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Carry-over effects of ocean acidification in a cold-water lecithotrophic holothuroid

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ABSTRACT: Ocean acidification (OA) due to anthropogenic CO_2 emissions is predicted to affect the world's oceans in the near future. To date, knowledge remains limited on the response of species that are weakly calcified, produce lecithotrophic (non-feeding) larvae and live in polar/subpolar environments. The cold-water sea cucumber *Cucumaria frondosa* represents an ideal model for such investigations. 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 yr. The present study examined 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 wk using a fully open flow-through design allowing for natural fluctuations in pH, light, temperature, salinity and nutrient levels. There were 2 treatment groups, ambient pH (7.9–8.2; pCO_2 430–470 µatm) and low pH (7.5–7.7; pCO_2 1330–1530 µatm). Results indicate that low pH/high pCO_2 interferes with gamete synthesis, leading to discrepancies in oocyte/embryo buoyancy and morphology and developmental tempo, translating into 100% mortality before the blastula stage. Differences in the microstructural appearance of ossicles and the lipid contents of muscles, gonads and spawned oocytes were also highlighted. 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.

KEY WORDS: Ocean acidification · pH · Sea cucumber · North Atlantic · Reproduction · Lecithotrophic development · Polar environments

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 & Wickett 2003). The overall effect of this equilibrium shift is the depletion of available carbonate and the dissolution of deposited forms of carbonate. Reports have predicted a ~0.4 unit decrease in pH by 2100, which could have widespread impacts on marine life (Caldeira & 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 (Micael et al. 2009). Most echinoderms utilize high-magnesium calcite to form skeletal ossicles, and thus are hypothesized to be severely impacted by OA (McClintock et al. 2011). However, research thus far indicates that the impact of OA on echinoderms is species and life-stage specific. While studies have

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suggested that the early life stages may be more susceptible to OA than adults (reviewed by Byrne 2011), the available evidence remains ambiguous (Orr et al. 2005, Guinotte & Fabry 2008).

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 & Pörtner 2013). Although these experiments provide valuable insights, they do not necessarily reflect representative scenarios, as recently highlighted by Uthicke et al. (2016). 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 (reviewed by Ross et al. 2016). 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) at the community and ecosystem levels (see reviews by Dupont & Pörtner 2013, Ross et al. 2016). 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 dissolved organic matter 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. Siikavuopio et al. 2007, Stumpp et al. 2011, 2012, Catarino et al. 2012, Dupont & Thorndyke 2012, Kurihara et al. 2013, Moulin et al. 2014, 2015, Uthicke et al. 2014, 2016, Yuan et al. 2016). One of the main issues associated with OA is hypercapnia (CO_2 accumulation in the internal fluids) leading to acidosis (Collard et al. 2013), which can interfere with calcification, nutrition and metabolism (Pörtner 2008). 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 (reviewed by Dupont & Pörtner 2013). Species with different life-history strategies must also be investigated. For instance, although an estimated 68% of echinoderms produce non-feeding lecithotrophic (yolk-sustained) larvae (Uthicke et al. 2009), only a handful of studies have examined the effects of OA on species with this development mode. Moreover, these studies 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. 2010, 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 been the subject of only a few OA studies so far (Yuan et al. 2016). In one study, *Holothuria* sp. showed a decrease in percent motile spermatozoa with decreasing pH (Morita et al. 2010). Moreover, acid-base physiology in the sea cucumbers *H. scabra* and *H. parva* was negatively impacted during short-term exposure (6 to 12 d) to low pH, causing extracellular acidosis of the coelomic fluid (Collard et al. 2014). In *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). Furthermore, a reduced growth rate with decreasing pH was documented in small adults of the same species (Yuan et al. 2016). At another level, sea cucumbers may help offset ocean acidification. Studies on the species *Stichopus herrmanni* and *H. leucospilota* on the Great Barrier Reef showed that when calcium carbonate ($CaCO_3$) is dissolved through digestion, sea cucumbers have the ability to aid in 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 & Mercier 1996a,b). It has been commercially exploited in the North Atlantic for over 35 yr and has a growing commercial market (Hamel & Mercier 2008a, Nelson et al. 2012). Its gametogenesis, spawning, embryonic and larval development, settlement, growth, and feeding habits have all been studied extensively (Hamel & Mercier 1996b,c, 1998, 1999, Singh et al. 1998, So et al. 2010, 2011). Because its biomass may reach millions of tons in some locations of eastern Canada (Hamel & Mercier 2008a), population declines due to shifting environmental conditions, including OA, could have cascading im-

pacts on fundamental ecosystem functions (Hamel & Mercier 1998) and associated economic activities (Nelson et al. 2012).

The purpose of the present study was to examine the effects of predicted near-future ocean acidification, namely a decrease in pH by ~0.4 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 (1) overall health, (2) lipid synthesis and (3) the abundance, microstructural composition and shape of ossicles were analysed. The study explored potential transgenerational responses under naturally fluctuating temperature, light, phytoplankton abundance, salinity and pH, true to nearby oceanic conditions where extensive populations of *C. frondosa* are found. A transgenerational response, or transgenerational plasticity (TGP), is a non-genetic carry-over effect defined as phenotypic change in offspring in response to the environmental stress experienced by the parents, especially during gamete development (Ross et al. 2016). Such responses can occur over rapid time scales because they do not involve genetic modification, unlike adaptation, which implies increased fitness through heritable genetic traits. Nevertheless, determining immediate short-term effects will hopefully aid in developing a mechanistic understanding of adaptation and locating areas of high conservation concern that may incur biodiversity loss under near-future ocean conditions.

MATERIALS AND METHODS

Specimen collection

Adults of *Cucumaria frondosa* were collected in December 2013 by scuba divers in Admiral's Cove (47.0969° N, 52.9092° W), southeastern Newfoundland, from a depth of 15–20 m (water temperature 3 to 4°C). Sea cucumbers were kept in the laboratory for less than 1 mo before the study began. Only healthy individuals (attached firmly to the substrate, feeding, exhibiting proper coloration and undamaged body wall) were selected.

Experimental setup

Sea cucumbers were experimentally tested in the laboratory using a continuously flowing system of unfiltered seawater pumped directly from the ocean for a period of 19 wk, from December 26, 2013, to May 7, 2014. The beginning of the trial immediately preceded the initiation of gametogenesis in *C. frondosa* (Hamel & Mercier 1996b) and the study encompassed the periods of gamete synthesis and spawning (Hamel & Mercier 1996b, Mercier & 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 3 individuals, i.e. a total of 24 sea cucumbers per treatment. At the onset of the experiment, the immersed weight (measured while the animal is suspended underwater) and contracted length (from mouth to anus) of each sea cucumber were recorded. Average total wet weight (±SD) was 148.2 ± 30.8 g in ambient pH and 150.8 ± 38.1 g in low pH, and length varied from 10 to 14 cm in both treatments.

To incorporate natural carbonate chemistry variability (Reum et al. 2014), the experimental system used in the present study was fully open, unlike most setups described previously as 'flow through' (Cornwall & Hurd 2016), while designed to achieve a suitable level of independence among experimental units. A common source of running seawater was continuously fed to all the experimental tanks (no storage tank was used), either directly (ambient pH) or via secondary in-line treatment (low pH). The latter consisted of electronically-controlled injection of CO_2, via a CO_2 regulator (Milwaukee Instruments MA957) and reactor (AquaMedic CO_2 Reactor 500), adjusted to −0.4 units relative to ambient pH, based on near-future predictions for global and northern oceans (Meehl et al. 2007, Denman et al. 2011). The supply of both ambient and acidified seawater was provided through intermediate header tanks (40 l), in and out of which seawater flowed at a rate of ~150 l h^{-1}, and was continuously fed to each experimental tanks (~7.2 l h^{-1}). The intermediate header tanks had flow rates that were 700% those of the experimental tank and they had no biological activity in them, thus minimizing any header effects relative to effects in experimental units. All individuals were initially acclimated to the multi-tank system at ambient pH (no secondary treatment) for 1 wk before gradually switching over to automatically-adjusted pH for the low-pH tanks, allowing the animals to acclimate to the decreasing pH over a period of ~12 h.

Water temperature followed local conditions; flow rates kept temperature below 10°C, mimicking conditions in the natural habitat of *C. frondosa*. Photoperiod corresponded to natural sunrise/sunset hours with maximum light intensity ~150 lx (spring condi-

tions). The setup allowed for natural fluctuations in both ambient and experimental pH levels, following 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 food supplement (to standardize food availability between treatments) in the form of phytoplankton cells (Phyto-Feast® Live, Reef Nutrition) composed of *Pavlova, Isochrysis, Thalassiosira weissflogii, Tetraselmis, Nannochloropsis,* and *Synechococcus* (~25 000 cells ml^{-1}) 3 mornings a week for the duration of the trial. Phytoplankton is known to dominate the diet of *C. frondosa* in nature (Hamel & 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 & Hamel 2010).

Water chemistry monitoring

The physical and chemical parameters of the water (pH, temperature, salinity, and dissolved oxygen [DO]) were monitored in each tank twice daily (morning and evening) using a YSI 556 MPS multiprobe. Total alkalinity (TA) was measured twice a week in 4 randomly chosen tanks (2 from ambient and 2 from low pH conditions) using a TA test kit (Orion; accuracy ± 50 µmol kg^{-1}). Temperature, salinity, pH_{NIST}, and TA were used to estimate pCO_2, saturation state of aragonite (Ω_{arag}) and saturation state of calcite (Ω_{ca}) using CO2SYS software (Lewis & 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 2 h in 1 randomly selected tank per treatment.

Behavioural monitoring

Overall health and behaviour of sea cucumbers were recorded on a weekly basis. 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 2 tentacles with a sterile pipette. The retraction and eventual re-extension of the tentacles post-contact was recorded for 2 min using an Olympus Stylus TG-2 digital camera. Videos were later analysed and compared between the 2 groups (n = 4 to 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. As with many other species of holothuroids, cloacal opening (cloacal respiration) rates will increase as an indicator of stress (Gianasi et al. 2015). Rates were determined by counting the number of cloacal openings within a 2 min period in all individuals where the cloaca was visible without disturbing the animal (n = 7 to 18 individuals per treatment per week).

Sample collection

Three sampling periods were established during the experiment. At the onset of the trial (T_0), a subsample (n = 22) of the initial sea cucumber population was analysed to determine their baseline status. Subsequently, 4 out of 8 tanks per treatment were randomly sampled after 10 wk, during the pre-spawning period (T_{10}; March 11 and 12, 2014; n = 11 to 12 individuals per treatment), and the remainder were sampled at the end of the trial, after 19 wk during the post-spawning period (T_{19}; May 6 and 7, 2014; n = 11 to 12 individuals). At T_{10} and T_{19}, sea cucumbers were photographed and their wet weight and contracted 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, 2 sets of 3 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 & Mercier 1996a). One set of 3 tubules was preserved in 4% formaldehyde for a histological study of gametogenesis (at T_{19} only) and the other group was used to determine fecundity at both time points. Additionally, at T_{10} and T_{19}, 3 gonad tubules and the muscle bands from each individual (n = 11 to 12 per tissue type; n = 3 individuals per sex per treatment) were wrapped in aluminum foil (previously heated to 450°C for 5 h), and kept at −80°C for a maximum of 3 mo before lipid analysis (see method below).

Sample analysis

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 gamete development, 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 to minimize fluctuations (Hamel & Mercier 1996a). (2) Oocyte abundance and size (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 T_{19} were used. Gonads were processed using standard methods (Havenhand & Schlegel 2009), i.e. dehydrated using a series of ethanol and xylene baths. 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 within the field of view were analysed with the software ImageJ (n = 9 sections per individual). (3) Histological sections were also used to assign a qualitative stage of oogenesis to females (Hamel & Mercier 1996a) based on oocyte size and number of phagocytized cells. (4) Finally, a quantitative maturity stage index (MSI) was ascribed to each female (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:

$$\text{MSI} = \frac{}{(\text{oocyte density}) \times (\text{oocyte diameter}) \times \text{CV} \times 0.001} \quad (1)$$

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

Fecundity

To assess differences in gamete abundances (in males and females), 3 gonad tubules from each individual (n = 11 to 12 individuals per treatment per sampling period) were weighed and their length was measured (see fecundity metrics described below). A 1-cm segment of each tubule was isolated from the tip of the tubule and its gamete contents were collected by gently squeezing down the length of the tubule (tubules were thoroughly examined for residual gametes). The weight of the empty tubule and its contents (male or female gametes) were recorded separately. The gametes were preserved in 20% ethanol in filtered seawater (15 ml). For female samples, the total number of oocytes was calculated and size (Feret diameter) was measured. For males, a 10 µl aliquot per tubule (n = 3 tubules per individual) was placed in a hematocytometer to count 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 immediately before spawning (T_{10}). Total fecundity was extrapolated for the whole gonad.

Spawning and development

C. frondosa is a broadcast-spawning species that releases large bright-orange lecithotrophic oocytes (Hamel & Mercier 1996b). For the duration of the spawning period, the intensity of any gamete release was monitored several times a day. Upon sighting of oocytes, a sample was taken to determine if they had been fertilized (through elevated fertilization envelop or cleavage). At the end of the spawning, 3 water samples of 10 ml were taken to estimate the number and 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 relative to the total number of oocytes per subsample. This procedure was repeated every 2 h for the first 24 h to determine developmental kinetics. Embryos/larvae were then assessed twice a day. Development was monitored under the respective pH conditions in which spawning occurred. A new stage was scored when ~50% of the propagules reached it (Hamel & Mercier 1996b). Development was assessed and pictures were taken using the previously described equipment. Subsamples of oocytes (100 to 300 depending on the total released)

from spawning events (n = 2 in each treatment) were preserved for lipid analysis using the aforementioned methods.

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 treatments (as per sampling described above), following previous work (Sun et al. 2012). A modified Folch procedure was used to extract lipids (Folch et al. 1957, Parrish 1999), and total lipid classes were determined using a series of developing and conditioning sequences routinely used for the separation of aquatic lipid classes (Parrish 1987). Fatty acids were extracted using a fatty acid methyl ester (FAME) derivatization. The detailed protocol can be found in the in the Supplement at www.int-res.com/articles/suppl/m557p189_supp.pdf.

Ossicles

To determine whether calcification of *C. frondosa* was affected by low pH, frozen body wall samples were thawed and cut into 3 regions (anterior, middle and posterior), 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) as the number of ossicles from all regions divided by total wet body wall weight. Three subsamples of ossicles from each region were rinsed 3 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 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 (860× to 20 000×). 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 analysed. Elements present were determined using the Phenom ProSuite elemental identification (EID) software.

Statistical analysis

The effects of time (T_{10}, T_{19}) and pH treatment (ambient, low) on the 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, in cases where the assumption of equal variance was violated. The latter tests were also used to compare the proportions of phagocytized and vitellogenic oocytes between the 2 pH treatments at T_{19}, the frequency of cloacal movement between treatments at various time points and the total number and elemental composition of ossicles between treatments. These analyses were performed in Sigmaplot software (v. 11.0; Systat). In addition to *t*-tests (or Mann-Whitney *U*-test), lipid data were also compared using semi-parametric permutation multivariate analyses of variance (PERMANOVA), similarity percentage (SIMPER) and multidimensional scaling (MDS). Criteria for inclusion of various lipids and fatty acids was determined by a correlation set at 0.8 to 0.9 in the software Primer version 6.1.16 (Primer-E). Statistical significance for all tests was set at $p < 0.05$.

RESULTS

Water chemistry

Average daily pH measurements showed that a 0.41 ± 0.14 unit decrease relative to ambient pH was maintained during the study (Table 1). However, due to in-line acidification, pH levels in the low-pH tanks were more variable and the difference between the 2 treatments increased slightly during the study, not perfectly following the seasonal increase in ambient pH from winter to spring (Fig. 1A). Temperature and salinity in both treatments also increased towards the end of the study period, whereas dissolved oxygen was relatively constant (Fig. 1B). Calculated water parameters associated with differences in pH showed

Table 1. Experimental conditions for investigation of the effects of ocean acidification on the sea cucumber *Cucumaria frondosa*. All values are mean ± SD. Values of pH_{NIST}, temperature, salinity and dissolved oxygen (DO) were measured twice daily over the 19-wk experimental period in the 2 treatments (n = 1700). Mean daily differences between treatments are also shown for these variables. Total alkalinity values are based on weekly measurements from 2 tanks per treatment (n = 34). pCO_2 and saturation states of calcite (Ω_{ca}) and aragonite (Ω_{arag}) were calculated in CO2SYS using weekly measures of pH, total alkalinity, salinity and temperature

Parameter	Treatment		Mean daily difference
	Ambient pH	Low pH	
pH	8.03 ± 0.12	7.63 ± 0.09	0.41 ± 0.14
Temperature (°C)	4.5 ± 1.5	4.5 ± 1.3	1.0 ± 0.9
Salinity	36.6 ± 0.37	36.6 ± 0.34	0.15 ± 0.16
DO (%)	98.1 ± 9.2	98.6 ± 7.9	1.4 ± 3.2
Alkalinity (µmol kg^{-1})	2398 ± 82	2498 ± 10	–
pCO_2 (µatm)	446 ± 22	1427 ± 100	–
Ω_{ca}	2.19 ± 0.22	0.85 ± 0.06	–
Ω_{arag}	1.38 ± 0.14	0.53 ± 0.03	–

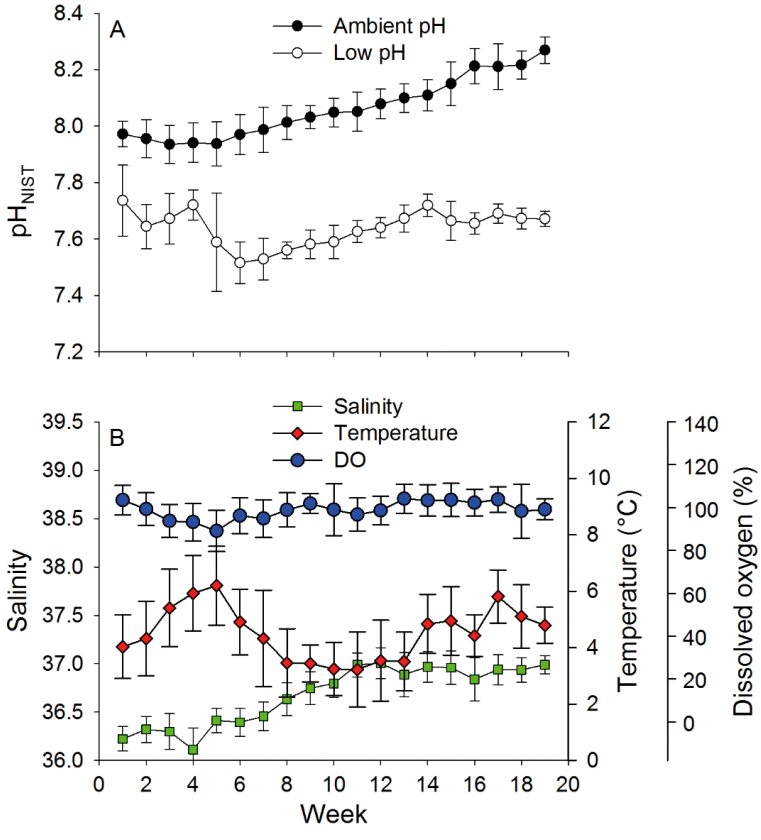

Fig. 1. Water chemistry for investigation of the effects of ocean acidification on the sea cucumber *Cucumaria frondosa* over a 19-wk experimental period (December 26, 2013, to May 7, 2014): (A) ambient and low pH_{NIST}; (B) temperature, salinity and dissolved oxygen (DO). All data are shown as weekly mean ± SD (n = 4 to 8)

that pCO_2 was approximately 3 times higher in low pH (average pCO_2 of 446 µatm in ambient pH and 1427 µatm in low pH) and that saturation states of calcite and aragonite were both higher in ambient pH (Table 1).

Gonad indices, gametogenesis and fecundity

There was a significant interaction between time and pH on the male GI ($F_{1,16}$ = 4.61, p = 0.047; Fig. 2A). Based on independent *t*-tests, there was a significant decrease in GI from T_{10} to T_{19} in ambient pH conditions (U = 0, df = 9, p = 0.004); but not in low pH (t_7 = 1.05, p = 0.327). In females, the GI (Fig. 2B) was not significantly affected by time ($F_{1,21}$ = 0.86, p = 0.366) or pH ($F_{1,21}$ = 0.001, p = 0.970).

There was a significant decrease in spermatozoa density over time (Fig. 3A) in both ambient and low pH treatments ($F_{1,17}$ = 61.53, p < 0.001) and no interaction between time and pH ($F_{1,17}$ = 2.62, p = 0.124). Similarly, there was no significant interaction between time and pH on oocyte density ($F_{1,21}$ = 0.64, p = 0.434) but there was a significant decrease in oocyte density ($F_{1,21}$ = 75.43, p < 0.001) over time (Fig. 3B). Decrease in gamete density was visually more pronounced in ambient pH in both sexes (Fig. 3A,B); as was the number of individuals (gonads) showing signs of spawning.

Gonad histology at T_{19} revealed that the density of phagocytized oocytes, an indicator of the gonad recovery stage (Fig. S1 in the Supplement), was significantly higher in ambient than in low pH (Fig. 2C; t_4 = 5.07, p = 0.007). Oocyte size frequencies based on freshly extracted material at T_{10} and T_{19}, and on histological material at T_{19}, did not show any clear pattern apart from the persistence of larger size classes under low pH (Fig. S2). Histological sections from T_{19} 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 T_{19} (Fig. 4A).

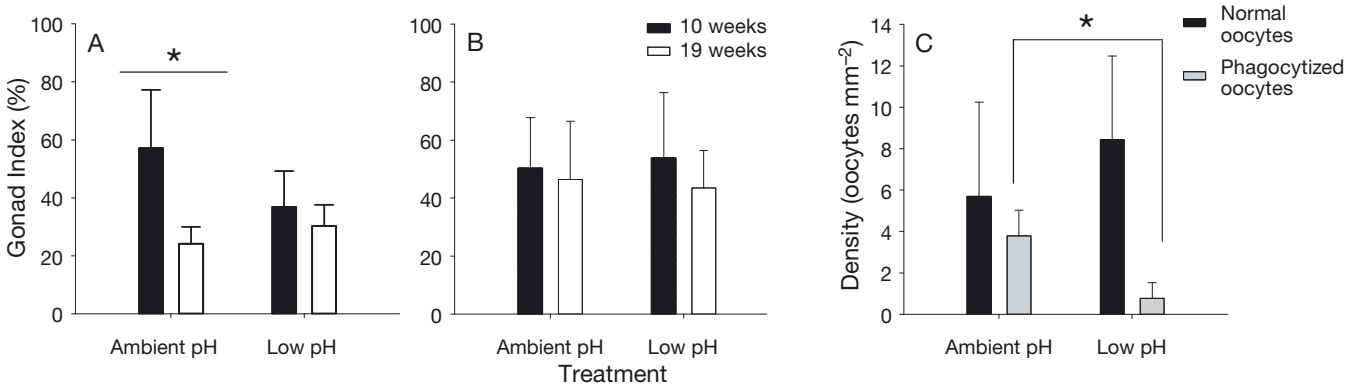

Fig. 2. *Cucumaria frondosa.* Gonad index in (A) males and (B) females at 10 wk (T_{10}; pre-spawning) and at the end of the experiment after 19 wk (T_{19}; post-spawning) under ambient and low pH treatments (n = 5 to 7); (C) number of normal and phagocytized oocytes at T_{19} in both treatments (n = 3). All data are shown as mean ± SD. The asterisk (*) in (A) identifies statistically significant differences between time points for that treatment; refer to text for statistical results. The asterisk in (C) shows statistically significant differences between treatments in the number of phagocytized oocytes; refer to text for statistical results

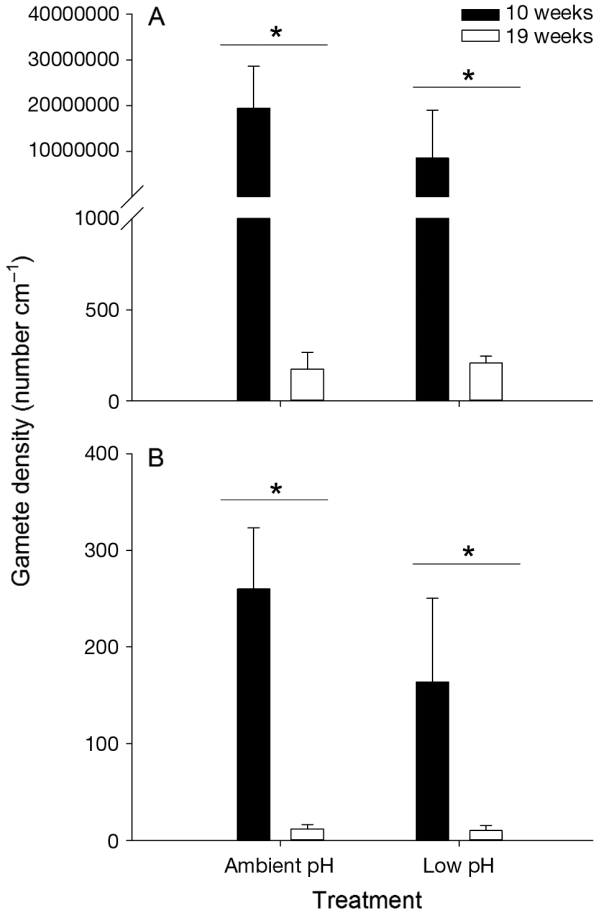

Fig. 3. *Cucumaria frondosa.* Gamete density at T_{10} and T_{19} for (A) males and (B) females (n = 5 to 7). Data are shown as mean ± SD. Asterisks (*) identify statistically significant differences between time points; refer to text for statistical results

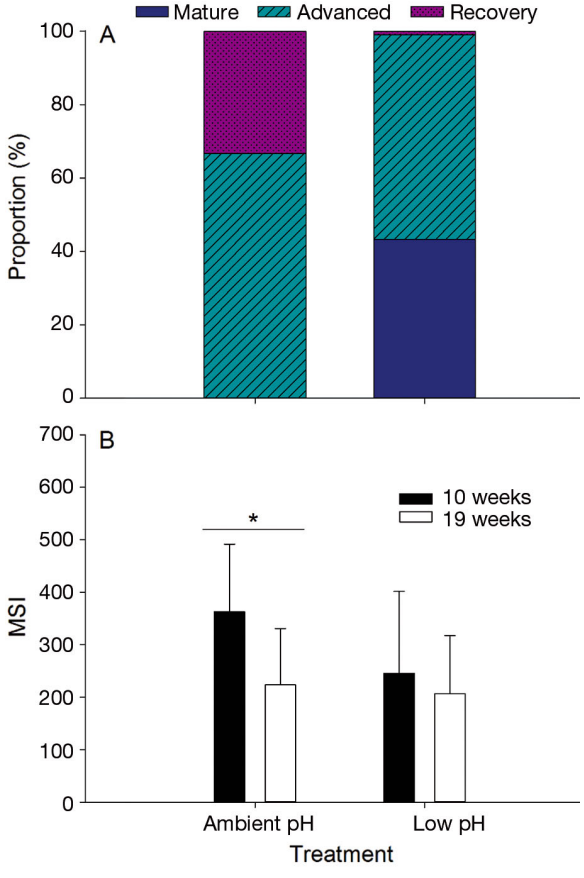

Fig. 4. *Cucumaria frondosa.* (A) Percent gonad tubules in each gametogenic stage for females at ambient and low pH (n = 3) at T_{19}. (B) Female maturity stage index (MSI) at T_{10} and T_{19}. Data in (B) are mean ± SD (n = 5 to 7). The asterisk (*) identifies statistically significant differences between time points for that treatment; refer to text for statistical results

There was no significant interaction between the effects of time and pH on the MSI ($F_{1,21}$ = 1.46, p = 0.240) but there was a significant effect of time ($F_{1,21}$ = 4.59, p = 0.044). Because the effect appeared visually stronger in the low pH group, independent *t*-tests were conducted, showing a significant difference in MSI at T_{10} compared to T_{19} in ambient pH (t_{10} = 2.28, p = 0.046) but not in low pH (t_{11} = 0.69, p = 0.506; Fig. 4B).

Spawning and development

More spawning events were detected 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 (Fig. 5A). However, nearly all the oocytes (~98%) released under low pH conditions were negatively buoyant and sank to the bottom (Fig. 5B,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 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 low pH was 0% (all oocytes were found decaying on the bottom). Eggs or embryos in ambient pH had a smooth, round shape and uniform orange color (Fig. 6A–C), whereas those released in low pH were similarly colored but irregular in shape (Fig. 6D–H). Additionally, some of the latter eggs appeared to have a rough, uneven surface and dimpled cytoplasm (Fig. 6I).

Behaviour

Rates of cloaca opening were higher in low pH for the first 4 wk but stabilized for the remainder of the experiment, with significant differences between treatments restricted to Week 1 (mean of 5.6 vs. 4.3 cloaca openings in 2 min; t_{12} = −4.63, p < 0.001) and Week 4 (5.1 vs. 4.4; t_{19} = −2.47, p = 0.023). The average tentacle reaction time across the entire 19-wk 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 = −0.19, p = 0.856) and neither was the average number of individuals actively feeding at a given time (ambient pH 4.2 ± 2.4 individuals vs. low pH 4.8 ± 2.9 individuals; t_{22} = −0.54, p = 0.592).

Lipids

PERMANOVAs revealed significant differences when comparing the lipid profiles between treatments and between tissues for T_{10} males (pseudo-$F_{1,8}$ = 2.63, p = 0.050 and pseudo-$F_{1,8}$ = 12.67, p = 0.002, between treatment and tissues respectively), T_{10} females (pseudo-$F_{1,8}$ = 6.06, p = 0.001 and pseudo-$F_{1,8}$ = 107.36, p = 0.004), T_{19} males (pseudo-$F_{1,8}$ = 2.24, p = 0.022 and pseudo-$F_{1,8}$ = 24.03, p = 0.003) and T_{19} females (pseudo-$F_{1,8}$ = 3.82, p = 0.014 and pseudo-$F_{1,8}$ = 29.91, p = 0.002). Furthermore, the SIMPER analysis showed that for both sexes and both sampling periods, muscles were 25 to 33% dissimilar, and gonads were 25 to 34% dissimilar between ambient and low pH (Fig. 7A–D). Different lipid classes and fatty acids contributed to this dissimilarity. Values were reported when contribution was greater than 5% in at least 1 treatment group and these contributors were compared between treatments using *t*-tests (Table 2).

In male muscles at T_{10}, 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). Similarly, female muscles 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 (Table 2). Tritriacylglycerol (TAG) and ai15:0 were higher in female gonads from ambient pH while 20:5ω3 was higher in low pH (Table 2).

At T_{19}, muscles 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). In male gonads, 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). Female gonads showed slightly different trends, with proportions of ai15:0 being higher in ambient pH, and proportions of sterols and 20:5ω3 higher in low pH (Table 2).

Lipid profiles of naturally spawned oocytes were 39% dissimilar between treatments. The major contributors included a higher proportion of 16:1ω7 in ambient pH and slightly higher proportions of sterols, 16:1ω5, ai15:0, and 20:5ω3 in low pH, although none were significantly different in *t*-tests (Table 2).

Ossicles

There was no significant difference between treatments in the total number of ossicles (t_8 = 1.60, p = 0.149). However, there was a difference in their

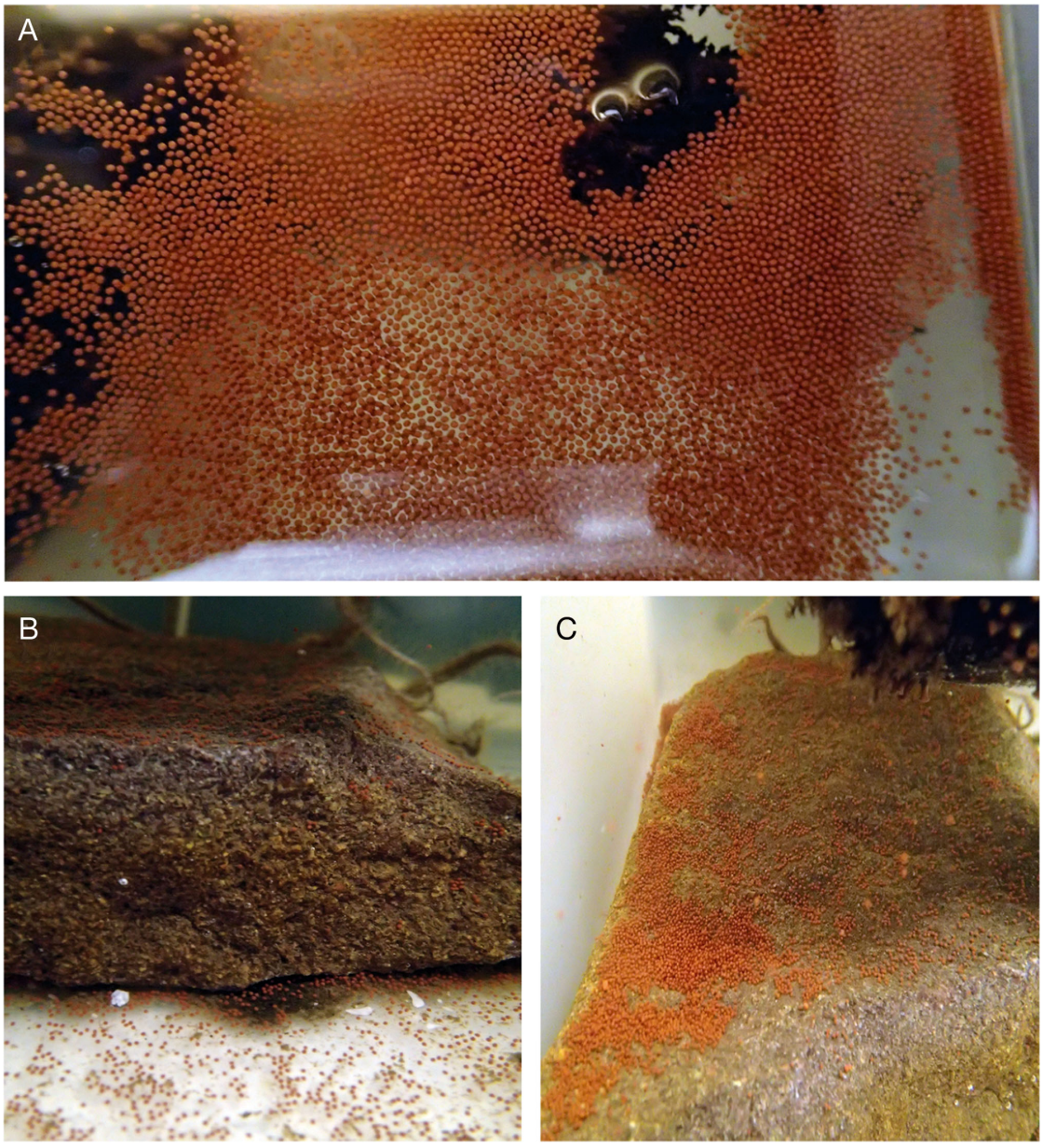

Fig. 5. *Cucumaria frondosa*. Spawning observed during the study: (A) positively buoyant oocytes released under ambient pH floated to the water surface; (B,C) negatively-buoyant oocytes sank to the bottom of the tanks under low pH conditions. The red-orange oocytes measure between 500 and 600 µm

appearance. Ossicles extracted from individuals under ambient pH showed consistently smooth and uniform surfaces (Fig. 8A–E), whereas several ossicles sampled from low pH (Fig. 8F–J) had a rougher surface (Fig. 8G,H), with the occasional occurrence of larger calcified protuberances (Fig. 8I) and/or tinted areas (Fig. 8J). Based on elemental analysis, there was no significant difference between treatments in the content of calcium (t_4 = 0.15, p = 0.889) or magnesium (U = 0, df = 4, p = 0.100) in ossicles.

DISCUSSION

The sea cucumber *Cucumaria frondosa* showed both resilience and vulnerability to predicted near future OA conditions. Several indicators, including rates of cloaca opening, tentacle deployment and feeding, and timing of spawning activity, suggest that *C. frondosa* was able to cope, at least superficially, with a ~0.3 to 0.6 decrease in pH. Importantly, the experimental setup was demonstrated to satisfy

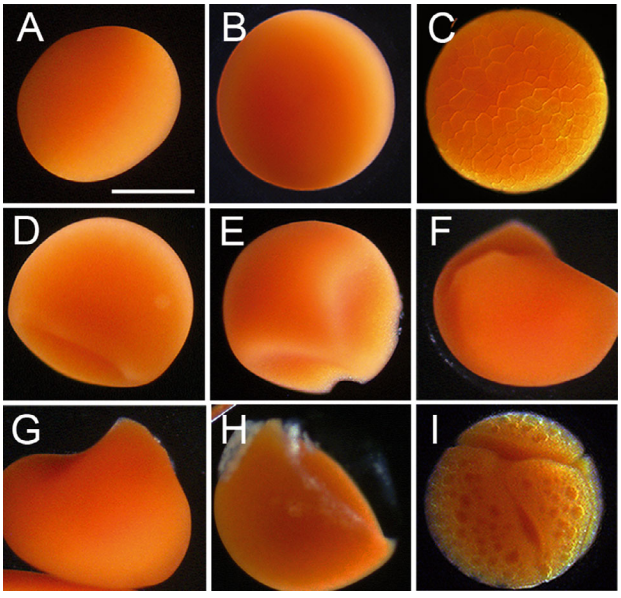

Fig. 6. Comparison of development of *Cucumaria frondosa* under ambient pH (A–C) and low pH conditions (D–I): (A) unfertilized egg; (B) newly fertilized egg; (C) late blastula; (D–H) irregularly shaped oocytes; (I) dividing oocyte showing irregular dimpled surface. Scale bar in (A) represents 300 µm and applies to all panels

the conditions necessary for successful reproduction to occur. However, finer investigations revealed preliminary evidence of calcification issues, and important impacts on gametogenesis, oocyte morphology, and embryo development and survival in individuals exposed to low pH for 4 mo. The latter findings highlight unsuspected transgenerational effects, indicating that, contrary to early assumptions from work on larvae, lecithotrophic species may not be more robust to OA than planktotrophic counterparts.

The combined analysis of GI, gamete abundance (including relative fecundity) and MSI in *C. frondosa* indicated that a decrease in pH (increase in pCO_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 T_{10} (pre-spawning) and weaker indicators of recent gamete release at T_{19} (post-spawning). Exposure of sea urchins to elevated pCO_2 caused a similar decrease in gonad development in *Strongylocentrotus droebachiensis* (Siikavuopio et al. 2007, Stumpp et al. 2012, Dupont et al. 2013) and a 1-mo delay in gonad maturation and spawning in *Hemicentrotus pulcherrimus* (Kurihara et al. 2013), al-

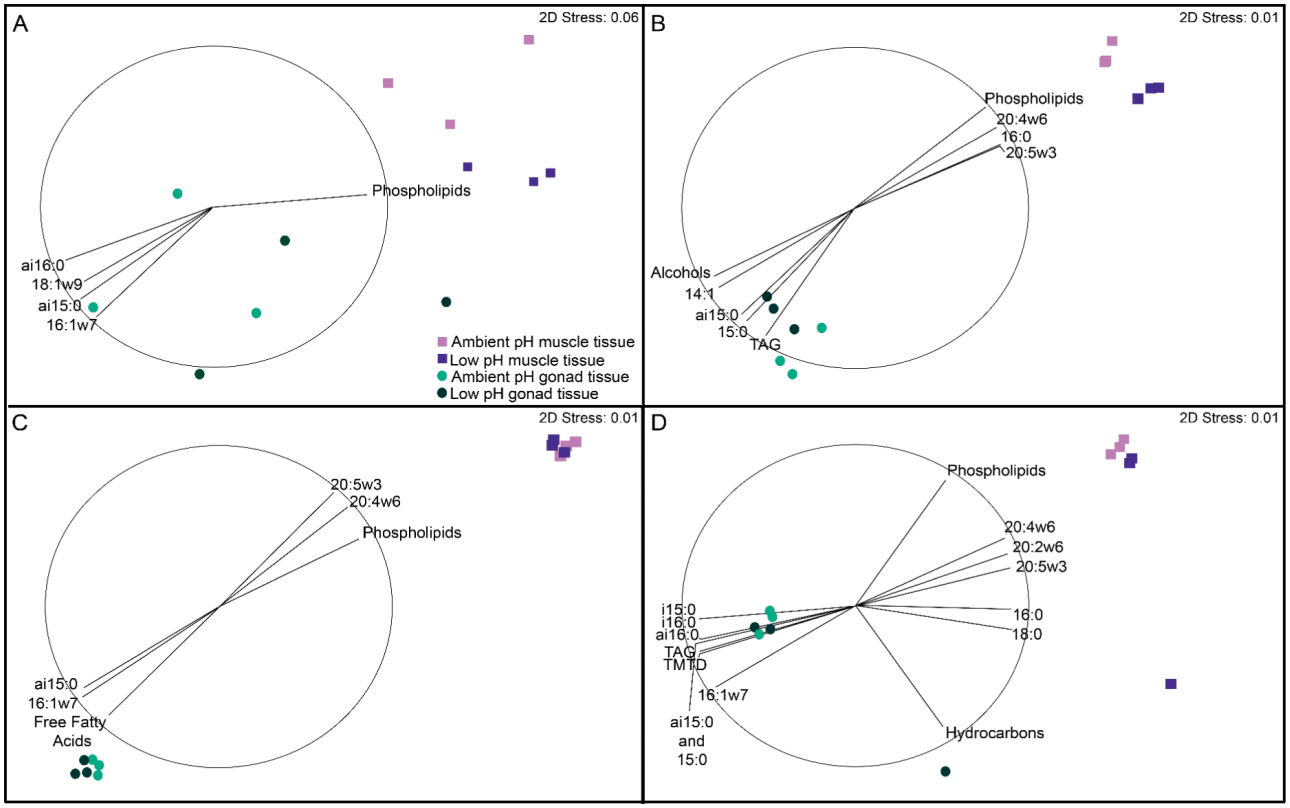

Fig. 7. *Cucumaria frondosa*. Multidimensional scaling (MDS) comparison of lipid classes and fatty acid composition in muscles and gonads of males and females (n = 3): (A) male at T_{10}, correlation set at 0.9; (B) female at T_{10}, correlation set at set at 0.9; (C) male at T_{19}, correlation set at 0.8; (D) female at T_{19}, correlation set at 0.9

Table 2. Results of exposure of sea cucumbers *Cucumaria frondosa* to low pH conditions, showing mean proportions (%) of fatty acids and lipid classes in gonads and muscles at T_{10} (after 10 wk; pre-spawning) and T_{19} (after 19 wk; post-spawning) under ambient and low pH (n = 3 per sex per treatment). Median data are shown for oocytes. P: phospholipids, S: sterols, TAG: triacylglycerol. Weak contributors were omitted (–); asterisks (*) identify significantly higher proportions in a treatment

Time point	Sample	Fatty acid/ lipid class	Female					Male				
			Ambient pH	SD	Low pH	SD	p	Ambient pH	SD	Low pH	SD	p
T_{10}	Muscle	P	66.34	7.9	68.66	4.2	0.678	58.08	16.2	61.57	13.4	0.788
		20:5ω3	33.48	2.7	47.88*	7.6	0.036	29.18	10.9	47.62*	2.7	0.047
		22:4ω6	12.36*	0.57	0	0	<0.001	12.12	11.7	0	0	0.147
		20:1ω11	6.05*	0.21	0	0	<0.001	6.84*	0.65	0	0	<0.001
		20:1ω9	0	0	6.69*	0.54	<0.001	1.13	2.0	8.80*	1.0	0.004
T_{19}	Muscle	P	62.07	9.9	40.48	31.6	0.322	68.68	19.8	53.84	7.1	0.289
		20:5ω3	34.67	2.1	46.01*	1.8	0.002	35.16	6.2	44.06	7.9	0.200
		22:4ω6	11.84*	1.572	0	0	<0.001	8.53	7.4	0	0	0.117
		20:1ω11	6.74*	0.40	0	0	<0.001	6.79*	0.31	0	0	<0.001
		20:1ω9	0	0	8.71*	0.72	<0.001	0	0	8.45	0.70	<0.001
T_{10}	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
		20:5ω3	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	–	–	–	–	–
T_{19}	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
		20:5ω3	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
		16:1ω7	–	–	–	–	–	19.48	1.1	22.89*	0.23	0.006
N/A	Ooctyes	S	9.73		43.11		0.245					
		20:5ω3	19.5		23.48		0.245					
		ai15:0	34.36		40.69		0.245					
		16:1ω7	11.96		0		0.245					
		16:1ω5	24.59		33.11		0.245					

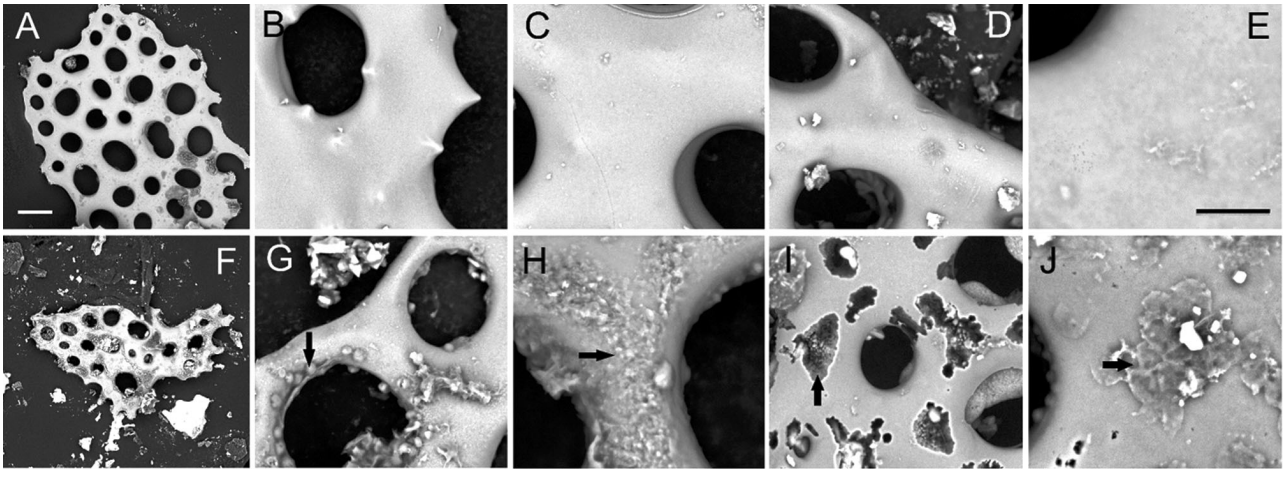

Fig. 8. Ossicles of *Cucumaria frondosa* under ambient pH (A–E), and low pH conditions (F–J). Arrows highlight abnormalities in low pH, showing (G) large calcified bumps and thinning centers, (H) an etched, texturized surface, (I) erosion of ossicle surface and (J) a texturized surface. Scale bar in (A) represents 40 µm and applies to (A) and (F); scale bar in (E) represents 5 µm and applies to all other panels

though potential experimental flaws of the latter finding have been highlighted (Moulin et al. 2015). Interestingly, *C. frondosa* individuals collected at 1200 to 1450 m depth, below their typical depth of occurrence, exhibited impaired oocyte maturation (Ross et al. 2013), indicating that natural environmental stress can reduce reproductive output in this species. Accordingly, 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 (Hamel & Mercier 1996c). A similar process was reported in the sea urchin *S. droebachiensis* where energy storages were used to mitigate 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 the field.

Impairment of gamete development by exposure to low pH/high pCO_2 may relate to the coelomic fluid, which is suspected to play a key role in nutrient and hormone translocation in echinoderms (reviewed by Mercier & Hamel 2010). OA conditions elicited changes in the pH of the coelomic fluid in several species of sea urchin (Spicer et al. 2011, Dupont & Thorndyke 2012, Stumpp et al. 2012, Calosi et al. 2013, Holtmann et al. 2013, Moulin et al. 2014, 2015, Uthicke et al. 2014) and sea cucumber (Collard et al. 2014), some of which compensated for acidosis by eliminating protons (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 (Stumpp et al. 2012, Collard et al. 2013, Moulin et al. 2014, 2015, Yuan et al. 2016). While some species of sea urchin appear to be unable to compensate for coelomic fluid acidosis (e.g. Catarino et al. 2012, Kurihara et al. 2013, Spicer et al. 2011), contradicting findings exist (e.g. Calosi et al. 2013, Collard et al. 2013). The exposure of 2 holothuroid species to low pH resulted in a decrease in coelomic fluid pH, which was not overcome within a 6 to 12-d period (Collard et al. 2014). Moreover, when exposed to a pH of 7.41, the sea cucumber *Apostichopus japonicus* experienced significant shifts in energy budget, resulting in lowered allocation to somatic growth (Yuan et al. 2016). Changes in the coelomic fluid pH can be hypothesized to have occurred in *C. frondosa* during exposure to OA in the present study. Whether directly or indirectly, through compensation mechanisms, acidosis likely 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*. Exposure to elevated pCO_2 led to the presence of deep holes and etching on the surface of the ossicles of the brittle star *Ophionotus victoriae* (Walker et al. 2013), and led to non-uniform ossicle pore size, as well as signs of surface degradation in the sea urchin *Arbacia lixula* (Bray et al. 2014). Similar structural changes were noted here in *C. frondosa*, evoking ossicle dissolution. It has been suggested that some species of sea urchin can dissolve and reabsorb calcium carbonate structures to create a bicarbonate ion buffer (Stumpp et al. 2012, Calosi et al. 2013, Holtmann et al. 2013, Moulin et al. 2014,). What was scored as ossicle degradation in the present study may be the result of such a response, rather than a direct effect of contact with acidified seawater, given the location of ossicles inside a matrix of connective tissue. However, this hypothesis has been countered, stating that if compensation is occurring, skeletal dissolution likely plays a small role in the global mechanism (Dubois 2014). Overall, calcification effects need to be further explored in echinoderms, especially in sea cucumbers where they have yet to be adequately documented. Perhaps dissolution of ossicles plays a greater role in compensation mechanisms within weakly-calcified sea cucumbers compared to what has been determined in sea urchins, which possess a more developed skeleton.

Minor differences in the 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 in the muscles of both sexes under low pH/high pCO_2 in the pre-spawning period suggest that they experienced a delay in the translocation of important fatty acids towards reproduction, as suggested for other marine 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 used for energy storage in echinoderms are TAG and diacylglycerol ether (DAGE) (Allen 1968, Hayashi & Kishimura 1997). For species with lecithotrophic (yolk-sustained) larvae, like *C. frondosa*, DAGE is typically dominant over TAG (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 more 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 pCO_2 in *C. frondosa.* Low-pH oocytes were characterized by morphological anomalies/deformities such as uneven shapes and non-uniform cytoplasms. Disruption of vitellogenesis was reported in the brittle star *Ophuria ophuria* (a planktotrophic species) exposed to low pH, according to Lowe et al. (unpubl. data, reported 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 delayed development when exposed to elevated pCO_2 prior to hatching (Schiffer et al. 2014). When adults of the sea urchin *Echinometra* sp. A were exposed to elevated pCO_2, spawning and oocyte size were not affected; however, the number of spermatozoa released, percent normal larvae and larval size all decreased, independently of adult pre-acclimation (Uthicke et al. 2013). In fertilization trials using gametes of the sea cucumber *A. 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 auricularia stage increased with decreasing pH, while the duration of the mid-auricularia stage decreased and 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 planktotrophic species *A. japonicus*, although the present study included carry-over 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 of successive life stages (Dupont et al. 2013, Uthicke et al. 2014, Ross et al. 2016).

Normal fully-mature oocytes of *C. frondosa* are positively buoyant and develop near the water surface until metamorphosis (Hamel & Mercier 1996b). In the present study, nearly all oocytes and embryos developing under low pH/high pCO_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; as 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 compared to ambient pH. Such essential fatty acids are required for growth, reproduction (Phleger 1998, Santos et al. 2002) and buoyancy control (Pond & 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 & 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, and this aspect requires further study under OA conditions. Moreover, lipids are the primary components in gonads of *C. frondosa*, but protein and glycogen are also seasonally abundant (David & MacDonald 2002). Therefore, oocyte buoyancy and shape could be 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. It remains impossible to predict whether these effects would persist or be attenuated during evolutionary adaptation to a changing ocean. In the former case, the impact of OA on oocyte buoyancy in *C. frondosa* would have major consequences on its ability to reproduce successfully.

In this regard, the present study on *C. frondosa* highlights potentially important and previously overlooked impacts of OA on species that produce lecithotrophic oocytes, which 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. 2010, see reviews by Ross et al. 2011, 2016). Studies on lecithotrophic species to date have focused only on fertilization or embryonic/larval development. For instance, larvae of the sea star *Crossaster papposus* exposed to low pH exhibited faster growth rates (Dupont et al. 2010). 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). A 0.4 unit decrease in pH negatively impacted

fertilization success in the sea urchin *Heliocidaris erythrogramma* (Havenhand et al. 2008). Contrastingly, in the same species, fertilization and early development were unaffected by low pH (0.3 to 0.6 unit decrease from ambient) (Byrne et al. 2009). In general, species with lecithotrophic development were previously 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. 2010, review by Ross et al. 2011, Nguyen et al. 2012). However, this assumption overlooked the possibility that larvae can be affected by OA through transgenerational effects (oocyte provisioning), which had apparently not been explored prior to the present study.

A brief word should be said on the benefits and drawbacks of conducting long-term studies under realistic conditions. Importantly, monitoring must include meaningful life-history events, which implies a good basic knowledge of the focal species. The experimental setup used during the present study strove to incorporate the biotic and environmental factors (and fluctuations thereof) that mediate gametogenesis and spawning in the focal species (Hamel & Mercier 1996a,c), which is not possible 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 wk, some of the finer negative impacts might have gone unnoticed. On the other hand, maintaining low-pH conditions that parallel ambient conditions over long periods in open flow through is challenging and thus rarely attempted; unlike the present work, most previous studies classified as 'flow through' have used storage tanks undergoing periodic water changes (Cornwall & Hurd 2016). 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. Experimental approaches such as the one employed here have definite advantages in OA research (e.g. allowing for realistic fluctuations in water chemistry, and assessing potential carry-over effects over longer exposures), but future studies will need to further elucidate notions of acclimation, adaptation and synergy of environmental stressors (Cornwall & Hurd 2016, Ross et al. 2016).

Long-lived marine animals such as echinoderms, with lifespans reaching ≥100 yr (Ebert & Southon 2003), will likely be exposed to significant climate change within a single generation. The lifespan of *C. frondosa* is at least in the order of decades (Hamel & Mercier 1996b). Since the species is a major component of temperate, subarctic and arctic benthic communities, 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 & 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 & Mercier 2008a,b). Despite this, the current study suggests 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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