

Severe seawater acidification causes a significant reduction in pulse rate, bell diameter, and acute deterioration in feeding apparatus in the scyphozoan medusa *Cassiopeia* sp.

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ABSTRACT: The detrimental effect of ocean acidification (OA) on marine animals with carbonate exoskeletons or shells is an issue drawing increased attention in marine biology and ecology, yet few studies have focused on the impact on gelatinous organisms like scyphozoan medusae. Here, we examined the physiological tolerance of *Cassiopea* sp., an abundant genus of scyphozoans valuable for their role as bioindicators and for having similarities to other cnidarians, to OA by conducting three, 12-week trials using CO_2 diffusers and electronic pH controllers to incrementally lower the water to test pHs of 7.5 and 7.0. The impact of reduced pH on the survival, pulse rate, bell diameter, and reorientation and settlement abilities of *Cassiopea* sp. medusae were measured weekly. *Cassiopea* sp. was tolerant to pH 7.5 while further reduction of the pH to 7.0 resulted in 22.22% mortality rate, which was significantly different from the control and treatment pH 7.5. Significant differences between the treatment pH 7.0 and control first occurred on day 23.5 with a 50% reduction in the pulse rate, and on day 36 with a 16.6% reduction in bell diameter, while pH 7.5 had no effect. By the final time point of 66 days in treatment pH 7.0, there was an 87% reduction in pulse rate and a 36% reduction in bell diameter versus control. Reduced pH 7.0 caused bell malformations, inhibited swimming abilities, and deterioration of the oral arm feeding apparatus, but had no effect on the orientation and settlement assay. Observations indicate that asexual reproduction via planuloid production and strobilation was unaffected by pH reduction, though polyps in treatment pH 7.0 gave rise to ephyrae with inverted bells. Combined, findings from this study demonstrate *Cassiopea* sp. to be resilient to the end of century ocean acidity prediction of pH 7.6, and vulnerable to more severe OA to pH 7.0.
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KEY WORDS: Ocean acidification, *Cassiopea* sp., medusa, climate change, Scyphozoa, pulse rate.

Сильное закисление морской воды вызывает значительное уменьшение пульсации, диаметра колокола и ухудшение состояния ротового аппарата у сцифоидной медузы *Cassiopea* sp.

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РЕЗЮМЕ: Пагубное воздействие закисления океана на морские животные с известковым экзоскелетом или панцирем — проблема, привлекающая значительное внимание в морской биологии и экологии. Однако только немногочисленные исследования посвящены изучению влияния изменения кислотности среды обитания на такие желеобразные организмы как сцифоидной медузы. Нами проведено исследование физиологической устойчивости к закислению сцифоидных медуз-биоиндикаторов, относящихся к *Cassiopea* sp. Исследование включало продолжавшиеся 12 недель три эксперимента с использованием рассеивателей CO_2 и электронных измерителей pH, для понижения кислотности воды (pH) до значений 7,5 и 7,0. Влияние пониженного pH на выживаемость, пульсацию, диаметр колокола, а также способность к переворачиванию и оседанию медуз *Cassiopea* sp. измеряли еженедельно. Медузы *Cassiopea* sp. показали устойчивость к pH 7,5. Снижение pH до 7,0 приводило к увеличению смертности до 22,22%. Значительные различия между условиями pH 7,0 и контрольными отмечены на 23-й день и проявлялись в снижении частоты пульсации на 50%. На 36-й день отмечено уменьшение диаметра колокола медуз на 16,6%. На 66 день эксперимента в условиях pH 7,0 отмечено снижение частоты пульсации на 87% и уменьшение диаметра колокола на 36%. Понижение кислотности pH до 7,0 вызывало изменения в развитии колокола, снижало плавательную активность и ухудшало работу пищевого аппарата, но не влияло существенно на способность к переворачиванию и оседанию. Способность к бесполому размножению планулами и стробиляции не менялась заметно при снижении pH, однако в условиях низкого pH (7,0) формировались эфиры с перевернутыми колоколами. Таким образом, медузы *Cassiopea* sp. устойчивы к прогнозируемым на конец века условиям кислотности океана (pH 7,6), но уязвимы при более существенном закислении океана (pH 7,0).

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КЛЮЧЕВЫЕ СЛОВА: закисление океана, *Cassiopea* sp., медуза, изменение климата, сцифоидные, пульсация.

Introduction

Ocean acidification (OA), the gradual decrease of the average pH of the ocean over time, is an issue of increased importance in marine biology and ecology. Since the Industrial Revolution, the oceans have absorbed approximately 28% of anthropogenic CO_2 and the average pH of the ocean has decreased by about 0.1 units, becoming 30% more acidic (Kleypas *et al*., 1999, Sabine *et al*., 2004; Feely *et al*., 2009; Kennedy 2010; Jiang *et al*., 2019). Determining the ecological effects of reduced sea pH levels is crucial to predict future changes in marine ecosystems at every level of the food web, and their societal impacts.

The dissociation of carbonic acid in the sea leads to increasing bicarbonate concentrations at the expense of carbonate concentrations, creating problems for many shell and skeleton builders (Kleypas *et al*., 1999; Kroeker *et al*., 2013), therefore, much of the focus of OA research has been on marine animals with calcium carbonate exoskeletons or shells including corals, crustaceans, and zooplankton such as pteropods (Orr *et al*., 2005, Gazeau *et al*., 2007; Comeau *et al.,* 2009; Kleypas, Yates 2009; Hofmann *et al*., 2010; Whitely, 2011; Andersson, Gledhill, 2013; Jokiel *et al*., 2016; Campoy *et al*., 2020). Fewer groups have examined the biological effects of OA on gelatinous organisms, and most lines of evidence suggest that jellyfish are more resistant to OA than many taxa, though they are not entirely unaffected. Previous research has focused on the genus *Aurelia* (common name: moon jellyfish) planulae larvae, polyps, and ephyrae and, viewed collectively, demonstrate the resistance of the genus to this environmental stressor (Kikkawa *et al*., 2010; Winans, Purcell, 2010; Alguero-Muniz *et al*., 2016; Tills *et al*., 2016; Treible *et al*., 2018; Goldstein *et al*., 2017; Dong, Sun, 2018).

Jellyfish are vital parts of their ecosystems and are prey for several species, including sea turtles, ocean sunfish, teleosts and seabirds (McInnes *et al.,* 2017; Hays *et al*., 2018). *Cassiopea* sp. (common name: upside-down jellyfish), a member of the Class Scyphozoa and Order Rhizostomeae, reside in shallow, benthic environments in the Atlantic Ocean, Caribbean Sea, Gulf of Mexico, and the Pacific Ocean (introduced) (Holland *et al*., 2004). *Cassiopea* sp. are involved in nutrient cycling of reefs and mangrove habitats, as their bell pulsing pulls nutrients up from the sediment on which they lay (Jantzen *et al*., 2010). *Cassiopea* sp. are eukaryotic mixotrophs, with both polyp and medusae life stages containing photosynthetic endosymbiont zooxanthellae, the dinoflagellate Symbiodiniaceae (LaJeunesse *et al.*, 2018), which provides its host with a source of energy. Since they can harbor more than one clade, *Cassiopea* sp. are models for studying the uptake of Symbiodiniaceae by corals, as the planulae do not yet contain endosymbionts, and later life stages acquire them from surrounding waters (Lampert, 2016). *Cassiopea* sp. is a prolific species that is easily cultured in captivity. For this reason, and the aforementioned likenesses to other cnidarians, *Cassiopea* sp. was chosen for this study.

While the current ocean pH in native ranges of *Cassiopea* sp. is between pH 7.6–8.3, with average global surface pH of 8.1 (Barbero *et al*., 2019, Jiang *et al*., 2019), and the projected average surface pH of the ocean in the years 2050 and 2100 is 7.8 and 7.6, respectively (Feely *et al*., 2009; Kennedy, 2010), these values indicate averages across the ocean as a whole, and samples in the native ranges are already measuring pH 7.6 (Barbero *et al*., 2019), with global pH likely to continue to decrease with projected CO_2 emissions (Jiang *et al*., 2019). The fluctuations in pH can be more drastic in areas with known upwelling, bringing water saturated with dissolved inorganic carbon (DIC) up to the surface (Wu *et al*., 2019), the nearest to the native range of *Cassiopea* sp. being the Southern Caribbean upwelling system (Rueda-Roa, Muller-Karger, 2013).

Published research on the effect of OA on the medusae stage of any scyphozoan, cubozoan, or hydrozoan species is lacking. A previous study by Chelsky *et al*. (2015) examined the effects of low pH on frozen specimens of *Catostylus mosaicus*, to determine the effects on decomposition. Chuard *et al*. (2019) presented the first evidence of the potential lethal effects of OA on the medusae of the wild-caught cubozoan *Carybdea xaymaca.* One group has previously reported the effect of OA on *Cassiopea* sp., where treatment (pH 7.9, 7.6) of medusae for four weeks did not impact endosymbiont density or the bell diameter, while the effect on the pulse rate, orientation ability, and the polyp and ephyrae stages were not examined (Weeks *et al*., 2019). To test the hypothesis that exposure of *Cassiopea* sp. to seawater acidification for 12 weeks would cause increased mortality rates, a decrease in bell diameter and pulse rate and a disruption of the reorientation and settling abilities, we conducted three trials in which CO_2 gas diffusers and electronic pH controllers were used to incrementally lower the pH of the water to reach test pHs of 7.5 and 7.0. These pH values were chosen based on current pHs in areas of upwelling where *Cassiopea* sp. is found, and to ensure that there was no overlap in pH between the test groups based on the accuracy of the pH controller used. The effect of pH treatment (7.5, 7.0) on the pulse rate, bell diameter, orientation and settling abilities in medusae were measured weekly and compared to animals in the control tanks maintained at pH 8.0–8.3, that of normal seawater. Since the polyp and medusae stages of the life cycle are equally necessary for the survival of the species (Lucas *et al*., 2012), the condition of polyps and the ephyrae was also monitored.

Materials and Methods

EXPERIMENTAL DESIGN. *Cassiopea* sp. have been cultured at the Tennessee Aquarium since 2008, sourced from multiple captive raised populations over the years that they have been maintained at the Aquarium. They have produced an unknown, but presumably large, number of generations as they readily produce new polyp colonies in captivity, which strobilate frequently throughout the year. Their use for this research was approved by Tennessee Aquarium Conservation Institute Animal Health and Welfare Committee (AHW Approval Number: 18-07). Jellyfish used in these studies were approximately the same age since they came from the same group of ephyrae captively raised at the Tennessee Aquarium. The pH of the water in the holding system is maintained at 8.0–8.2.

Three trials of 12–13 weeks in duration were conducted. Nine 10-gallon (37.85 liters) aquariums were used per trial and were placed in groups of three tanks that fit under one aquarium light, referred to as the A, B, and C groups. Each set of three A tanks, three B tanks, and three C tanks included one tank maintained at control pH and two test tanks, where the pH was incrementally lowered to either pH 7.5 or pH 7.0 by diffusing CO_2 into the test tanks using large micro CO_2 bubble diffusers and Milwaukee MC122 pH controllers. CO_2 delivery was activated when water pH levels climbed above the set value and shut off when the pH reached the set value. The test pHs of 7.5 and 7.0 were chosen based on current global pH projections and to ensure there would be no overlap between test groups, given the accuracy of the pH controllers was +/– 0.2 pH units. The pH probes were calibrated using pH standards 4 and 7. Any variation in control pH occurred naturally and was recorded, giving an overall control pH range of 8.0–8.3.

Five jellyfish of similar bell diameter (approximately 60 mm) were placed in each tank containing cycled artificial sea water prepared in deionized water at 35 ppt (parts per thousand) and baseline data was recorded. The test tanks were kept at control pH of 8.0–8.3 for ~2 weeks before lowering the pH to allow the animals to acclimate to the experimental systems and data was not collected during this time. The pH reduction was performed incrementally, to allow the subjects to acclimate to changes in pH, by decreasing the pH controller dial setting by 0.2 units pH every approximately 3–4 days. Once the test pHs were reached for their respective tanks, the water was maintained at that pH for the duration of the experiment. Data were pooled for the three trials for statistical analyses. The average number of days since the initial lowering of the pH was calculated and these are the values seen on the x-axis in Figures 1–3. After first introducing CO_2, pH 7.5 was reached

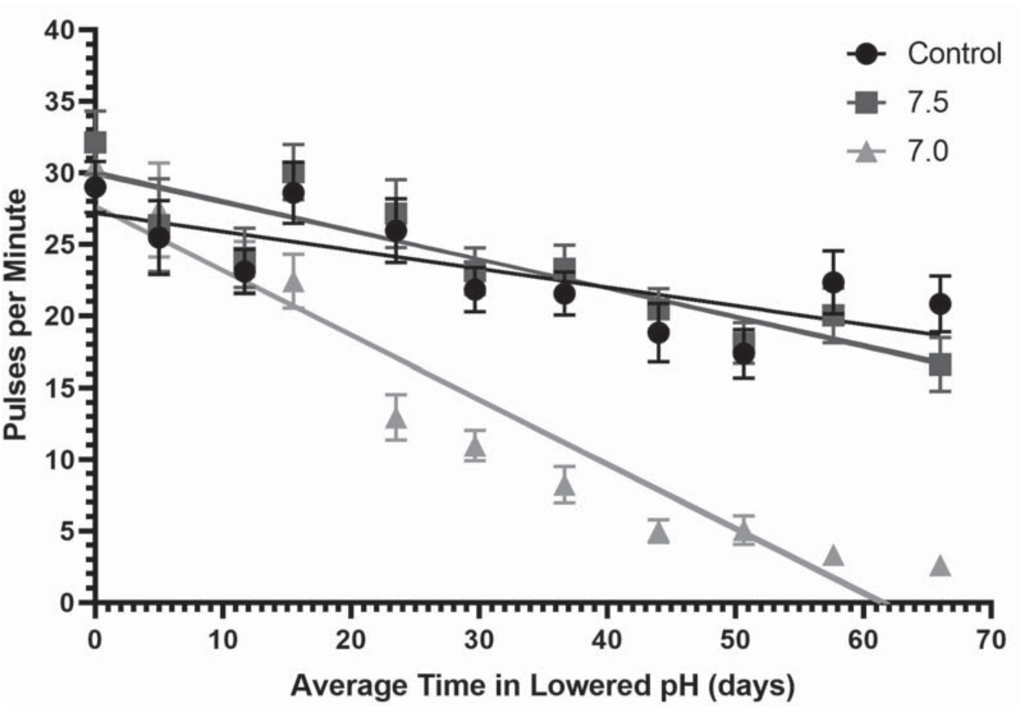

Fig. 1. Pulse rate of *Cassiopea* sp. as a function of lowering of the pH over time. Average pulse rate of subjects in three trials (y-axis) vs. average number of days exposed to lowered pH (x-axis); baseline represents the first day of induction of CO_2. Each data point represents the mean values ± S.E.M. of $n = 45$, except for day 5 ($n = 15$) and days 15.5 and 23.5 ($n = 30$). The regression line for each treatment group and control group is shown. Control group — black circles, pH 7.5 group — medium grey squares, pH 7.0 group — light grey triangles. pH 7.5 was reached by day 7.67. pH 7.0 was reached by day 22.34.

Рис. 1. Изменение пульсации *Cassiopea* sp. в зависимости от снижения pH с течением времени. Средняя частота пульсации в трех экспериментах (ось Y) в сравнении со средним количеством дней нахождения в условиях пониженного pH (ось X); исходный уровень — первый день насыщения CO_2. Каждая точка данных — средние значения ± стандартная ошибка среднего из $n = 45$, за исключением 5-го дня ($n = 15$) и дней 15,5 и 23,5 ($n = 30$). Линия регрессии показана для экспериментальных и контрольной групп. Обозначения: контрольные условия — черные кружки, pH 7,5 — серые квадраты, pH 7,0 — светло-серые треугольники. pH 7,5 был достигнут к дню 7,67; pH 7,0 к дню 22,34.

at an average of 8 ± 1.5 (S.E.M.) days and pH 7.0 was reached at an average of 22.7 ± 3.3 (S.E.M) days for all trials. Trial 1 included two additional end testing dates (average days 73 and 80 in lowered pH) not used in any statistical analysis, though photomicrographs from trial 1 day 80 are included in Fig. 4 and Supplementary file 1.

Water temperature was maintained at 25–26 °C and lighting was set on a standardized photoperiod cycle of 12:12. Subjects were fed 48-hour old *Artemia* nauplii (enriched with Reed Mariculture Shellfish Diet 1800) and frozen rotifers. A mesh divider was secured with aquarium grade silicone to separate the subjects from the tank filter. Weekly water quality testing was performed to measure salinity, ammonia, nitrates, and nitrites, to eliminate any extraneous effects on the subjects or the pH of the test tanks. It should be noted that jellyfish show more growth when housed in larger volume aquatic systems, with larger filters and protein fractionators, all of which allow for more food to be added to the system without deleterious effects to water quality (AZA Aquatic Invertebrate TAG, 2013). Underfeeding in sub-optimal hous-

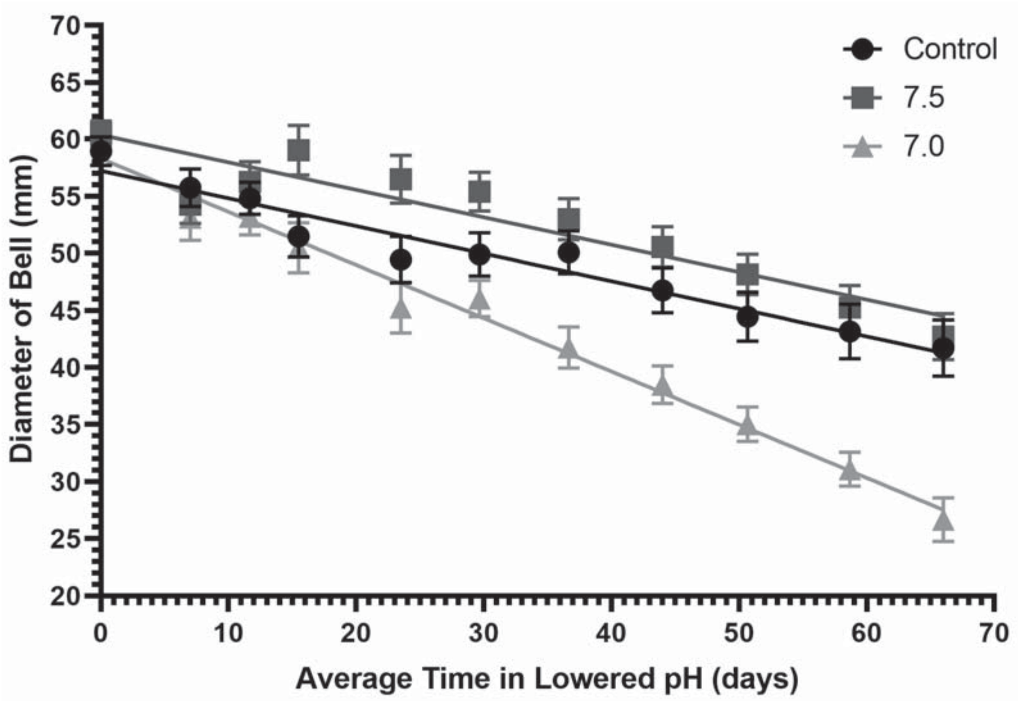

Fig. 2. Bell diameter of *Cassiopea* sp. as a function of lowering of the pH over time. Average bell diameter (mm) of subjects from three trials (y-axis) vs. average number of days exposed to lowered pH (x-axis); baseline represents the first day of induction of CO_2. Each data point represents the mean values ± S.E.M. of $n = 45$, except for days 7, 15.5, and 23.5 ($n = 30$). The regression line for each treatment group and control group is shown. Control group — black circles, pH 7.5 group — grey squares, pH 7.0 group — light grey triangles. pH 7.5 was reached by day 7.67. pH 7.0 was reached by day 22.34.

Рис. 2. Диаметр колокола *Cassiopea* sp. в зависимости от снижения pH с течением времени. Средний диаметр колокола (мм) медуз в трех испытаний (ось Y) в отношении к среднему количеству дней нахождения в условиях пониженного pH (ось X); исходный уровень представляет собой первый день насыщения CO_2. Каждая точка данных представляет средние значения ± стандартная ошибка среднего из $n = 45$, за исключением трех дней: 7, 15,5 и 23,5 ($n = 30$). Линия регрессии показана для экспериментальных и контрольной групп. Обозначения: контрольные условия — черные кружки, pH 7,5 — серые квадраты, pH 7,0 — светло-серые треугольники. pH 7,5 был достигнут к дню 7,67; pH 7,0 к дню 22,34.

ing conditions of the experimental tanks could result in inhibited or reverse growth (Hatai, 1917).

CARBONATE CHEMISTRY. Carbonate chemistry was assessed for each tank. In the case of anthropogenic sources of OA, a clear trend is observed: as CO_2 dissolves into the ocean, the pH decreases, the [CO_2 (*aq*)] and [HCO_3^-] increases, and [CO_3^{2-}] decreases (Barker, Ridgwell, 2012). Once test pH levels were reached, the total CO_2 in each tank was measured using the HACH Carbon Dioxide Test Kit (Model CA-23). Total dissolved inorganic carbon (DIC) is defined as the total amount of carbon dioxide, carbonic acid, and bicarbonate in seawater and is expressed as:

$$DIC = [H_2CO_3] + [HCO_3^-] + [CO_3^{2-}]$$

The average amount of CO_2 was used to determine the dissolved inorganic carbon (DIC), pCO_2, and $\grave{U}_{Ar}$ for each tank using the USGS CO_2calc software (Robbins *et al*., 2010) and using constants by Mehrbach *et al*. (1973) and pH on a total scale.

PULSE RATE. A one-minute video was taken with an iPhone camera weekly of undis-

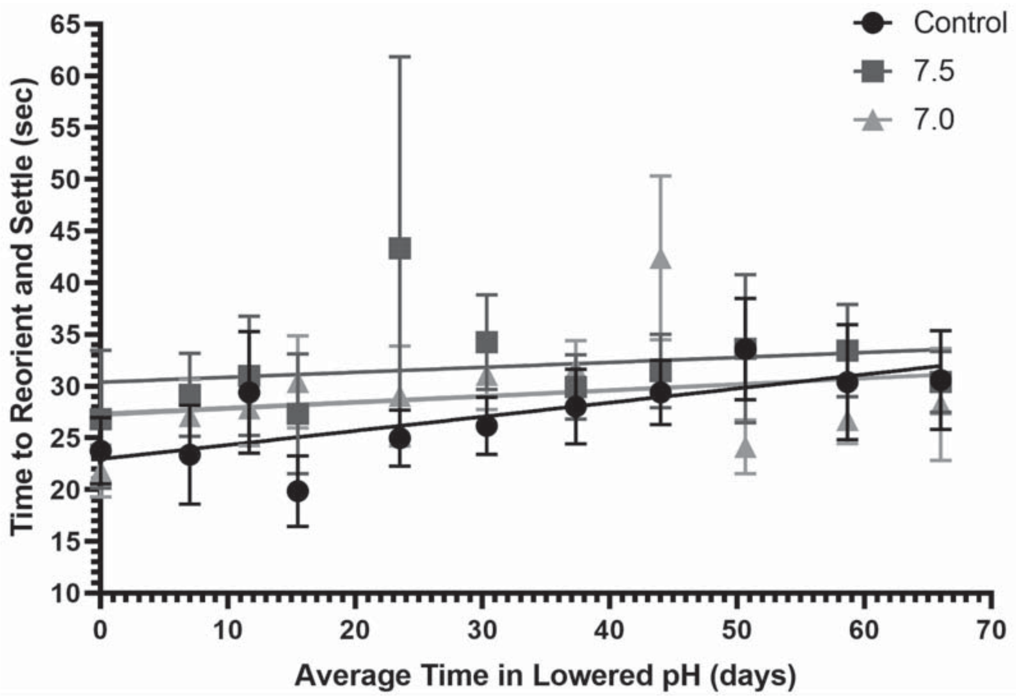

Fig. 3. Reorientation and settling ability of *Cassiopea* sp. as a function of lowering of the pH over time. Average time (sec) for the group to reorient and settle from three trials (y-axis) vs. average number of days exposed to lowered pH (x-axis); baseline represents the first day of induction of CO_2. Each data point represents the mean values ± S.E.M. for nine tanks (three tanks per treatment pH (7.5, 7.0) for a total of three trials) except for the following day 7: *n* = 6; day 11.6: *n* = 7; day 15.5: *n* = 6; day 23: *n* = 6 (except for pH 7.5 *n* = 5); day 30: *n* = 8; control day- 58: *n* = 8. The regression line for each treatment group and control group is shown. Control group — filled black circles, pH 7.5 group — medium grey squares, pH 7.0 group — light grey triangles.

Рис. 3. Изменение способности к переворачиванию и оседанию *Cassiopea* sp. в зависимости от снижения pH. Среднее время (с) для группы, переворачивание и оседание после трех опытов (ось ординат) в зависимости от количества дней нахождения в условиях пониженного pH (ось абсцисс); исходный уровень представляет собой первый день насыщения CO_2. Каждая точка — средние значения ± стандартная ошибка среднего для девяти резервуаров (три резервуара для разных условий pH (7,5, 7,0), всего три испытания), за исключением дня 7го: *n* = 6; а также дня 11,6: n = 7; дня 15.5: *n* = 6; для 23-й: *n* = 6 (кроме pH 7,5 n = 5); дня 30-го: *n* = 8; контрольного 58-го дня: *n* = 8. Линия регрессии показана для экспериментальных и контрольной групп. Обозначения: контрольные условия — черные кружки, pH 7,5 — серые квадраты, pH 7,0 — светло-серые треугольники.

turbed subjects and the video was reviewed to count the number of bell pulses per minute (ppm) for each animal. In healthy jellyfish, pulse rate can be affected by the size of the jellyfish, surrounding currents, presence of food in the water, proximity to conspecifics, periods of rest, or if the jellyfish is actively swimming or relocating (Hamlet *et al*., 2011; Hamlet, Miller, 2014; Ohdera *et al*., 2018). To control for these variables, videos were taken at 4:00 p.m. before food was given to the subjects.

BELL DIAMETER. Each week, jellyfish were gently removed from their tanks and placed subumbrellar side up in glass bowls containing their respective tank water to measure diameter (mm) of the fully open bell in real time. After animals had settled within 2–3 minutes, each jellyfish was positioned in the bowl over a thin plastic ruler and was observed for several pulse cycles to visually assess the difference between

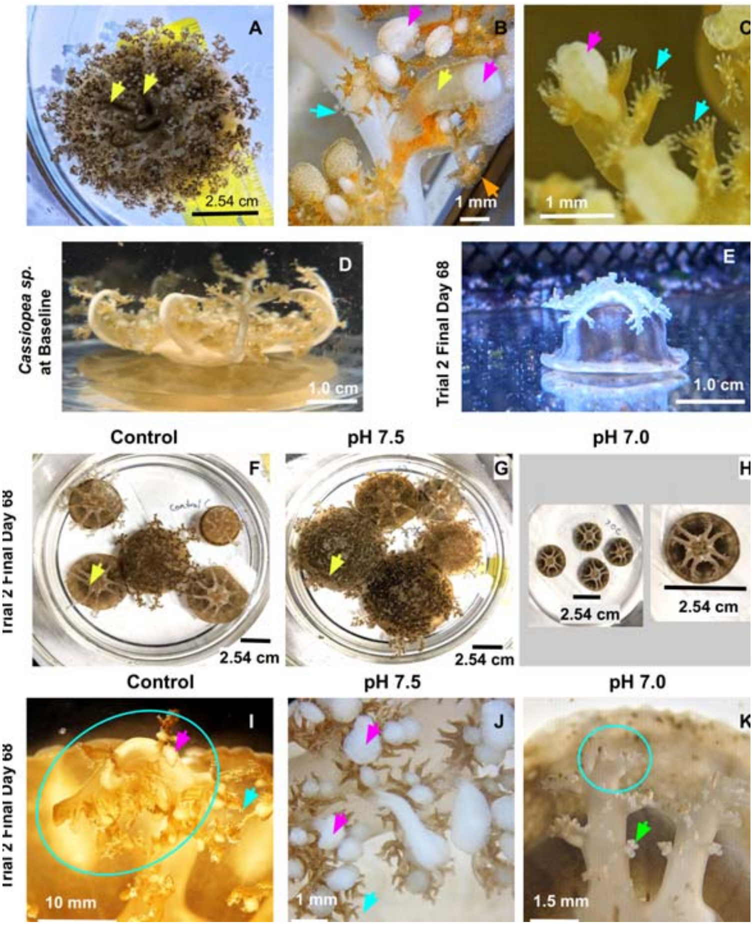

Fig. 4. Deterioration in the feeding apparatus of *Cassiopea* sp. in response to treatment pH 7.0. A–C — images of baseline subjects in good condition; A — exumbrellar photograph of baseline subject; B — food internalization following capture by digitate cirri at a mid-oral arm furcation; C — digitate cirri and cassiosome nests at distal end of oral arm; D — photograph of lateral view of baseline subject; E — photograph of lateral view of gum dropped subject from trial 2C tanks on final day 81. F–H — scaled group photographs of exumbrellar view of trial 2 final day 81 subjects; F — control; G — pH 7.5; H — pH 7.0 with inset of subject. I–K — photomicrographs of subjects on trial 2 final day 81; I — feeding appendages on distal end of an oral arm of a control subject; J — cassisome nests on oral arm of a pH 7.5 subject; K — "t-rexed" oral arms from a pH 7.0 subject.

Yellow arrows — ornamental vesicular appendage; cyan circles — distal end of oral arm, cyan arrows — digitte cirri lining oral collar of secondary mouths; fuchsia arrows — cassiosome nests; orange arrow — food internalization at secondary mouth; neon arrow — exposed oral discs.

Рис. 4. Деградация пищевого аппарата *Cassiopea* sp. в ответ на содержания при pH 7,0. A–C — исходное состояние медуз; A — вид медузы со стороны эксумбреллы; B — поглощение пища после захвата пальцевидными усиками; C — пальцевидные усики и гнезда кассиосом на дистальном конце ротовой лопасти; D — фотография исходного состояния медузы сбоку; E — вид медузы на 81-й день эксперимента 2C. F–H — эксумбрелла медузы в эксперименте 2, 81 день эксперимента; F — контроль; G — pH 7,5; H — pH 7,0. I–K — медузы, эксперимент 2, день 81; I — питающие придатки на дистальном конце ротовой лопасти медузы в контрольных условиях; J — гнезда кассисомы на ротовой руке медузы, pH 7,5; K — ротовые лопасти медузы, pH 7,0.

Желтые стрелки — везикулярный придаток; кружки — дистальный конец ротовой лопасти, голубые стрелки — пальцевидные цирры, выстилающие ротовые воротнички вторичных ртов; пурпурные стрелки — скопления кассиосом; оранжевая стрелка — положение пищи во вторичном рте; зеленая стрелка — ротовой диск.

the diameter of the fully open bell during the recovery vs. the actively contracted phase of the pulse. In some cases, where the individual did not settle on the bottom or did not pulse, the subject was gently moved to the bottom of the bowl and placed over the ruler for measurement.

REORIENTATION AND SETTLEMENT ASSAY. Each week, a plastic pitcher was used to collect one liter of water from the surface of each tank and then poured back into the tank from a consistent distance of approximately 5 cm above the surface of the water in order to create a current strong enough to lift the jellyfish from their resting locations on the bottom of the tank. The elapsed time (sec) until all animals in the tank settled normally (subumbrellar side up on the bottom of the tank, exumbrellar side suctioned to the walls of the tank, or exumbrellar side up at the surface of the water) was recorded in real time. Settling on the surface of the water was defined as when a subject appeared to have come to rest on the surface tension of the water and was no longer actively swimming away from that position. Any abnormalities including landing exumbrellar side up, not pulsing to reach the bottom, or inability to settle before or after the test were noted.

ANATOMICAL OBSERVATIONS. To track morphological features over time, a weekly group photo was taken (iPhone or Google Pixel Camera) of subjects from each tank after they had been placed in glass bowls. Photomicrographs were taken using an iPhone camera through the ocular of a Zeiss Stemi 2000 dissection microscope or using a Google Pixel camera through the ocular of a Leica dissecting microscope. Anatomical features observed include the feeding apparati (secondary mouths and the digitate cirri lining the oral collar), whether the distal ends of the oral arms extended past the margin of the bell or were drawn in closer to the center (referred to as "t-rexing"), vesicular appendages (ornate appendages, sometimes housing cassiosome nests, that extend from the oral arms and are believed to play a role in hydrodynamics in *Cassiopea* sp.), flattening/uncurling of the margin of the bell during both the active phase of the pulse cycle, bell malformations including inversion or the doming shape of the bell (the "gumdrop" effect), presence of rhopalia and statocysts, presence of zooxanthellae, and polyp stalk length and oral disc width. Feeding was documented by imaging jellyfish 5 minutes after being fed *Artemia* while in a glass bowl.

POLYP OBSERVATIONS. Polyps were grown on filtration BioBalls or plastic grating placed in each tank. To assess growth of polyp colonies, the number of polyps present on the BioBalls placed in the A tanks and 7.0 B tank of trial 1 were counted from photomicrographs (taken with an iPhone camera through the ocular of a Zeiss Stemi 2000 dissection microscope) at baseline and the final day. Since polyps were counted only for trial 1, data were not statistically analyzed. Planuloid production and strobilation were noted, but not quantitated. Photographs (taken with Nikon D3S camera and Micro-Nikkor 105 mm f/2.8 lens) of polyps growing on plastic grating on the final day in trial 3 C tanks were used to measure polyp stalk length (from the base of calyx to the base of polyp stalk) and across the oral disc using ImageJ (National Institutes of Health, https://imagej.nih.gov/ij/). Since this analysis was performed on polyps in trial 3 only, data were not statistically analyzed.

STATISTICAL ANALYSIS. Subject mortality rate and pH treatment effect on bell diameter, pulse rate, and reorientation and settlement assay were analyzed using GraphPadPrism, version 9.2.0. Statistical significance was evaluated at 0.05 alpha level.

MORTALITY RATE. Overall mortality per trial (9 tanks per trial, 5 subjects per tank) was calculated by adding the number of deceased subjects on the final day of each trial and dividing by the total starting number ($n = 45$). Animals were considered to be living if they pulsed at the rate of one pulse per minute (ppm). A one-way ANOVA test was used to test for the effect of pH treatment (control, pH 7.5, and pH 7.0) on subject mortality rate. When significant difference was found, a Tukey's multiple comparison post hoc analysis was performed to identify significance difference between pairs of factors. The adjusted p-values and 95% confidence intervals were reported.

BELL DIAMETER, PULSE RATE, REORIENTATION AND SETTLEMENT ASSAY. To test for the effect of pH treatment on bell diameter, pulse rate, and the reorientation and settlement assay, we first ran an ordinary 2-way ANOVA based on the factors of 1) pH reduction and 2) trial and reported the p-value, degrees of freedom (DF), and the F-value (if relevant). When significance was found, a Tukey's multiple comparison post hoc analysis was performed with adjusted p-values and DF reported. To determine the timing of when the pH reduction caused significant changes, we performed a multiple comparisons unpaired t-test on the test points and listed the p-values for each in a table. In the pulse rate analysis, each data point represents the mean values ± the standard error of the mean (S.E.M.) for n = 45, except for day 5 (n = 15) and days 15.5 and 23.5 (n = 30). In the bell diameter analysis, each data point represents $n = 45$, except for days 7, 15.5, and 23.5 ($n = 30$).

REORIENTATION AND SETTLEMENT ASSAY. Each data point represents the mean values ± S.E.M. for nine tanks (three tanks per treatment pH (7.5, 7.0) for a total of three trials) except for the following day 7: $n = 6$; day 11.6: $n = 7$; day 15.5: $n = 6$; day 23: $n = 6$ (except for pH 7.5 $n = 5$); day 30: $n = 8$; control day 58: $n = 8$. To determine if there was a difference in the rate of change in the bell diameter, pulse rate, or orientation and settling ability between the pH treatments over time pH (7.5, 7.0), we performed a simple linear regression and reported the equations, the p-value, the R^2 value, and the 95% confidence intervals (C.I.). A basic t-test was performed to determine whether the slopes of the linear regression lines were significantly different between the treatment and control groups. The p-value and F-value were reported.

Results

CARBONATE CHEMISTRY. Water carbonate chemistry measurements for each test pH were taken and are in accordance with the patterns of DIC redistribution between its various species as in anthropogenic driven OA (Table 1). As CO_2 was added to the systems, the pH decreased (to experimental levels of 7.5 and 7.0), the [CO_2 (*aq*)] and [HCO_3^-] increased, and [CO_3^{2-}] decreased, in all pH 7.5 and 7.0 tanks compared to all trial 2 control tanks. We observed the same trend in trial 3 for all pH 7.5 and 7.0 tanks compared to all trial 3 controls.

MORTALITY RATES. The mortality rate for treatment pH 7.5 was 2.20% (± 0.11% S.E.M.), pH 7.0 was 22.22% (±0.31% S.E.M.), and control was 2.50% (± 0.13% S.E.M.). To test the hypothesis that pH reduction causes a difference in the mortality rates, we performed a one-way ANOVA test between the three factors of control, pH 7.5, and pH 7.0 for the nine tanks and found a significant difference between the mortality rate based on the pH treatment factor (p= 0.0027, DF = 2, F = 7.720). To determine which pH treatment had an effect on the mortality rate, we performed a Tukey's multiple comparison post hoc test (control v. pH 7.5, control v. pH 7.0, and pH 7.5 v. pH 7.0) and found that mortality rates were significantly different between treatment pH 7.0 and control group (adjusted p = 0.0083, [95% confidence interval: –0.7325 to 0.7603]) and between treatment pH 7.0 and pH 7.5 (adjusted p = 0.0058, [95% confidence interval: –1.724 to –0.2759]). One outlier (trial 3C control tank) was removed from the data set (*z*-score = 2.53), decreasing the control group *n* from 45 to 40 for calculating the mortality rates and number of tanks from nine to eight for the ANOVA and post hoc tests. Three deaths were observed in the trial 3C control tank due to factors independent of the treatment. Water quality for this tank was within normal parameters and the cause of the mortality is unknown.

PULSE RATE. To test the hypothesis that pH reduction would cause a decreased pulse rate, we performed an ordinary 2-way ANOVA based on the factors of 1) pH reduction and 2) trial. The factor of pH had a significant effect on the pulse rate (p = 0.001; DF= 2; $F_{(2,18)}$ = 0.001). The trial number did not have a significant effect on the pulse rate (p= 0.0646; DF = 2;

Table 1. Summary of carbonate species in trial 2 and 3 tanks. Data computed from measurement of average total CO_2 in each tank over multiple weeks at experimental pH.
Таблица 1. Содержание углекислого газа и ионов карбонатной системы в опытных резервуарах 2 и 3. Данные рассчитаны на основе измерения среднего общего CO_2 в каждом резервуаре в экспериментальным условиях pH.

	TCO_2 (µmol/ kg SW)	pCO_2 (µatm)	CO_3 (µmol/ kg SW)	HCO_3 (µmol/ kg SW)	CO_2 (µmol/ kg SW)	Ω_{Ar}
Trial 2 Control A	526.698	116.852	49.558	473.748	3.392	0.782
Trial 2 7.5 A	561.706	414.998	17.6	532.058	12.048	0.278
Trial 2 7.0 A	720.62	1642.63	6.967	665.967	47.687	0.11
Trial 2 Control B	563.249	96.333	40.856	390.56	2.797	0.644
Trial 2 7.5 B	498.891	368.589	15.632	472.558	10.7	0.247
Trial 2 7.0 B	600.509	1368.841	5.805	554.965	39.738	0.092
Trial 2 Control C	533.536	118.369	50.201	479.898	3.436	0.792
Trial 2 7.5 C	830.098	613.291	26.01	786.283	17.804	0.41
Trial 2 7.0 C	1003.324	2287.044	9.7	927.23	66.395	0.153
Trial 3 Control A	519.678	115.294	48.897	467.434	3.347	0.771
Trial 3 7.5 A	533.536	394.186	16.718	505.375	11.443	0.264
Trial 3 7.0 A	720.62	1642.63	6.967	668.967	47.687	0.11
Trial 3 Control B	266.075	59.031	25.035	239.326	1.714	0.395
Trial 3 7.5 B	699.833	517.049	21.929	662.894	15.01	0.346
Trial 3 7.0 B	1143.291	2606.95	11.053	1056.582	75.657	0.174
Trial 3 Control C	461.928	102.482	43.464	415.489	2.975	0.686
Trial 3 7.5 C	554.323	409.543	17.369	525.065	11.889	0.274
Trial 3 7.0 C	637.471	1453.094	6.163	589.124	42.184	0.097

$F(2, 8) = 0.064$). To determine which pH treatment (7.5, 7.0, or both) had an effect on the pulse rate, we performed a Tukey's multiple comparison post hoc test. There was no significant difference between the control and pH 7.5 (adjusted p-value = 0.0895, DF= 0.23) or between pH 7.5 and pH 7.0 (adjusted p-value = 0.0801, DF= 0.23). A significant difference was found between the control and pH 7.0 (adjusted p-value = 0.0007, DF= 0.23). To determine when the pH reduction caused a significant difference in pulse rate between the control and pH 7.0 group, we performed a multiple comparisons unpaired *t*-test (Table 2) and found that significant differences first occurred at day 23.5 with a 50.19% reduction in the pulse rate (Fig. 1, 22.43 vs. 12.93 ppm, p = 0.000068). In addition, by the final time point of 66 days in reduced pH (7.0), there was a 87.43% reduction in pulse rate (Fig. 1, 20.82927 vs. 2.61765 ppm, p = <0.000001).

To determine if there was a difference in the rate of change in the pulse rate over time for the different treatments pH (7.5, 7.0), we performed a simple linear regression (Table 3). The high R^2 values indicate that the equations for treatment pH 7.5 and 7.0 highly predict test results shown in the data, while the equation for control has less of a fit to the data. Then we used a basic *t*-test to determine whether the slopes of the linear regression lines were significantly different (Table 4). There is a significant difference in the slopes between control and pH 7.0 (p = <0.0001) and between pH 7.5 and pH 7.0 (p = <0.0001).

Table 2. Multiple comparisons unpaired *t*-test for control vs pH 7.0 for pulse rate, bell diameter, and re-orientation assay.
Таблица 2. Сравнение параметров пульсации, диаметра колокола и способности к переворачиванию для контрольной группы и условий с pH 7,0 методом множественного сравнения независимых переменных при помощи *t*-критерия.

Test Point (days)	**p-value**		
	Bell Diameter	**Pulse Rate**	**Reorientation and Settlement Assay**
Baseline	0.53096	0.896472	0.637612
7	0.31566	0.896472	0.544815
11.67	0.45332	0.896472	0.827486
15.5	0.72744	0.133302	0.089557
23.5	0.16663	0.000068	0.483468
29.67	0.12661	<0.000001	0.276113
36.67	0.00194	<0.000001	0.424257
44	0.00174	<0.000001	0.146041
50.67	0.00064	<0.000001	0.107585
58.67	6.9E-05	<0.000001	0.537995
66	1.3E-05	<0.000001	0.749215

However, there is no significant difference between the slopes of the control and pH 7.5 (p=0.1753).

BELL DIAMETER. To test the hypothesis that pH reduction would cause a decrease in bell diameter, we performed an ordinary 2-way ANOVA based on the factors of 1) pH reduction and 2) trial. The factor of pH had a significant effect on the bell diameter (p = 0.0063; DF= 2; F (2, 18) = 6.800). The trial number had a significant effect on the size of the bell diameter (p-value = 0.0011; DF = 2; F (2, 18) = 10.21). To determine which pH treatment (7.5, 7.0, or both) had an effect on the bell diameter, we performed a Tukey's multiple comparison post hoc test. There was no significant difference between the bell diameter of control and pH 7.5 (adjusted p-value = 0.9929, DF= 0.23), while significant differences were found between the bell diameter of control and pH 7.0 (adjusted p-value = 0.0150, DF= 0.23) and between pH 7.5 and pH 7.0 (adjusted p-value = 0.0118, DF= 0.23). To determine which trial number (1, 2, or 3) had an effect on the bell diameter, we performed a Tukey's multiple comparison post hoc test. There was no significant difference found between trials 1 vs. 2 (adjusted p-value = 0.422, DF= 0.23), while significant differences were found between trial 1 vs. 2 (adjusted p-value = 0.0158, DF= 0.23) and between trial 2 vs. 3 (adjusted p-value = 0.0010, DF= 0.23). Trial 3 is likely different because of the mortality (*n* =

Table 3. Linear regression analysis of all pH levels for pulse rate, bell diameter, and reorientation assay.
Таблица 3. Линейный регрессионный анализ всех уровней pH для пульсации, диаметра колокола и способности к переворачиванию.

Test	Analysis Result	pH Level		
		Control	7.5	7.0
Pulse Rate	Best Fit Line Equation	Y = –0.1296*X + 27.18	Y = –0.1985*X + 29.89	Y = –0.4486*X + 27.69
	R^2	0.5806	0.8081	0.9193
	Slope Confidence Interval	–0.2126 to –0.04653	–0.2714 to –0.1256	–0.5489 to –0.3484
Bell Diameter	Best Fit Line Equation	Y = –0.2417*X + 57.24	Y = –0.2404*X + 60.38	Y = –0.4662*X + 58.31
	R^2	0.9492	0.8689	0.9849
	Slope Confidence Interval	–0.2839 to –0.1996	–0.3108 to –0.1700	–0.5097 to –0.4227
Reorientation and Settlement Assay	Best Fit Line Equation	Y = 0.1363*X + 22.95	Y = 0.04791*X + 30.38	Y = 0.05695*X + 27.35
	R^2	0.5598	0.05428	0.05556
	Slope Confidence Interval	0.04517 to 0.2275	–0.1029 to 0.1987	–0.1201 to 0.2340

3) that occurred in the trial 3 control A tank. Though this tank was excluded from the data for mortality calculations (outlier *z*-score = 2.53), it was not removed from any other data set. To determine when the pH reduction caused a significant difference in the bell diameter between the control and pH 7.0 group, we performed a multiple comparisons unpaired *t*-test (Table 2) and found that significant differences first occurred at day 36 with 16.63% reduction in the bell diameter (Fig. 2, 42 vs. 50 mm, p = 0.001935) and that by the final time point of 66 days in reduced pH (7.0), there was a 36.04% reduction in bell diameter (Fig. 2, 42.77 vs. 41.77, p = 0.000013).

To determine if there was a difference in the rate of change in the bell diameter over time for the different treatments pH (7.5, 7.0), we performed a simple linear regression test (Table 3). The high R^2 values indicate that the equations for the control, pH 7.5, and pH 7.0 fall relatively in line with the data. Next, we used a basic *t*-test

Table 4. *T*-test analysis between the regression lines for each test pH vs. control and for pH 7.5 vs. 7.0 for pulse rate and bell diameter.

Таблица 4. *T*-тест линий регрессии для каждого тестируемого pH в сравнении с контролем и для pH 7,5 и 7,0 для скорости пульсации и размера колокола.

Test	**Analysis Result**	**pH Level Comparison**		
		Control vs 7.5	**Control vs 7.0**	**7.5 vs 7.0**
Pulse Rate	**P value**	0.1753	<0.0001	<0.0001
	F value	1.991	30.74	49.04
Bell Diameter	**P value**	0.9709	<0.0001	<0.0001
	F value	0.001366	70.26	38.11

to determine whether the slopes of the linear regression lines were significantly different (Table 4). There was a significant difference in the slopes between the control and pH 7.0 (p = <0.0001) and between pH 7.5 and pH 7.0 (p = <0.0001). The high p-value between the control and pH 7.5 (p = 0.9709) suggests that the control and pH 7.5 bell diameter declined at a similar rate.

REORIENTATION AND SETTLEMENT ASSAY. To test the hypothesis that pH reduction would cause longer times to reorient and settle, we performed an ordinary 2-way ANOVA based on the factors of a) pH reduction and b) trial. The factor of reduced pH had no significant effect (p = 0.9430; DF= 2; F (2, 18) = 0.05888) and trial number had no significant effect on assay performance (p = 0.0011; DF = 2; F (2, 18) = 10.21). Settling times ranged between 20–40 seconds for each group (Fig. 3). To determine if there was a linear relationship with respect to time for each treatment for reorientation and settling ability, we performed a simple linear regression (Table 3). The relatively low R^2 values indicate that — for the control, pH 7.5, and pH 7.0 — the pH level does not explain much of the variation observed in this assay. One outlier value (trial 3B control tank value of 147.8 seconds on day 58 (*z*-score = 2.42) was removed from the reorientation and settlement assay data set.

ANATOMICAL OBSERVATIONS. Group photographs of all subjects taken at baseline and on the final day of the experiment are arrayed in Supplementary files 1–3 to draw comparisons of any anatomical changes that occurred within groups over time and between groups and trials. Baseline *Cassiopea* sp. specimens of good condition have abundant vesicular appendages (Fig. 4, yellow arrows), oral arms that extend past the margin of the bell (Fig. 4A) with numerous cassiosome nests (Fig. 4, fuchsia arrows) and secondary mouths (Fig. 4B), the latter encircled by digitate cirri (Fig. 4C, cyan arrows). Fig. 4B depicts internalization of *Artemia* following capture by a secondary mouth (orange arrow) located at a middle oral arm furcation. Baseline subjects maintain a mostly flat bell, typically with the exumbrellar side against the substrate or walls of the tank, with the bell margin slightly curled inward during the resting phase of the pulse cycle (Fig. 4D), while subjects in pH 7.0 treatment exhibited inversion of the bell to an unnatural gumdrop shape (Fig. 4E).

Compared to control and treatment pH 7.5, final day pH 7.0 subjects were smaller and had t-rexed oral arms that were bare in appearance (Fig. 4 F–H, images to scale, except for zoomed in pH treatment 7.0 animal in Fig. 4H on right). Photomicrographs (Fig. 4 I–K) show that final day pH 7.0 subjects had a loss of feeding appendages (cyan circles) and cassisosome nests

Table 5. Number of polyps counted growing on the spherical cap of a BioBalls in Trial 1.
Таблица 5. Количество полипов, растущих на сферической поверхности в опыте 1.

	Number of Polyps			
Day	**Control A Tank**	**pH 7.5 A Tank**	**pH 7.0 A Tank**	**pH 7.0 B Tank**
Day 1 (Baseline)	108	131	109	119
Day 92	107	142	136	179

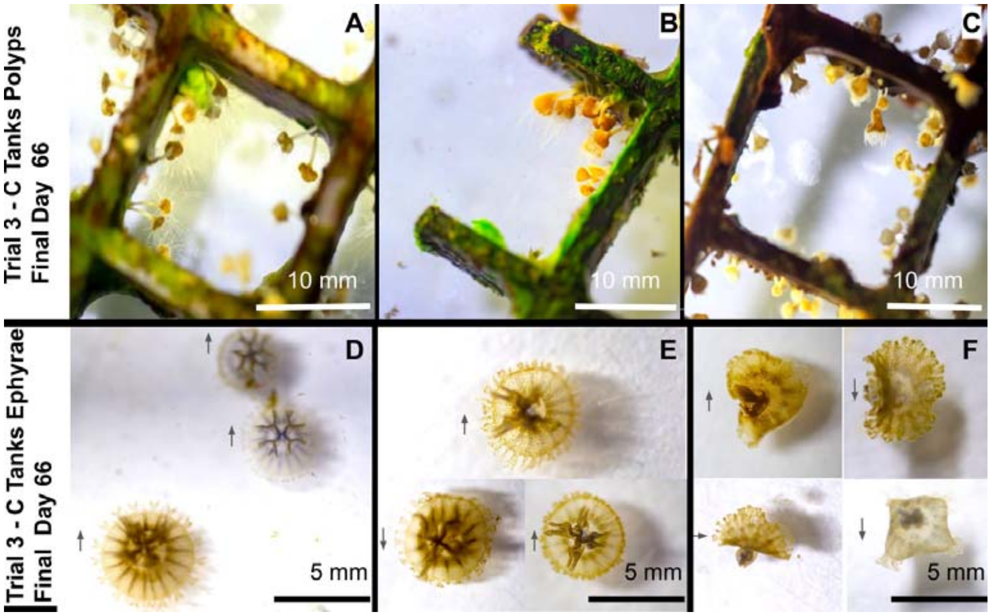

Fig. 5. Polyp and ephyrae health in response to treatment (pH 7.5, 7.0). A–C — photographs of polyps attached to plastic grating from trial 3 C tanks on day 78: A — control; B — pH 7.5; pH 7.0. D–F — photomicrographs of ephyrae retrieved from trial 3 C tanks on day 78: D — control; E — pH 7.5; F — pH 7.0. Grey arrows indicate whether the oral side of the animal is facing up, down, or sideways.
Рис. 5. Изменения полипов и эфиров в эксперименте (pH 7,5, 7,0). A–C — фотографии полипов, прикрепленных к пластиковому основанию в резервуарах на 78-й день эксперимента 3C: A — контрольные условия; B — pH 7,5; pH 7,0. D–F — эфиры, извлеченные из опытных резервуаров (3C) на 78-е сутки: D — контрольные условия; E — pH 7,5; F — pH 7,0. Стрелки указывают направление ротовой стороны.

(fuchsia arrows) seen in the control and pH 7.5 groups, and secondary mouths with little to no digitate cirri leaving the underlying oral discs exposed (green arrow).

Photomicrographs of the bell margin show that, even in t-rexed pH 7.0 subjects, the rhopalia (Supplemental file 4A–B, red circles) and the statocysts (composed of statoliths) (Supplemental file 4C–D, red arrows) were present, and zooxanthellae (small golden-brown cells) were abundant throughout this tissue. No animals experienced bleaching as an effect of lowered pH, and shrunken pH 7.0 subjects often appear darker in color as their zooxanthellae condensed in the remaining tissue (Fig. 4H and Supplementary files 1–3).

In feeding, the oral arms of the control and pH 7.5 groups were splayed out providing maximum exposure of the secondary mouths to the water column for prey capture (prey seen as clumps of orange *Artemia* nauplii), while the pH 7.0 subjects curled their oral arms inwardly towards the central manubrium with prey concentrating at the proximal portion of oral arms while ingesting (Supplementary file 5).

POLYP OBSERVATIONS. An increase in the number of polyps growing on Bio Balls in test tanks (pH 7.5, 7.0) occurred from the baseline to the final day 92 in trial 1, whereas the number of polyps in the control tank remained the same (Table 5). Polyps grown on plastic grating in the trial 3C control tank had elongated, healthy tentacles, elliptical to round calyxes, and elongated stalks (Fig. 5A), treatment pH 7.5 had slightly contracted tentacles, elliptical to round calyxes, and shortened stalks (Fig. 5B), and treatment pH 7.0 had shortened or constricted tentacles, irregularly shaped calyxes, and shortened stalks (Fig. 5C). No difference in oral disc diameter between test groups and control was observed. Planuloid production was observed in all groups throughout the experiment and polyp colonies were present on the final day in all groups in all trials. Strobilation occurred in all groups throughout the experiment in all trials. Ephryae collected from the control and pH 7.5 tanks had normally shaped bells with a flat exumbrellar side (Fig. 5D–E), while some ephyrae in the pH 7.0 tanks had inverted bells (Fig. 5F).

Discussion

It has been suggested that gelatinous organisms are more tolerant to acidifying and warming waters than other marine taxa leading to perceived increases jellyfish blooms occurring over wider geographic ranges (Attrill *et al.*, 2007), though other causal factors implicated include the loss of prey or competitors and that jellyfish populations are subject to worldwide oscillations with an ~20-y periodicity (Condon *et al.*, 2013). This study adds to the line of evidence that jellyfish are more resistant to OA than many taxa, with 98% of *Cassiopea* sp. medusae surviving and in good health after 66 days of exposure to low pH. These findings corroborate the previous body of research that describes the tolerance of *Aurelia* sp. planulae larvae, polyps and ephyrae to OA, and the only previous research on the effect of OA (pH 7.6 for 4 weeks) on *Cassiopea* sp. (Weeks *et al.*, 2019). In contrast, treatment of wild caught box jellyfish with pH 7.5 (12 hours) resulted in 35% mortality rates with surviving animals presenting with retracted tentacles and totally inhibited swimming abilities (Chuard *et al*, 2019). This study is the first to examine the effect of pH<7.6 on *Cassiopea* sp. Lethal effects occurred with treatment pH 7.0 for 66 days with 22% mortality rates and with surviving jellyfish in poor condition.

After introducing CO_2, pH 7.5 was reached by an average of 8 days and pH 7.0 was reached by an average 22.67 days. Significant differences between the treatment pH 7.0 and control first occurred on day 23.5 with a 50% reduction in the pulse rate and was followed by a 16.6% reduction in bell diameter ~2 weeks later, while pH 7.5 had no effect. The decline in pulse rate preceded the day that the test pH 7.0 level was reached. By the final time point of 66 days in treatment pH 7.0, there was an 87% reduction in pulse rate and a 36% reduction in bell diameter versus control. The pulsing behavior is the primary mechanism by which jellyfish draw in and catch prey, so if a jellyfish loses its ability to pulse properly, its feeding ability will be compromised and will reduce in size.

The feeding strategy of *Cassiopea* sp. employs the anatomy of the oral arms and various appendages to create vortices that swirl around the secondary mouths during each pulse (Hamlet *et al.*, 2011). Here, t-rexing, loss of vesicular appendages, inversion and gumdropping of the bell in pH 7.0 medusae weakened the pulse, and therefore the currents which deliver prey to the secondary mouths. While the zooxanthellae were retained, the nutrition was inadequate as significant size reduction occurred in treatment pH 7.0. In the only previous work to examine the effect OA on the pulse rate, treatment of *A. aurita* ephyrae at pH 7.6 for 7 days resulted in slower pulse rates and smaller surface area, central disc area, and lappet length (Tills *et al*., 2016) and treatment with pH 7.28 for 7 days caused slower ephyrae growth (Alguero-Muniz *et al*., 2016), however, no previous research has examined the effect of pH reduction on the pulse rate of any scyphozoan medusae. Additional research to explore the effects of reduced pH on the metabolism of *Cassiopea* sp. could examine the difference between how much prey is ingested at pulse rates affected by reduced pH, and which subjects are expending versus conserving energy. Since *Cassiopea* sp. cycles nutrients in the substrates of their native environments through the pulsing behavior, a decline in pulse rate in native populations would have negative impacts on the nutrient cycles of these benthic environments.

Having adapted to a benthic lifestyle, *Cassiopea* sp. lacks lengthy stinging tentacles typically found in other scyphozoans, and instead uses oral arms lined with numerous secondary mouths equipped with digitate cirri and cassiosomes, both of which release stinging nematocysts. Treatment pH 7.0 caused damage to the feeding apparati of medusae with a loss of digitate cirri, thereby limiting the effectiveness of the secondary mouths. These subjects were observed to curl the oral arms more inwardly than the normal position during feeding. Loss of vesicular appendages and cassiosome nests in pH 7.0 animals may have also impacted their ability to capture prey, as they are an integral part of the *Cassiopea* sp. feeding strategy (Weeks *et al*., 2019; Ames *et al*., 2020). As the brachial cavities and secondary mouths are involved in sexual reproduction of this species, damage to these structures in severe OA to pH 7.0 could also inhibit the species' ability to brood planulae produced by fertilized eggs, making the survival of the species dependent on the asexual reproduction of polyps, thereby limiting genetic diversity over time. A lack of genotypic variation in future generations caused by the lethal effects of OA on the sexually reproductive medusae stage of *C. xaymacana* has also been proposed by Chuard *et al*. (2019).

Previous work on the effects of OA on *A. aurita* polyps found that treatment with pH 7.62 for 36 days had no effect (Treible *et al*., 2018) and treatment with pH <4.5 for 240 days caused tissue degradation (Goldstein *et al*., 2017). Here, *Cassiopea* sp. polyps cultured in reduced pH (7.5 and 7.0) for 66 days had shortened tentacles, irregularly shaped calyxes, and shortened stalks, but the specimens appeared in good condition. The effect of these anatomical changes on prey capture efficiency was not examined. Exposure of *Aurelia aurita* planulae larvae to pH 7.4 for 240 days in combination with low temperature of 4 °C (Goldstein *et al.,* 2017) and of *Aurelia coerulea* to pH 7.3 for 7 days (Dong, Sun, 2018) increased planulae settlement rates. Though we did not examine planulae larvae here, exposure of polyps to 66 days of pH 7.0 did not impact their ability to reproduce asexually through release of planuloids, which occurred throughout the experiment, even on the final days. Planuloid tolerance to reduced pH is demonstrated by the increase in polyp colony size over time in reduced pH environments (as observed in trial 1). Though able to strobilate throughout the experiment, polyps exposed to treatment pH 7.0 gave rise to some ephyrae with inverted bells and inhibited swimming abilities, in support of the work of Kikkawa *et al*. (2010) in which *Aurelia* sp. ephyrae exposed to pH 6.15 for 96 hours had inverted oral arms and inhibited swimming abilities.

While treatment had no significant effect on the reorientation and settlement assay, the manner in which the pH 7.0 subjects settled was different from the other groups. Subjects with gumdropped bells were observed to have ineffective bell contractions during the pulse cycle which affected the hydrodynamics of their swimming, causing them to sink rather than swim to reach the bottom, but in about the same amount of time as controls. A more accurate assessment would be to record the proportion of animals per tank that settle normally by pulsing to the bottom (as was observed in the control and pH 7.5 groups) versus sinking (as was observed in the pH 7.0 groups) and how many are able to recover to a normal position in a given amount of time after disturbance.

The reorientation test was intended to discern whether OA would cause deterioration to the only mineralized part of a jellyfish's anatomy, the statoliths, small "stones" composed of calcium sulfate subhydrate within the statocyst, which functions in sensory balance to detect positioning in the water (Becker *et al*., 2005; Sötje *et al*., 2017). Winans and Purcell (2010) reported that exposure of *Aurelia labiata* polyps to pH 7.2 for 122 days gave rise to ephyrae with smaller statoliths. Though the statoliths of ephyrae released from polyps in experimental tanks were not examined in this experiment, the statoliths of *Cassiopea* sp. medusae were intact following 66 days of treatment pH 7.0, suggesting that the inability of the pH 7.0 subjects to actively pulse or swim to settle was unlikely due to dysfunctional balance machinery. Future work should determine if pH reduction diminishes the size of the statocyst or the number of statoliths present.

Conclusions

Cassiopea sp., and possibly other scyphozoans, are likely to be more resistant to OA to a pH of 7.5 than many taxa. Our results indicate that more severe OA to pH 7.0, which may be possible in the future in parts of this species' range exposed to increasing CO_2 pollution or upwelling, would be lethal. While the polyp stage of *Cassiopea* sp. can reproduce asexually and even strobilate at pH 7.0, they do not always produce a healthy new generation of *Cassiopea* sp. Future OA research on gelatinous zooplankton should implement experimental designs with stepwise incremental pH reduction and longer exposure times to each pH unit change with data collected at each step for each stage of the life cycle, including effects on gamete production and planulae larvae and Symbiodiniaceae density. Incremental pH reduction not only mimics the gradual change happening in nature, but also negates the effects of "shocking" test subjects by not providing enough time for them to acclimate to test conditions (sometimes seen in captive populations), and unintentionally skewing results. Furthermore, since *Cassiopea* sp. are found in shallow lagoons subject to high temperatures, a known stressor of Symbiodiniaceae in the coral-algae relationship (Hoegh-Guldberg, 1999), future studies should include the effect of both OA and rising temperatures related to climate change on survival.

Compliance with ethical standards

CONFLICTS OF INTEREST: The authors declare that they have no conflicts of interest.

Supplementary data. The following materials are available online.

Supplementary file 1. Photographs of trial 1 subjects taken at the baseline and final day. Columns: Left — A tanks, middle — B tanks, right — C tanks. Rows: top — control, middle — pH 7.5, bottom — pH 7.0. Scale bars 2.54 cm.

Supplementary file 2. Photographs of trial 2 subjects taken at the baseline and final day. *Day number adjusted for time spent in acidic water. Columns: Left — A tanks, middle — B tanks, right — C tanks. Rows: top — control, middle — pH 7.5, bottom — pH 7.0. Scale bars 2.54 cm.

Supplementary file 3. Photographs of trial 3 subjects taken at the baseline and final day. *Day number adjusted for time spent in acidic water. Columns: Left — A tanks, middle — B tanks, right — C tanks. Rows: top — control, middle — pH 7.5, bottom — pH 7.0. Scale bars 2.54 cm.

Supplementary file 4. Intact rhopalia and statocysts observed in pH 7.0 treatment day 71. A–B — photomicrographs of the bell margin of two subjects. C–D — photomicrographs of statocysts in two subjects. Red circles — rhopalia. Red arrow — statocysts containing statoliths. C, D shown in 40x magnification. Scale bars 1 mm.

Supplementary file 5. Modified feeding behavior observed in pH 7.0 treatment day 71. A — control subjects; B — pH 7.5; C — pH 7.0. Images taken of jellyfish in trial 1B tanks on day 71. Scale bar 10 mm.

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