive output. Impacts on spawning, larval development, overall health and microstructural composition and shape of chaetae were also analysed.

3.3 Methods

3.3.1 Specimens Collection

Specimens of *Ophryotrocha* were collected with the sea pens *Anthoptilum grandiflorum* and *Halipteris finmarchica*, as by-catch from research surveys performed by Fisheries and Oceans Canada (DFO) in December 2010 off Newfoundland, Canada (48°52'N–45°51'W) between 500 and 1500 m depth. Once transferred to the Ocean Sciences Centre (Logy Bay, Newfoundland, Canada), worms were kept in beakers within darkened flow-through tanks (5-50 L h^{-1} pumped directly from the ocean). Individuals were collected from depth but kept under shallow water pressure conditions as seen in other deep-sea OA research (see Taylor et al. 2014). All tanks were supplied with a thin layer of marine sediment (see Mercier et al. 2014 for details). The individuals of *Ophryotrocha* sp. (n=128; 10-15 mm long) used for the present experiment are from the third generation of worms that reproduced and reached sexual maturity in the laboratory.

3.3.2 Experimental Setup

Deep-sea worms were exposed to either ambient or low-pH treatments for a period of 26 weeks, from December 26, 2013 through June 25, 2014, which encompassed the period of gamete synthesis leading to mating, spawning, egg laying and development (as per Mercier et al. 2014). Individuals were kept in 50 ml perforated plastic tubes, with meshed-covered openings (100 µm), within16-L tanks under dark conditions. A small sample of marine sediment was incorporated to each tube. There were two tanks per treatment group, each containing 4 tubes and 8 worms per tube, for a total of 64 worms per treatment (ambient vs low pH). Initially,

worms were acclimated to the tank system at ambient pH for one week. Seawater with a decreased pH (-0.4 units relative to ambient, see below) was then slowly trickled into the experimental tanks, providing a gradual change in pH over 12 hours before the onset of the study.

The pH was maintained by electronically-controlled injection of CO_2, via a CO_2 regulator (Milwaukee Instruments MA957) and reactor (AquaMedic CO_2 Reactor 500) into an experimental header tank. Both ambient and acidified seawater were continuously distributed to the tanks at a flow rate of ~7.2 L h^{-1}. Average water temperature varied between 3 and 9°C over the duration of the experiment, similar to the temperature range recorded along the continental slope (DFO 2009) where the specimens were collected. The setup allowed for fluctuations in ambient pH following daily and seasonal cycles, with the experimental pH being maintained proportionally lower by ~0.4 units. Additional parameters such as salinity and temperature also followed natural fluctuations throughout the experiment, as did the planktonic food present in the unfiltered seawater. It should be noted that, although *Ophryotrocha* sp. is a deep-sea species, bathyal depths in its native geographic still experience moderate environmental fluctuations compared, for instance, to abyssal depths. The area of the North Atlantic where these worms were collected can undergo fluctuations in temperature from -1.5 to 8 °C at depths down to 600–800 (DFO, 2009). Because *Ophryotrocha* sp. was shown to be a facultative carnivore (Mercier et al. 2014), the experimental animals were fed small pieces of gonads from the blue mussel *Mytilus edulis* bi-weekly (~0.02 g wet weight per tube).

3.3.3 Monitoring

The physical and chemical parameters of the water (pH, temperature, salinity, and DO) were monitored in each tank twice daily (morning and evening) using a YSI 556 MPS multi-

probe. Total alkalinity (TA) was measured twice a week in each of the tanks (two from ambient and two from low pH conditions) using a total alkalinity test kit (Orion; accuracy ± 5 ppm). Temperature, pH_{NIST}, and TA were used to estimate ρCO_2, saturation state of aragonite (Ωarag) and saturation state of calcite (Ωca) using CO2SYS software (Lewis and Wallace 1998) with constants of Mehrbach et al. (1973) as refitted by Dickson & Millero (1987). Data loggers (HOBO Pendant®, Onset Computer Corp.) were used to measure the temperature and light intensity every two hours in one randomly selected tank per treatment.

3.3.4 Sample Collection

Two sampling periods were established during this experiment. One tube per tank (2 tubes per treatment) were randomly sampled in early February after 6 weeks of exposure (T6), during the pre-spawn period (as per Mercier et al. 2014). From each tube, five worms were preserved in 1% formaldehyde for 48 hours (to maximize their integrity), then transferred to 4% formaldehyde for long-term preservation until they were processed for histological examination of gamete development. At the end of the trial in late June, after 26 weeks of exposure (T26), all remaining tubes were sampled (5 tubes per treatment). From each tube, five worms were placed in 70% ethanol for analysis of body wall and chaetae fine structure and elemental composition using scanning electron microscopy (SEM; see below). It should be noted that between T6 and T26 one tube per treatment was sampled to examine egg masses laid (see below).

3.3.5 Gametogenesis

To establish potential fecundity (number of oocytes just before spawning), worms sampled at T6 (n=5 per treatment) were used. Complete worms were processed using standard histological methods (Havenhand and Schlegel 2009), i.e. dehydrated using a series of ethanol baths, followed by xylene to remove the alcohol. Samples were then embedded in methacrylate,

sectioned (7 µm), and stained with hematoxylin and eosin. Photographs taken using a Nikon Eclipse 80i microscope coupled with a Nikon DXM1200F digital camera were analysed with the software ImageJ. Oocytes were counted and their maximum diameter (Feret diameter) measured in all gonads. Potential fecundity was calculated as (1) number of oocytes per ovary and (2) total number of oocytes, calculated as the mean number of oocytes per gonad multiplied by total number of gonads (standardized to an overall size of 32 segments or 64 gonads as per Mercier et al. 2014). Spermatozoa where counted from an area of 1 mm^2 (n=2 gonad per individual or 10 gonads per treatment). Only cells entirely within the field of view were counted.

3.3.6 Spawning and Development

Once every three days, each tube was carefully examined under low light intensity (~ 50 lux) for the presence of courtship behaviour (pairs attaching head to tail) and presence of egg masses (transparent, cylindrical, gelatinous mass containing small off-white propagules; as per Mercier et al. 2014). When egg masses were detected their locations and the spawning date were noted. The masses were left undisturbed for as long as possible but collected before the juveniles hatched, at which time they were gently removed from the tube. Overall, only one egg mass was found in each treatment. At time of sampling, juveniles were photographed using a Nikon SMZ1500 microscope coupled with a Nikon DXM1200F digital camera and preserved in 70% ethanol for further imaging (n=3-48 juveniles per treatment). Adult worms were also preserved (n=5 in 4% formaldehyde; n=3 in 70% ethanol per treatment). Photographs were used to determine and compare stages of embryonic development between treatments. Effective fecundity (number of hatchlings produced) was assessed as the number of propagules per egg mass.

3.3.7 Scanning Electron Microscopy

The morphology of the body wall and the elemental composition of chaetae (n=5 worms per treatment collected at T26) were assessed via scanning electron microscopy (SEM; Phenom ProX). Samples were attached to stubs using freezing gel and frozen to -18 °C with the temperature-controlled sample holder. To compare fine morphology, photos from each treatment were taken at a high magnification (220x- 15000x) and the surface structure of the body wall and chaetae compared. For the body wall, number of surface pore openings per mm^2 were compared; only pores entirely within the field of view were counted.

Elemental composition of chaetae was determined using energy-dispersive X-ray spectroscopy (EDS). To determine the characteristic composition from a specific sample, an electron beam is focused on the sample, the number and energy of x-rays being emitted from the sample is measured via the EDS detector. Three points of analysis (base, middle, tip) were taken along each chaeta. To standardize measurements, chaetae attached to the sixth segment from the anterior end on the right side were used for all photos and elemental comparisons. This specific location was chosen because, upon initial microscopic comparison, it was intact (no broken chaetae or deformities to the body wall) in all samples. Elements present were determined using the Phenom ProSuite elemental identification (EID) software.

3.4 Results

3.4.1 Water Chemistry

Average daily pH measurements showed that the ~0.4 unit decrease relative to the naturally fluctuating ambient pH was maintained throughout the experiment (Figure 3-1A). Over the 26 week period, pH increased progressively from the beginning to the end of the trial in June and daily pH typically increased from morning to evening (Figure 3-1B). Over time, temperature

briefly increased over the first four weeks, then decreased back to colder temperatures, before rising towards the end in June (Figure 3-1C). Salinity increased slightly towards February-March (Figure 1C) and dissolved oxygen stayed consistent over the 26 weeks (Figure 3-1C). Other water parameters associated with changes in pH showed that ρCO_2 was approximately three times higher in tanks under low pH conditions and the saturation states of calcite and aragonite were higher in tanks under ambient pH (Table 3-1).

3.4.2 Gametogenesis

Overall, potential fecundity was higher in worms exposed to low pH/high ρCO_2 when compared to individuals from ambient pH (Figure 3-2). Statistical analysis using t-tests or Mann-Whitney tests showed that the number of oocytes per ovary (t_9=-1.926, p=0.086) and total number of oocytes per individual (t_9=-1.926, p=0.086) were not significantly affected by pH condition, although both indices were visually higher in low pH (Figure 3-2). Oocytes in the small size classes, 20-25 µm and 30-35 µm, were significantly more abundant in ambient pH (Figures 3-3, 3-4C; t_8=2.781, p=0.024 and t_8=2.645, p=0.029, respectively). However, oocytes in larger size classes, 55-60 µm and 60-65 µm, were significantly more abundant in low pH (Figures 3-3, 3-4F; t_8=-5.819, p=<0.001 and U=2.5, p=0.032, respectively). All other size classes of oocytes did not differ significantly between treatments (Figure 3-3). Overall, mean oocyte size was also significantly larger in low pH (36.9 µm ± 3.8) compared to ambient pH (31.4 µm ± 2.4) (t_8=-2.721, p=0.026). Spermatozoa were significantly less abundant in low pH than in ambient pH conditions (Figures 3-4B, E and 3-5; t_8=2.359, p=0.046).

3.4.3 Behavioural Monitoring

Courtship behaviour (pairs of worms attached head to tail) was observed in all tanks and in both treatments from January 27, 2014 to March 5, 2014. There appeared to be no difference in the onset of this behaviour as it was noticed simultaneously and went on for a similar duration.

3.4.4 Spawning and Development

From opportunistic preliminary observations, effective fecundity (number of eggs laid) and juvenile development were both negatively impacted by low pH/high ρCO_2. Two egg masses were discovered: one from ambient pH on February 17, 2014, and one from low pH on February 23, 2014. Both egg masses were sampled on March 21, 2014. Effective fecundity was 48 juveniles in low pH. However, juveniles from the egg mass discovered in ambient pH hatched much faster, prior to sampling, thus effective fecundity could not be directly determined.

After 33 days of development the larvae from ambient pH were at the 3-chaetiger stage and possessed chaetae (Figure 3-6A); taking temperature in account, their development was consistent with previously published data (Mercier et al. 2014). Under similar water temperature, after 27 days of development the larvae from low pH were ~90% at the segmented stage (no chaetae present, Figure 3-6B); the remaining 10% were even less developed, i.e. at the trochophore stage (Figure 3-6C), showing delayed development compared to the ambient pH group, as well as to previously published work (Mercier et al. 2014)

3.4.5 Scanning Electron Microscopy

Based on SEM examination, there was no noticeable morphological differences in the surface of the body wall or chaetae between treatments. Chemical composition of chaetae was not impacted by low pH conditions; there was no significant difference between percent

composition of oxygen, nitrogen, magnesium, calcium or carbon in chaetae when comparing individuals in ambient and low pH (Figure 3-7; t-tests: p=0.069-0.762).

3.5 Discussion

The deep-sea annelid under study (*Ophryotrocha* sp.) has shown adaptive abilities enabling it to reproduce for years under laboratory conditions, yet exposure to low pH for several weeks under otherwise realistic conditions elicited effects on the production of spermatozoa and oocytes. In addition, a potential trend towards a decrease in effective fecundity and slower larval development was highlighted.

Unexpectedly, effects on the production of male and female gametes by this hermaphroditic species differed. Spermatozoa production was reduced in individuals exposed to low pH/high ρCO_2. The vast majority of studies that assessed the effect of OA on gamete production have examined dioecious species. For instance, exposure to a 0.4 unit decrease in pH for several weeks resulted in lower numbers of gametes synthesized by both sexes in the shallow-water sea cucumber *Cucumaria frondosa* (Verkaik et al. in prep.). Several other studies have reported impacts of OA on gamete production in shallow-water species, showing that both spermatogenesis (Uthicke et al. 2013) and oogenesis (Dupont et al. 2013; Kurihara 2013) can be affected. In the only previous study of this type on a deep-water species, the deep-sea echinoid *Strongylocentrotus fragilis* exhibited lower gonad indices when exposed to very extreme pH values (6.6-6.7) over 4 months in the laboratory (Taylor et al. 2014).

The present study is the first to evidence the simultaneous intra-individual decrease in spermatozoa production and a potential tendency to increase in oocyte production following exposure to low pH. These results suggest that the former process was the first to be impacted,

whether directly or as a result of a trade-off. In hermaphrodites, it has been hypothesized that there is actually a three-way trade-off between gender allocation (male or female) and somatic growth, which can be altered by parameters such as environmental stress or mating group size (Aira et al. 2007; Lorenzi et al. 2006). Exposure to low pH has been associated with either increased or decreased metabolic rates in various shallow-water polychaetes (Calosi et al. 2013). From this, it can be hypothesised that more energy was used by *Ophryotrocha* sp. in an attempt to maintain growth, physiological processes and metabolism under low pH, depleting resources available for spermatozoa production. Concurrently, or alternatively, negative impacts on foraging abilities may have caused a decrease in energetic reserves. In the deep-sea crab *Pagurus tanneri*, prey detection was reduced during exposure to low pH (Kim et al. 2015). Should sensory abilities be similarly affected in *Ophryotrocha* sp., poor feeding may translate into decreased energetic reserves.

Whether dealing with low pH/high ρCO_2 somehow decreased the overall amount of energy available for spermatozoa production or interfered with the necessary nutrient translocation, *Ophryotrocha* sp. responded by producing a greater quantity of larger oocytes (better provisioned), to the detriment of spermatozoa. Some reports suggest that sex allocation should be biased to female gamete production, allowing them to benefit from the greater fitness return they would gain (Baeza 2007). However, little is actually known about the amount of energy required for producing gametes (spermatozoa versus oocytes) in hermaphroditic polychaetes. While eggs are larger than spermatozoa, increased sperm production has been linked to a reduction in body growth in *Ophryotrocha diadema*, indicating that spermatogenesis may be energetically costly (Sella 1990; Sella and Lorenzi 2003). Furthermore, lower spermatozoa counts are identified as a primary reaction to pollutants (e.g. copper, polycyclic

aromatic hydrocarbons, crude oil) and stress in many marine invertebrates (reviewed by Lewis and Ford 2012). It has also been hypothesized that species can alter energy investment to produce fewer but larger offspring as an adaptation to long term stress (Maltby 1994). For example, when exposed to low salinity levels, the crab *Chasmagnathus granulata*, produced eggs with a larger diameter and higher biomass, which was attributed an adaptive value by way of increasing the likelihood of survivorship during development (Gimenez and Anger 2001). Here, *Ophryotrocha* sp. exposed to low pH did not exhibit a trade-off between the quantity and quality of eggs, since they produced both more large oocytes and more oocytes altogether. However, being a hermaphroditic species, the trade-off was a simultaneous decrease in spermatozoa production.

Ultimately, preliminary data suggested a decrease in effective fecundity (number of eggs laid) and slower development rate under low pH/high ρCO_2, though this could not be analyzed statistically (see below). The onset of the reproductive season did not appear to be affected, as the timing and duration of courtship behaviour was consistent between treatments as well as with previous studies (Mercier et al. 2014). Although effective fecundity was only established for the low-pH treatment (in ambient pH juveniles had hatched much faster, prior to egg mass collection), comparisons to populations kept in the same conditions as ambient individuals had higher effective fecundity. This is also consistent with previous results obtained by Mercier et al. (2014), who showed that typical effective fecundity was between 80 and 110 propagules per egg mass, much higher than what was detected here under low pH. Development rate showed that individuals in ambient pH developed at consistent rates, whereas those from the single low pH egg mass developed at a much slower rate relative to both ambient pH and previous reports (Mercier et al. 2014). Contrastingly, the findings of Lane et al. (2013) revealed no change in

embryonic development of the tube-dwelling, shallow-water species *Hydroides elegans* exposed to pH levels of 7.9, 7.7 and 7.5, suggesting that the effects of low pH on annelids are species-specific and maybe habitat-specific. In some shallow-water dioecious species, delays in development rate have also been observed (see review Byrne et al. 2011). For example, in the Arctic spider crab, continued exposure (from adults to larvae) to elevated ρCO_2 resulted in a 20-day delay in development (Schiffer et al. 2014). While providing insight that remains rare in deep-sea species, the fact that only one egg mass per treatment could be studied here calls for cautious interpretation. Further investigation would be required to confirm the trend with a larger sample size.

It is still difficult to predict whether the trade-off between a greater quantity of larger oocytes and fewer spermatozoa produced under low pH in *Ophryotrocha* sp. can maximize fitness under such conditions since it translated into fewer and more slowly developing offspring in the egg mass examined. Only transgenerational studies could determine whether this strategy would persist and, if so, whether it would ultimately help the species maintain reproductive success. There is a possibility that low pH conditions may have directly impacted the spawning process and subsequent development. Perception of normal mating and spawning cues may have been altered leading to a decrease in the number of eggs effectively laid. Moreover, the production of the gelatinous egg mass may have been altered. Presumably this process requires energy; shifting allocations under OA conditions may have impacted the size or quality of the gelatinous cocoon, resulting in a decrease in the number of embryos able to develop. Furthermore, it was clearly established that fewer spermatozoa were available, which may have limited the ability to fertilize the increased number oocytes produced. Finally, reports have suggested that under OA conditions larval development may be more costly energetically

(Campbell et al. 2014) due to impeding processes such as ion regulation (Stumpp et al. 2011), compensation mechanisms (Thomsen and Melzner 2010), and maintaining homeostasis (Lannig et al. 2010). Therefore, trade-offs in parental investment and their roles in the overall response to OA remain difficult to fully understand. Over subsequent generations, spermatozoa production may recover or the fewer larvae produced may become more robust.

When comparing body wall morphology and the fine structure of chaetae in *Ophryotrocha* sp., and the elemental analysis of chaetae, low pH did not appear to have any structural impact. Calcification in many marine invertebrates, as well as some tube-dwelling polychaete species, has been shown to be impacted by low pH (Byrne 2011; Chan et al. 2012; Dupont et al. 2010; Kleypas et al. 2006; Kurihara 2008; Lane et al. 2013). While low pH was hypothesized to affect the hardened structures of *Ophryotrocha* sp., the lack of striking differences may be related to the composition of chaetae, which mainly includes sclerotized chitin, a polysaccharide-based substance (Briggs and Kear 1993). Thus, their formation may not depend on the amount of available calcium carbonate as seen in more calcified marine invertebrates. As this study was performed at ambient pressure, it did not incorporate the difference in carbonate mineral solubility typical of the deep sea. With increasing pressure, carbonate minerals (e.g. calcite, aragonite) become less soluble, compounding the effects of OA (Guinotte et al. 2006). If *Ophryotrocha* sp. does incorporate any of these minerals as part of the sclerotizing process, this may become impacted at depth. Thus, the lack of effects observed here on the hardened structures could be an underestimation of the potential impacts this species may face in its natural environment.

Overall, the results of the present study suggest that the deep-sea polychaete *Ophryotrocha* sp. may not be able to maintain normal reproductive output under predicted ocean

acidification scenarios, which supports the previously proposed hypothesis that species from the deep sea may be readily susceptible to OA (Barry et al. 2013). The fact that the deep sea is a relatively stable environment may explain this potential susceptibility to environmental changes (Guinotte et al. 2006; Orr et al. 2005; see review Wernberg et al. 2012). If slow development and a decrease in effective fecundity remain a trend under OA, detrimental effects on the number of individuals successfully maturing at the culmination of reproductive cycle in *Ophryotrocha* sp. would lead to population declines and could have cascading effects on the food webs, nutrient cycling and communities of the deep-sea environment of which this species is a crucial part.

3.6 Acknowledgements

We would like to thank the Department of Fisheries and Oceans Canada, the Canadian Coast Guard, as well as Sandrine Baillon for specimen collection and initial culture. In addition, we thank Iliana Dimitrova from the histology unit of the Medical Education and Laboratory Support Services (MELSS) at Memorial University for helping with histological slides. This study was funded by NSERC and CFI (grants to A. Mercier).

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3.8 Tables and Figures

Table 3-1: Experimental conditions. Average values of pH, temperature and salinity over the 26-week experimental period in the two treatments (±SD, n=491). Mean (±SD) daily differences between treatments are also shown since these factors were measured twice daily. Total alkalinity values provided as mean (±SD) based on weekly measurements from one tank per treatment (n=24). ρCO_2 and saturation states of calcite (Ωca) and aragonite (Ωar) were calculated in CO2SYS using pH, total alkalinity, salinity and temperature.

Parameters	**Treatments**		**Mean Daily Difference**
	Ambient pH	**Low pH**	
pH	8.13 ± 0 .1	7.67 ± 0.12	0.45 ± 0.12
Temperature (°C)	5.2 ± 3.4	5.1 ± 1.8	0.2 ± 2.2
Alkalinity (µmol kg^{-1})	2815 ± 128	2911 ± 151	-
Salinity	36.6 ± 0.28	36.6 ± 0.33	0.0032 ± 0.31
ρCO_2 (ppm)	474.9 ± 68.4	1508.2 ± 216	-
Ωca	5.1 ± 2.4	1.42 ± 0.56	-
Ωar	2.98 ± 1.6	0.98 ± 0.52	-

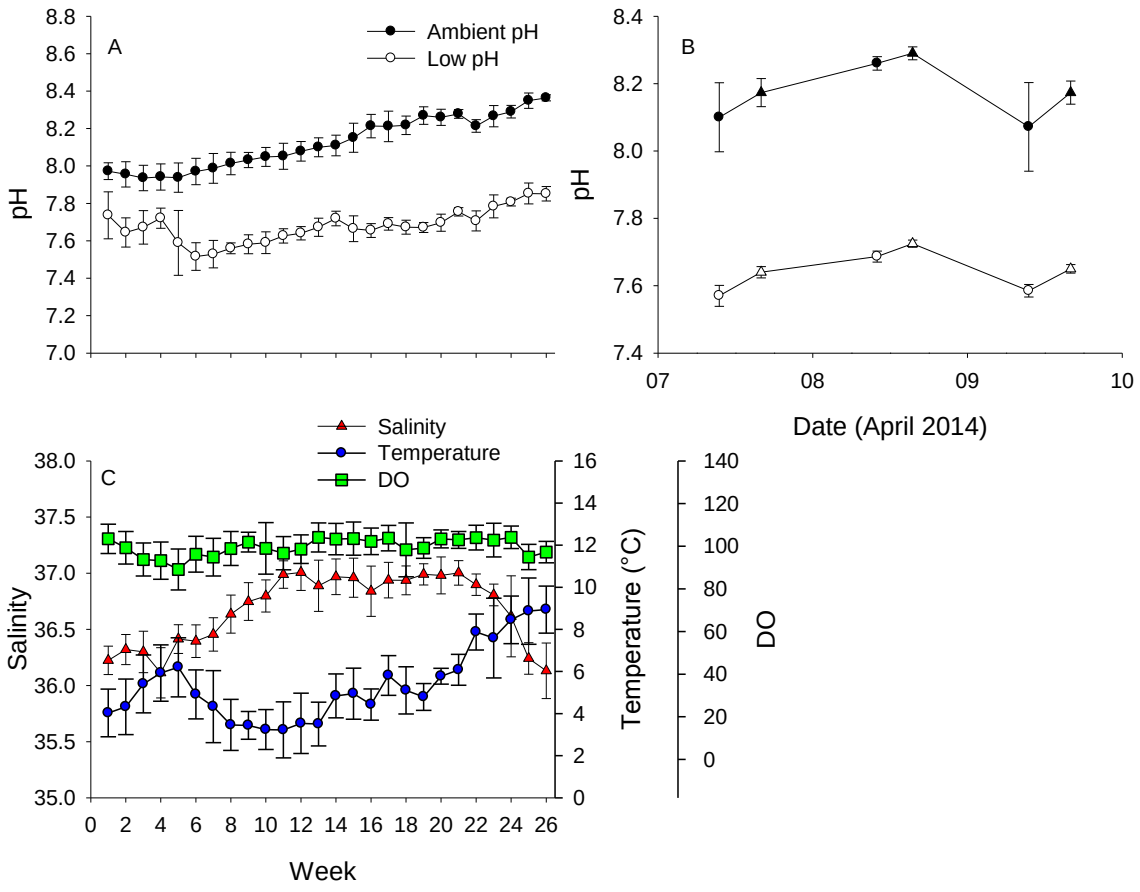

Figure 3-1: Water chemistry over the experimental period (Dec 26, 2013- June 25, 2014). A) pH B) Daily fluctuations shown over 3 days within the same month (circles show morning values and triangles show afternoon values). C) Temperature, salinity and dissolved oxygen over the experimental period. Data in A, B and C shown as weekly/daily mean ± SD (n=2-4).

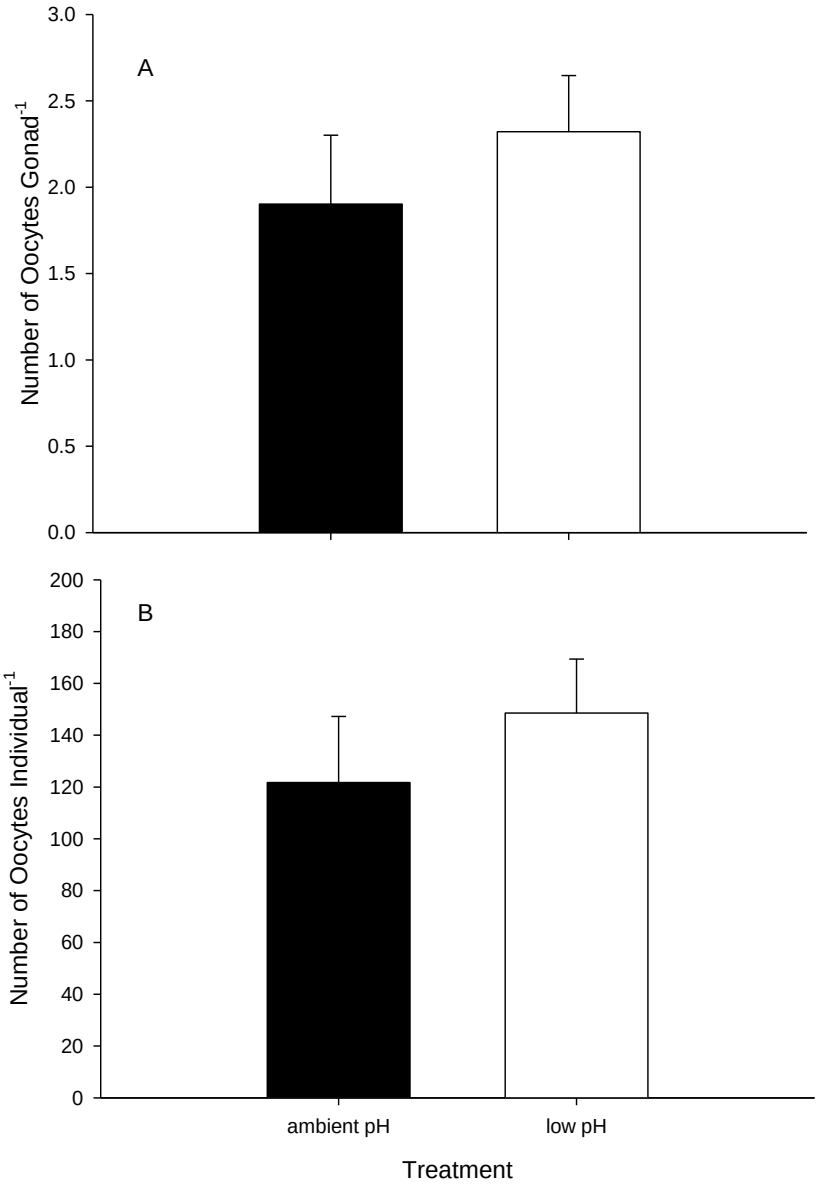

Figure 3-2: Comparison of potential fecundity from histological examination of *Ophryotrocha* sp. exposed to ambient and low pH treatments. A) Number of oocytes per gonad. B) Total number of oocytes per individual. Data shown as mean ± SD (n=5) at T6 (February 8, 2014).

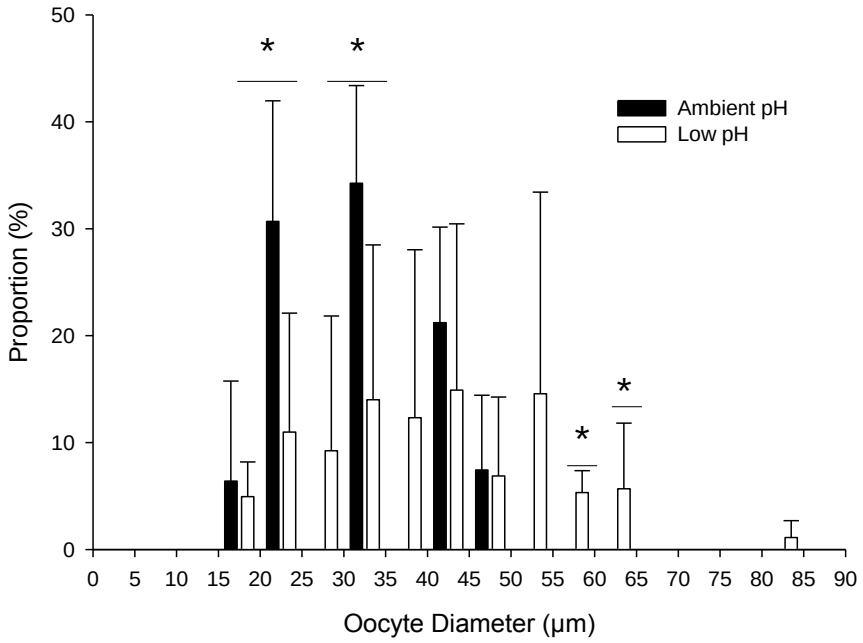

Figure 3-3: Oocyte size frequency distributions of *Ophryotrocha* sp. from histological examination at T6 (February 8, 2014). Data shown as mean ± SD (n=5). Asterisks (*) identify statistically significant differences between treatments for that oocyte size; refer to text for statistical results.

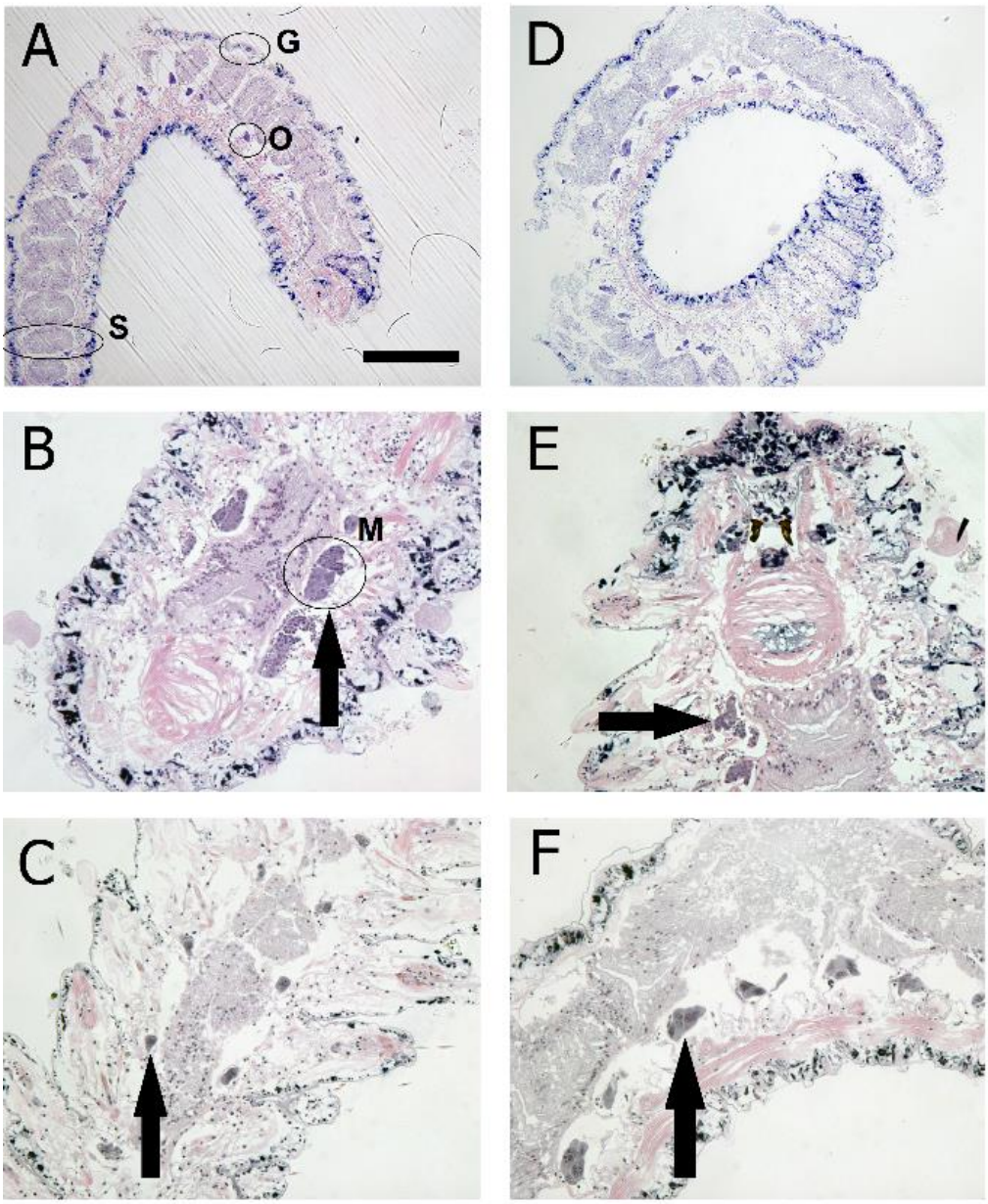

Figure 3-4: Longitudinal section of whole *Ophryotrocha* sp. A-C) Individuals from ambient pH. D-F). Individuals from low pH. o: oocyte, g: individual gonad, s: individual segment and m: male gametes. Arrows in B and E highlight the difference in number of spermatozoa produced. Arrows in C and F highlight the difference in the number of oocytes per gonad. Scale bar in A represent 7 µm and applies to all panels.

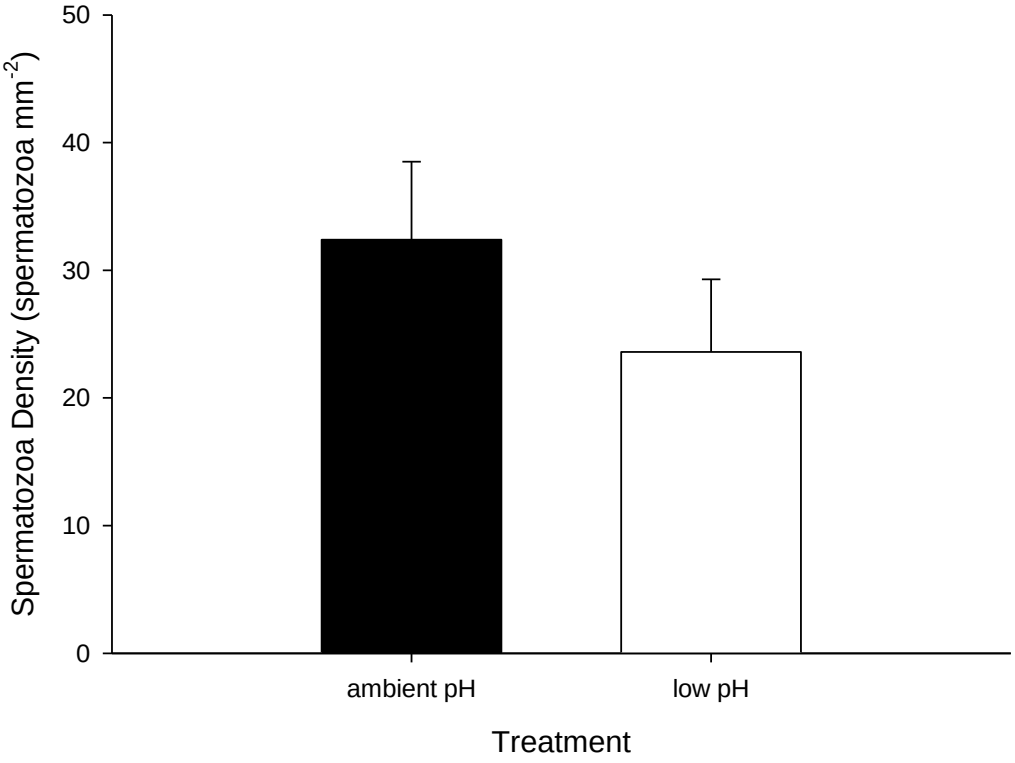

Figure 3-5: Number of spermatozoa produced by *Ophryotrocha* sp. at T6 (February 8, 2014), showing a statistical difference between ambient and low pH (refer to text for statistical results). Data shown as mean ± SD (n=5).

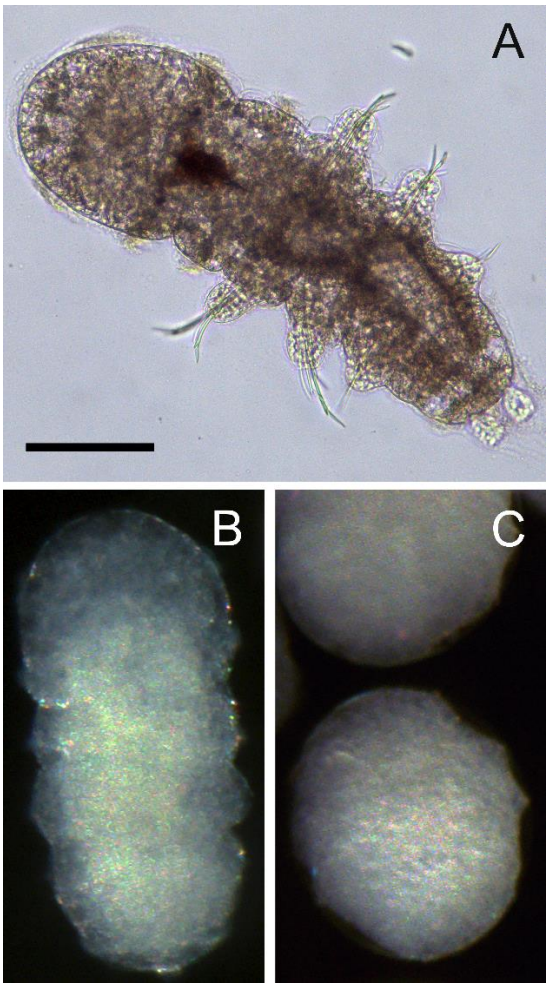

Figure 3-6: Comparison of larval development in *Ophryotrocha* sp. exposed to ambient or low pH. A) 3-chaetiger stage, 33 days, ambient pH. B) Segmented stage, 27 days, low pH. C) Trochophore, 27 days, low pH. Average water temperature during development was between 3 and 5 °C. Scale bar in A represents 13 µm and applies to all panels.

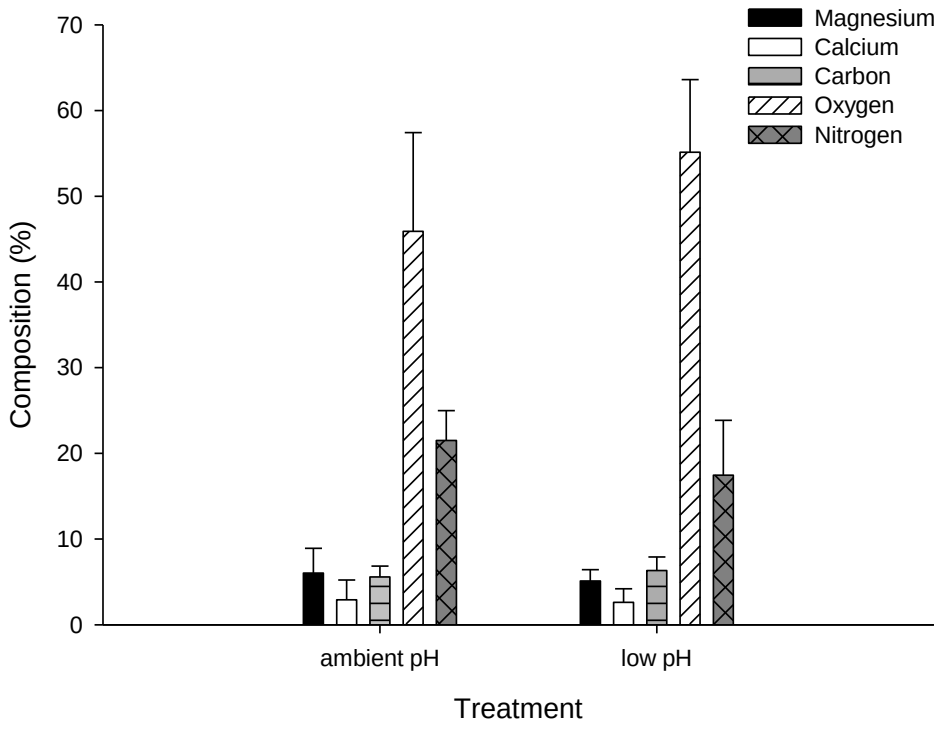

Figure 3-7: Elemental composition of chaetae in *Ophryotrocha* sp. at T26 after exposure to ambient or low pH. Data shown as mean ± SD (n=8-10).

Chapter 4: General Conclusions

4.1 Thesis summary

In this thesis, I provide information on the potential impact of predicted near future ocean acidification on reproductive output, development and other biological processes in the cold-water, lecithotrophic holothuroid *Cucumaria frondosa* and the hermaphroditic deep-sea polychaete *Ophryotrocha* sp.

In Chapter 2, I show that ocean acidification (OA) conditions elicited negative impacts on gametogenesis, fecundity, spawning and development in *C. frondosa*. Gonad index, gamete density and MSI indicate that a lower number of mature gametes were produced and released under OA. These impacts on gametogenesis are compounded by discrepancies in oocyte/embryo buoyancy, morphology and development, ultimately causing 100% mortality before the blastula stage. Discrepancies in the microstructural appearance of skeletal ossicles suggests reabsorption/dissolution. Furthermore, the lipid content and fatty acid signatures of muscle bands and gonads are also modified under low pH (high ρCO_2).

In Chapter 3, I outline the effects of a realistic OA scenario on the same processes in the deep-sea polychaete *Ophryotrocha* sp. In this hermaphroditic species, male and female gamete production are effected differently by low pH/high ρCO_2. From oocyte size structures I determined that larger, more abundant oocytes were produced under low pH/high ρCO_2. In contrast, the production of spermatozoa decreased. Ultimately, the analysis of a single egg mass provided indications of lower effective fecundity (number of eggs released) and slower development of the embryos/larvae. In this case, microstructure of the body wall as well as the appearance and elemental composition of chaeta were not affected.

The results of these two chapters show similarities and contrasts that underscore the species-specific nature of responses to OA, as well as the widespread diversity of organisms and

environments that are susceptible to its impacts. Gamete production in both species was affected by low pH/high ρCO_2. Presumably, these effects can be linked to stress associated with changes to environmental conditions. In *C. frondosa*, this is hypothesized to be due to potential alterations in the coelomic fluid pH causing extracellular acidosis. Coelomic fluid is suggested to play a role in nutrient and hormone translocation/function in echinoderms (Ferguson 1964); therefore, if the pH is altered, such processes can be disrupted. In turn, species must find ways to compensate for acidosis, which can shift energy investments. Hamel and Mercier (1996) observed that when *C. frondosa* was exposed to adverse environmental conditions and low food availability for long periods of time, the body wall was used as an energy reserve to sustain gametogenesis. Even more energy may have been used during my study in an effort to create a buffer against the physiological stress of acidosis, with negative impacts on gamete production and calcification.

The slightly different response in the gametogenesis of *Ophryotrocha* sp. may be linked to the fact that it is a hermaphroditic rather than a dioecious species. Oocyte production was enhanced at the detriment of spermatozoa production. Little is known about the energy requirements associated with producing gametes (spermatozoa versus oocytes) in hermaphrodite polychaetes, but it is suggested that a three-way trade off between male and female gamete allocation and somatic growth exists (Aira et al. 2007). While eggs are larger than spermatozoa in size (thus thought to be more energetically costly to produce), increased sperm production has been linked to a reduction in body growth in *Ophryotrocha diadema*, indicating that spermatogenesis may be equally, if not more, costly (Sella 1990; Sella and Lorenzi 2003). Under the assumption that low pH impaired foraging and/or required additional energy expenditure to combat acidosis, fewer resources were available for overall spermatozoa production.

Alternatively, the compensation processes might have interfered with the translocation of important nutrients to the gonads. Irrespective of the underlying mechanisms, *Ophryotrocha* sp. presumably used the limited energy/nutrients available to produce a greater quantity of larger oocytes and fewer spermatozoa. There was no trade-off between the quantity and quality of eggs produced to increase fitness under OA conditions; larger oocytes are consistent with what has been observed in the responses of other marine invertebrates when exposed to environmental stressors (Maltby 1994; Gimenez and Anger 2001).

In both species studied here, carry-over effects to subsequent life-history stages were detected, likely an outcome of the decreased energy allocated to gamete production. In the case of *C. frondosa*, a combination of altered oocyte provision (limited energy, modified lipid types) and impaired fertilization (lower spermatozoa quality) could explain these consequences of exposure to OA conditions. Negative buoyancy and oocyte deformities were observed, evoking altered lipid composition (or organization) and vitellogenesis**.** If not overcome, the marked impacts on oocyte buoyancy would have major consequences on the ability of *C. frondosa* to reproduce successfully. Negative buoyancy likely drove the differences in percent survival of embryos, as most propagules in the low pH treatment suffocated in the sediments.

In *Ophryotrocha* sp., based on the examination of a single egg mass, slightly different carry-over effects on development were detected under low pH/high ρCO_2, chiefly on effective fecundity (number of eggs laid) which showed signs of being reduced, as well as signs of delayed development tempo, both of which are just potential trends at this point. The fact that the greater quantity of larger oocytes produced under low pH during pre-spawning may not translate into increased effective fecundity and larval performance is both intriguing and preoccupying. It emphasizes the importance of measuring several indicators of reproductive output to get a clear

picture of the impacts of OA. In this case, impaired gamete release and/or fertilization may have voided the greater potential fecundity (possibly through sperm limitation) and higher environmental pH may have disturbed the development. In addition, the gelatinous egg mass produced in this species may have been affected by low pH/high ρCO_2. As with all processes, this requires energy; with energy allocations being altered, the size and or quality of this protective matrix may have been impacted. If so, this could have resulted in a decrease in the number of embryos able to develop. Reports have suggested that under OA conditions, larval development may be more energetically costly (Campbell et al. 2014) due to impeding processes such as ion regulation (Stumpp et al. 2011), compensation mechanisms (Thomsen and Melzner 2010), and maintaining homeostasis (Lannig et al. 2010). Therefore, while parental investment into larger, potentially better provisioned oocytes appeared to have occurred, OA may still have elicited problems that gametes and larvae were not energetically equipped to overcome. Importantly, the trade-off between a greater quantity of larger oocytes and fewer spermatozoa produced under low p/high ρCO_2H in *Ophryotrocha* sp. may not necessarily translate into decreased fitness. Only longer transgenerational studies will determine whether this strategy would persist and, if so, whether it would ultimately help the species maintain reproductive success.

Differences in the effects of OA on the hardened structures of the two species are likely related to their respective composition. Ossicles in *C. frondosa* showed signs of dissolution most likely consistent with reabsorbing/dissolving these skeletal elements to create a bicarbonate buffer to compensate extracellular acidosis. On the other hand, while low pH was hypothesized to effect the hardened structures of *Ophryotrocha* sp., the lack of visible impact may be related to the composition of chaetae, which mainly include sclerotized chitin, a polysaccharide-based

substance (Briggs and Kear 1993). This indicates that the formation of chaetae does not depend on the amount of available calcium carbonate as much as the ossicles of *C. frondosa*, which are composed entirely of highly soluble high-magnesium calcite (Morse et al. 2007). However, it should be noted that since the experiments were performed at ambient pressure, they did not account for the difference in carbonate mineral solubility found in the native deep-sea habitat of *Ophryotrocha* sp. As pressure increases, carbonate minerals (e.g. calcite, aragonite) become less soluble and even more difficult to deposit under the combined effect of OA (Guinotte et al. 2006). If *Ophryotrocha* sp. does incorporate any of these minerals as part of the sclerotizing process, this process may become impacted at native depths.

Overall, the results of my research suggest that cascading effects may be expected to occur in the ecosystems that harbour the focal species under near-future OA conditions. *C. frondosa* is a major component of arctic, subarctic and temperate benthic communities. It is a primary consumer that aids in nutrient cycling and is a food source to many predators, including marine mammals, fishes and predatory invertebrates. Moreover, it is the target of a growing fishery in the North Atlantic (Hamel and Mercier 2008b). Despite being widely distributed in the North Atlantic and Arctic oceans, including in areas that are affected by freshwater runoff and drastic environmental changes (Hamel and Mercier 2008a, b), *C. frondosa* was adversely impacted by a realistic pH decrease under otherwise natural and benign conditions. Impacts on reproductive success imply that population decline is a concern. This could jeopardize normal recruitment, generating ecological and commercial losses. Similarly, the impact of pH/ρCO_2 on the reproductive output of the deep-sea polychaete *Ophryotrocha* sp. suggests that it may not be able to maintain normal population dynamics under predicted ocean acidification scenarios. The hypothesis that species from the deep sea may be more readily susceptible to OA, as suggested

previously (Barry et al. 2013), was not entirely supported by the present study. While *Ophryotrocha* sp. did experience impacts from exposure to low pH/high ρCO_2, the shallow-water (subtidal) species *C. frondosa* also exhibited effects that appear to be equally or more drastic and concerning for the future generations of that species. In addition, the hypothesis that lecithotrophs should be more robust to OA was not supported by this study. Despite the fact that both species produce non-feeding larvae, they exhibited impaired gametogenesis and potential carry-over effects to subsequent life stages. This emphasizes the importance of preforming more studies on lecithotrophic species that would expose adults during gametogenesis (rather than just exposing the larvae themselves), as maternal investment is key to overall larval success.

4.2 Future directions

This study highlights the importance of diversifying OA research by exemplifying the species-specific nature of the responses. Because of this, making predictions becomes difficult and thus it is important to deploy further efforts to understand what marine organisms may face in a changing ocean environment. Ideally, to gain the most realistic understanding of the effects of OA on both species presented here, longer term studies over multiple generations would be necessary to determine the true consequences (negative or positive) of the observed carry-over effects. The current study has also highlighted the importance of knowing the biology of the target species biology in order to choose appropriate sampling times to avoid missing any underlying effects. As research on OA moves forward to assess realistic impacts, as close to real-life scenarios as experimentally possible, attention to experimental designs and choice of bioindicators will likely become instrumental. Here, the use of a flow-through system allowed for natural fluctuations in water chemistry parameters, providing a more realistic environment in which to study the effects of OA. This being said, as global climate change not only involves

OA, experimental trials will benefit from considering the combined effects of OA and ocean warming (see review by Byrne 2011). The two environmental factors could have interactive effects (i.e. additive, synergistic or antagonistic), possibly decreasing the chances of acclimation. However, combining temperature and OA in long-term realistic studies of species that already experience marked seasonal temperatures fluctuations (e.g. from -1 to ≥ 15°C in the case of *Cucumaria frondosa* and co-occurring subarctic taxa) will be a challenge. Furthermore, to gain an even better understanding of the potential differences between lecithotrophs and planktotrophs, species with a similar biology but differing development modes might be contrasted.

Future research with *C. frondosa* may take into account additional indicators. Major reduction in rate of fertilized eggs and cleavage have been reported in several echinoderms, including lecithotrophic species (Byrne et al. 2009; Havenhand et al. 2008; Kurihara and Shirayama 2004). In the sea cucumber *Holothuria* sp. a decrease in percent motile sperm occurred when exposed to low pH (Morita et al. 2014). Therefore, investigating fertilization success, sperm quality, and larval development until settlement in *C. frondosa* could provide insight into additional carry-over effects not immediately visible here. In addition, weight of ossicles could be analyzed as another means to compare effects on ossicle abundance and porosity under OA conditions.

As in any experimental study of deep-sea organisms, there were some limitations that should be addressed as follow-ups to the present study. Based on the size of the oocytes measured here, polychaetes from both treatments were in the first two weeks of gametogenesis in early February, whereas they should have been closer to mature stages (140-160 µm) based on previous results (Mercier et al. 2014). The overall number of egg masses produced in both

treatment conditions was lower than in non-experimental individuals of *Ophryotrocha* sp. held concurrently in the same laboratory. These differences may be due to the smaller holding containers (density effects) and the higher frequency of monitoring associated with the experimental setup (level of disturbance). While this does not have any bearing on the findings, since control and exposed polychaetes were treated (and were affected) similarly, I highlight this to propose that future studies should ideally adapt container size and design to facilitate monitoring without disruption. Future research should also seek to examine longer term exposure to elucidate the temporary or permanent nature of the potentially negative effects observed. In addition, newly collected polychaetes could be tested under native pressure, where possible. Since differences in the amount of spermatozoa produced were observed in the present study, future studies might look at fertilization success and sperm motility, which were determined to be reduced by low pH in other species of polychaetes (Campbell et al. 2014; Schlegel et al. 2014).

4.3 References

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