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Testing Multiple Climate Stressors at the Cold Range Limit of a Marine Calcifier

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ABSTRACT

Coastal marine ecosystems have been identified as at particularly high risk from global climate change. Laboratory mesocosm experiments with model organisms can be useful in elucidating the effects of multiple climate change stressors on marine species. Here I examine the combined effects of marine heatwaves (MHWs) and ocean acidification (OA) on early embryonic development of the sea urchin *Arbacia punctulata* taken from its cold (northern) range limit in the Northwest Atlantic. I observed additive effects of MHWs and OA on developmental rates, with rates enhanced by MHWs and hindered by OA as compared to ambient conditions. Hence, MHWs mitigated a negative effect of OA on development of the species at its cold range limit. My results provide an improved understanding of how MHWs and OA can combine to affect the sensitive early life-history stages of calcifying marine invertebrates and may be useful in predicting future shifts in species distributions.

MONTCLAIR STATE UNIVERSITY

Testing Multiple Climate Stressors at the Cold Range Limit of a Marine Calcifier

by

Christian R. Bojorquez

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INTRODUCTION

Anthropogenic activities are causing widespread environmental changes across the world's oceans (IPCC 2019). In particular, marine fauna and flora are threatened by ocean warming and ocean acidification (OA) (Harley et al. 2005, Orr et al. 2005). Ocean warming can manifest either as a gradual increase in sea temperature or as acute marine heatwaves (MHWs) that vary in spatial extent, timespan, and magnitude (Dunstan et al. 2018). Gradual warming on decadal scales has been implicated in the loss of marine species from warm range edges (Feehan et al. 2019) and has caused behavioral and phenological shifts in marine organisms (Edwards and Richardson 2004). Acute MHWs are relatively understudied, but also have been documented to cause fatal thermal stress, with associated localized extinctions (Thomsen et al. 2019) and broad shifts in ecosystem structure (Wernberg et. al. 2013). OA also adversely affects many taxa of marine organisms by interfering with calcification rates and physiological functioning (Logan 2010). For example, OA can affect physiological functions such as reproduction, metabolism, and acid-base regulation (Logan 2010). However, it is not well understood how combined stressors of MHWs and OA will impact marine species.

Laboratory mesocosm experiments can reveal whether a combination of climate change stressors produces interactive (synergistic or antagonistic) or additive effects on marine organisms (Crain et al. 2008). When the combination of stressors yields effects that are greater than the sum of the effects of the individual stressors, the effects are said to be synergistic (Folt et al. 1999). However, when the combination of stressors yields effects that are less than the sum of the effects of the individual stressors, the effects are said to be synergistic (Folt et al. 1999). Finally, when the combination of stressors affect separate physiological pathways and yield effects equal to the individual effects of the stressors, the effects are said to be additive (Folt et al. 1999). These studies

are important for understanding the impacts of multiple stressors on species, as interactive effects will cause lesser or greater impacts on species than expected based on observations of individual stressors. Studies that investigate the effects of multiple stressors often employ model species that are of interest for ocean conservation and management (Byrne et al. 2013). For example, echinoderms such as sea urchins and sea stars are often used as model organisms due to their role as keystone grazers and predators in coastal marine ecosystems (Feehan and Scheibling 2014).

Echinoderms experience a complete metamorphosis of body form as they develop from the planktonic (free-floating) embryonic and larval stage to the benthic (bottom-dwelling) adult stage. The development of embryos and larvae follows spawning of gametes by separate-sexes of benthic adults, and external fertilization occurs in the water column. While the larval stage allows for increased dispersal and reduced competition with adults (Metaxas and Young 1998), embryos and larvae also often are highly sensitive to changing environmental conditions (Catarino et al. 2012). For instance, the early embryos of echinoderms have been shown to often be the most sensitive life stage to environmental changes such as warming temperature (Catarino et al. 2010). Furthermore, exposure to low pH and warm temperature can alter rates of development and growth in echinoid larvae (Ericson et al. 2010, Feehan et al. 2018). Previous studies have shown that exposure to low pH causes delays in development and growth in sea urchin larvae (Catarino et al. 2012, Garcia et al. 2018). However, exposure to warm temperatures can either delay (Feehan et al. 2018) or accelerate (Garcia et al. 2018) development. Multi-stressor experiments on marine organisms from species' range limits should help in elucidating patterns of climate change impacts.

Ocean temperatures in the Northwest Atlantic are rising at twice the rate of the global average (Smith et al. 2010). In addition, the Northwest Atlantic is experiencing a decrease in pH at twice the rate of the global average (Snyder et al. 2019). These trends increase the urgency to

understand the combined effects of MHWs and OA on Northwest Atlantic marine species. MHWs are periods of anomalously warm ocean temperatures that last for at least 5 days (Hobday et al. 2018). In an effort to characterize the impacts of MHWs on ocean ecosystems, Hobday et al. (2018) categorized the intensity of these events from moderate (Category I) to extreme (Category IV), representing a range of anomalies of sea surface temperatures. The categories are defined by the extent to which temperature rises above the 90th percentile based on a 30-year historical baseline (Hobday et al. 2018). For example, a Category I MHW event would be an anomaly that is 1–2 times the difference between the 90th percentile and the climatological mean, a Category II MHW event would be 2–3 times the difference, a Category III MHW event would be 3–4 times the difference, and a Category IV MHW event would be greater than 4 times the difference (Hobday et al. 2018). This framework has allowed for broad comparisons of MHWs among disparate marine ecosystems (Hobday et al. 2018).

Currently, the average pH of the ocean is 8.1 (Garcia et al. 2018). However, it is predicted that by 2100, the average pH of the ocean will drop to between pH 7.8 and 7.6 (Clark et al. 2008). Ocean acidification can change the marine carbonate saturation state, which negatively impacts organisms that depend on oceanic carbonate chemistry (Fitzer et al. 2014). These changes adversely affect organisms that produce calcium carbonate exoskeletons and endoskeletons, such as molluscs and echinoderms (Sheppard Brennand et al. 2010, Clark et al. 2009, Dupont et al 2010). Analysis of seawater chemistry shows that the North Atlantic Ocean is progressively experiencing declines in pH, which could be detrimental to marine calcifying organisms (Bates et al. 2012, García-Ibáñez et al. 2016).

Here I use a model organism, the Atlantic purple sea urchin *Arbacia punctulata,* to gain insights into the individual and combined effects of climate stressors on a species at its cold range

limit, the Northwest Atlantic. I examine the combined effects of MHWs and OA in the laboratory on the sensitive embryonic stage of the sea urchin. I collected adult *A. punctulata* for spawning from Narragansett Bay, Rhode Island, USA. I test the hypothesis that *A. punctulata* will experience improved rates of embryonic development when exposed to MHWs at its cold range limit, but that this response will be modulated by negative impacts of OA in either an additive or interactive manner.

METHODS

Animal Collection and Maintenance

On 6 May 2019, 20 individuals of *A. punctulata* were collected from 2 m depth by divers with SCUBA at Fort Wetherill, Jamestown, at the mouth of Narragansett Bay, Rhode Island. Narragansett Bay is an estuary that includes the Sakonnet River, Mount Hope Bay, the Taunton River and opens into the Atlantic Ocean. Rhode Island is at the northernmost range limit of *A. punctulata*. Sea urchins were maintained in a closed saltwater aquarium system at Montclair State University containing reverse osmosis water mixed with Instant Ocean sea salt to a salinity of 30 ppt (hereafter "artificial seawater"). The saltwater aquarium system was maintained at a temperature of 21.5ºC with an AquaEuroUSA chiller. Filtration occurred with a sump containing Bio Balls. Effluent water was passed through a UV light sterilizer to minimize microbial growth. Sea urchins were fed *ad libitum* on blades of *Alaria esculenta* sourced from Maine, USA (Maine Coast Sea Vegetables).

Experimental Trials

Two experimental trials were performed in the laboratory to test the combined effects of a simulated MHW and OA on *A. punctulata* early embryonic development. In each trial, 2 levels of temperature (ambient and MHW) and pH (ambient and OA) were combined in a fully factorial experimental design. An "ambient" temperature of 21.8ºC was selected to represent the historical baseline sea temperature in Narragansett Bay. This baseline was defined as mean sea surface temperature over 30 years from 1983 through 2013 (Hobday et al. 2018) for the month of July since spawning in *A. punctulata* usually occurs between June and August (Lawrence 2013). A "MHW" temperature of 27.5ºC was selected to represent a Category III MHW in July in Narragansett Bay as defined by Hobday et al. (2018). The Category III MHW was calculated by finding the 90th percentile, subtracting the average July temperature, and multiplying this difference by three (Hobday et al. 2018). Category III MHWs are predicted to become more common with climate change (Hobday et al. 2018). Sea surface temperature data were accessed from the University of Rhode Island's Narragansett Bay Long-Term Plankton Time Series (https://web.uri.edu/plankton/data/). For the pH treatment, an "ambient" pH of 8.1 was selected to represent the current average pH of the ocean (Logan 2010), and an "OA" pH of 7.6 was selected as the average pH of the ocean is predicted to decline as low as pH 7.6 by the year 2100 (Clark et al. 2008).

For each trial, spawning was induced in adult *A. punctulata* via injection with 1–1.5 mL of 0.53 M KCL solution through the peristomal membrane (Strathmann 1987). In Trial 1 (12 July 2019), spawning occurred in five males (38.2 ± 2.59 mm test diameter, TD) and five females (37± 4.8 mm TD). In Trial 2 (29 July 2019), spawning occurred in three males (33.7 ± 4.9 mm TD) and four females (29.5 ± 1.9 mm TD). In each trial, the resultant eggs were rinsed with artificial

seawater at ambient conditions and combined from all females to a total volume of 400 mL. Eggs were then equally divided among replicate 4.0 L glass jars containing 500 mL of treatment water. To commence fertilization, one drop of concentrated sperm combined from all males was diluted in 10 mL of ambient artificial seawater and added to each jar containing eggs and homogenized by stirring (Strathmann 1987). In Trial 1, there were 3 replicate jars for each of the four treatment combinations (12 jars total) and in Trial 2 there were 2 replicates (8 jars total).

The "ambient" temperature treatment was achieved by placing treatment jars in a 170 L (50 cm x 53 cm x 93 cm) water bath maintained by an AquaEuroUSA chiller. The "MHW" temperature treatment was achieved with an identical water bath maintained by a combination of an AquaEuroUSA chiller and Eheim Aquarium heater. Digital thermometers and Onset Hobo temperature loggers (1-h interval) were used to monitor and record the temperature in the water baths. To achieve the "OA" pH treatment, carbon dioxide from a 22.7 L tank was bubbled directly into the jars via an IKS Aquastar pH stat system. IKS Aquastar pH probes connected to the pH stat system were used to monitor pH in the jars (5-min intervals). In addition, an Oaklon PCTS5 pH probe was used to measure the pH of all jars daily. The "ambient" pH treatment consisted of artificial seawater with no carbon dioxide added. All jars received constant aeration with a Pawfly brand air stone.

Embryonic Development

To assess development of the embryos, photomicrographs were taken with a Spot Insight microscope camera and Ziess Lab.A1 microscope at 2 hours post-fertilization in Trial 1, when >95% of embryos under ambient conditions were expected to have reached fertilization (Strathmann 1987), and at 1, 2, and 3 hours post-fertilization in Trial 2 to examine early temporal

patterns of development. For the photomicrographs, one aliquot of 1 mL of treatment water containing embryos was destructively sampled from each treatment jar. An average of 4 photographs were taken per treatment jar to visualize embryos in the aliquot. The 4 subsamples were pooled for all analyses (see Statistical Analysis). All photographs combined contained on average 33.4 ± 19.8 embryos per jar. A total of 668 embryos were photographed in both trials combined.

Embryos within each photograph were categorized into 5 developmental stages: not fertilized (unfertilized eggs), fertilized (fertilized eggs), 2-cell stage (when the first cleavage of the cell occurs), 4-cell stage (when the embryo divides to contain four cells), and >4-cell stage (when the embryo contains greater than four cells). The percentage of embryos at each developmental stage was calculated as the number of embryos at a given stage divided by the total number of embryos counted within a jar. In cases where the stage could not be discerned from a photograph (e.g. an embryo was out of focus), the embryo was not counted (< 0.1 % of embryos).

For all analyses, data were pooled within jars into 3 groupings, which included the: 1) percentage of embryos at the fertilized stage or a higher level of development (sum of percentage of embryos fertilized and at 2-cell, 4-cell, and >4-cell stage), 2) percentage of embryos at the 2-cell stage or a higher level of development (sum of percentage of embryos at 2-cell, 4-cell, and >4-cell stage), and 3) percentage of embryos at the 4-cell stage or a higher level of development (sum of percentage of embryos at the 4-cell and >4-cell stage) (see Statistical Analysis). The data were pooled in this fashion for ease of interpretation, since if grouping was not conducted, developmental success (e.g., percentage of "fertilized" embryos) would artifactually appear to decline through time as embryos transitioned from the "fertilized" to "2-cell" stage and so forth.

Seawater Physicochemical Parameters

For each trial, 307 ml of artificial seawater was treated with 153.5 µl mercuric chloride to prepare the sample for seawater physicochemical analysis. To calculate the total alkalinity (TA) for each treatment, a sample of seawater was titrated using an alkalinity titrator system. Dissolved inorganic carbon (DIC) was also measured. Carbon dioxide partial pressure (pCO_2), saturation level of calcite (Ωc), and saturation level of aragonite (Ωa) were calculated using the CO_2Calc software with measurements of TA, pH, salinity, and temperature based on the constants K1 and K2 and the NBS pH scale.

Statistical Analysis

To examine the combined effects of a simulated MHW and OA on the early development of embryos (2 hours post-fertilization), separate 3-way ANOVAs were conducted on the effects of temperature (2 levels, fixed: Ambient and MHW), pH (2 levels, fixed: Ambient and OA), and trial (2 levels, random) on the percentage of embryos reaching the 1) fertilized stage or a higher level of development, 2) 2-cell stage or a higher level of development, and 3) 4-cell stage or a higher level of development.

To examine the temporal patterns of the combined effects of a MHW and OA on the development of embryos, separate repeated measures ANOVAs were conducted on the effects of temperature (2 levels, fixed: Ambient and MHW), pH (2 levels, fixed: Ambient and OA), and hour (repeated measure: 1, 2, and 3 hours post-fertilization) on the percentage of embryos in Trial 2 reaching the 1) fertilized stage or a higher level of development, 2) 2-cell stage or a higher level of development, and 3) 4-cell stage or a higher level of development.

Due to a malfunctioning pH sensor within the pH stat system, one replicate of the ambient temperature and OA pH treatment combination in Trial 2 was excluded from the analyses.

Levene's test was used to test for assumptions of homogeneity of variances ($\alpha = 0.05$). Only minor violations were detected following arcsine transformation of the percentage data (assumptions were met at $\alpha = 0.10$). Given that ANOVA is robust to such minor violations, I proceeded with the analysis. For repeated measures ANOVA, the sphericity assumption was tested with Mauchly's test and no violations were detected ($\alpha = 0.05$). All statistical analyses were performed using TIBCO Statistica Desktop.

RESULTS

In both trials, the "ambient" and "MHW" temperatures were maintained within 0.35ºC of the desired treatment temperatures (Table 1). Furthermore, in both trials, "ambient" and "OA" pH were maintained within 0.11 units of the desired treatment pH (Table 1). The lower pH in the OA treatment as compared to the ambient treatment was associated with decreased aragonite and calcite in the seawater (Table 1).

Analysis of the early development (2 hours post-fertilization) of *A. punctulata* embryos when exposed to the treatments showed a significant main effect of pH for all three developmental stages (fertilized and a greater level of development, 2-cell and a greater level of development, and 4-cell and a greater level of development) (Table 2). Embryos that were exposed to OA experienced slower development as compared to embryos exposed to ambient pH (Figure 1).

There was also a significant main effect of temperature for the 2-cell and a greater level of development and the 4-cell and a greater level of development stages, and a marginally non-significant effect of temperature for the fertilized and a greater level of development stage (Table

2). Embryos that were exposed to a MHW temperature generally experienced faster development as compared to embryos exposed to ambient temperature (Figure 2).

An interaction between temperature and pH on development would indicate that the combination of treatments is non-additive (synergistic/antagonistic). However, there was no interaction observed between temperature and pH for any developmental stage (Table 2).

There was a significant effect of trial on development for the fertilized and a greater level of development and 2-cell and a greater level of development stages (Table 2). Development was slower overall in Trial 1 than in Trial 2 for these stages (Figure 2). There was a significant interaction between trial and temperature on development for the 4-cell and a greater level of development stage (Table 2), with a greater effect of temperature on development in Trial 2 (Figure 2).

Analysis of the temporal patterns of development (1, 2, and 3 hours post-fertilization) of *A. punctulata* embryos when exposed to the treatments showed main effects of both temperature and pH for all three developmental stages (fertilized and a greater level of development, 2-cell and a greater level of development, and 4-cell and a greater level of development) (Table 3). Embryos that were exposed to MHW temperature experienced faster development as compared to embryos exposed to ambient temperature and embryos exposed to OA pH experienced slower development as compared to embryos exposed to ambient pH (Figure 3). Again, there was no interaction between temperature and pH for any developmental stage (Table 3).

There was a significant effect of hour on development for the 2-cell and a greater level of development and the 4-cell and a greater level of development stages. There was a marginal effect of hour for the fertilized and a greater level of development stage. There was no interaction between hour and temperature or pH (Table 2). The percentage of embryos within each stage

generally increased through time consistently among treatments aside from cases where 100% development was already achieved by the 1st or 2nd hour (Figure 3).

DISCUSSION

I determined that *A. punctulata* embryos respond to multiple climate change stressors in an additive manner. Exposure to low pH significantly delayed rates of development for all three developmental stages at 2 hours post-fertilization. However, exposure to increased temperature significantly improved rates of development for both the 2-cell and a greater level of development and the 4-cell and a greater level of development stages; although this effect was weaker for the fertilized and a greater level of development stage, with a marginally nonsignificant effect on development. Examination of the effects of the treatments on development at 1, 2, and 3 hours post-fertilization confirms that *A. punctulata* embryos at all three stages of development experienced declines in rates of development when exposed to low pH and improved rates of development when exposed to high temperature.

These findings are consistent with previous studies on the effects of temperature and pH on larvae of other sea urchin species of the genus *Arbacia*. For example, Garcia et al. (2018) found that larval development of *Arbacia lixula* is delayed under low pH and increases under rising temperatures. Exposure to lower pH also caused lower fertilization rates and larval survival in *Arbacia lixula* (Garcia et al. 2018). In addition, a study on *Arbacia dufresnei* found that larvae exposed to low pH experienced a delay in development (Catarino et al. 2012). Exposure to acidified conditions caused a significant decrease in postoral arm length in larval *Arbacia dufresnei* which may increase susceptibility to predation and decrease recruitment success (Catarino et al. 2012).

The results of my study indicate that *A. punctulata* population dynamics could be positively impacted at the species' cold range limit by a future increase in the occurrence of Category III MHWs if the observed accelerated embryonic development yields improved larval recruitment. For example, recruitment pulses of green sea urchins (*Strongylocentrotus droebachiensis*) in Atlantic Canada have been linked to unusually warm spring temperatures for larval development (Hart and Scheibling 1988). However, if fertilization, and larval development, growth, or survival are significantly impacted at low pH, this could decrease recruitment success into the adult benthic population. Indeed, my finding that exposure to low pH delays development of *A. punctulata* embryos suggests that potentially improved recruitment of the species with MHWs is likely to be hindered by future OA.

Changes to marine environments are occurring rapidly and are not fully understood (Garcia et al. 2018). Coastal marine ecosystems have been identified as at particularly high risk from ongoing and future climate change (IPCC 2019). MHWs and OA can imperil marine ecosystems and their diverse inhabitants. Therefore, it is imperative to gain an understanding of the consequences of climate change on coastal ecosystems so that further impacts can be predicted and mitigated (IPCC 2019). Like many regions of the Northwest Atlantic, substantial ocean warming has occurred in Narragansett Bay (~1.2ºC from 1950 to 2010; Smith et al. 2010), indicating that this ecosystem is undergoing rapid change. The demonstrated tolerance of *A. punctulata* embryos to MHWs is likely due to the fact that the range of this species extends into the Caribbean Sea where it is exposed to temperatures as high as 31ºC (Hill and Lawrence 2006). This value may be near an upper thermal threshold for survival of this species and is well above my MWH treatment temperature (27.5ºC). Indeed, these sea urchins are likely persisting at a sub-optimal temperature in Narragansett Bay.

Not only is sea urchin development directly affected by the aforementioned stressors, but the changing marine environment also may have indirect effects on development through impacts on larval food sources. The pelagic larvae of *A. punctulata* feed on phytoplankton, and ocean warming has been shown to decrease the productivity of phytoplankton (Gittings et al. 2018). Importantly, for sea urchin larvae, there is a positive relationship between increasing temperature and increasing requirements of metabolic energy (Feehan et al. 2018). Therefore, a reduction in the available phytoplankton could negatively impact larval development and delay metamorphosis due to energetic limitation. A longer larval duration has been shown to increase mortality rates due to the increased time period of exposure to planktonic predators and likelihood of offshore advection away from settlement habitat (Vaughn and Allan 2010).

My seawater physicochemistry results show a pattern of decreased calcite and aragonite saturation levels in the OA treatments when compared to the ambient pH treatments. Calcite and aragonite are two crystalline forms of calcium carbonate, a mineral that marine calcifying organisms need to build their endoskeletons and exoskeletons (Dupont et al. 2010). The Hector v1.1 model predicts that future aragonite saturation levels will be reduced by fifty percent by the year 2100 (Hartin et al. 2016). This reduction in calcite and aragonite is predicted to negatively impact all marine calcifying organisms.

My results indicate a mitigating effect of MHWs on OA at the cold range limit of a marine calcifier. Thus, this local population of *A. punctulata* may experience a northern range expansion in response to combined ocean warming and OA. It is important to note that my examination of the effects of temperature and pH is limited to the earliest planktonic life-history stage (embryos) and it is therefore unknown how the later planktonic (larval) and benthic (adult) stages will respond to such conditions. Additionally, information on the recruitment dynamics of this species in the

field is very limited. Consequently, it remains to be determined exactly how climate change will impact populations of *A. punctulata*.

The ocean's ability to capture and sequester both heat and carbon is negatively impacting marine ecosystems (IPCC 2019). Benthic habitats in the Northwest Atlantic are particularly impacted, with observations that kelp forest ecosystems are shifting from a kelp-dominated to a turf algal-dominated ecosystem state (Filbee-Dexter et al. 2016, Feehan et al. 2019). *A. punctulata* has been observed grazing deleterious algal turfs in Narraganset Bay, opening up space for temperate corals *Astrangia poculata* (Grace and Feehan 2020). This evidence for an important ecological role of *A. punctulata* in the kelp/turf ecosystem, in combination with my observations that ocean warming and OA can affect its life history, make it a useful model for continued study of climate change in Northwest Atlantic marine ecosystems.

TABLES AND FIGURES

Table 1. Seawater physicochemical parameters in each treatment within each trial. Temp: seawater temperature (mean ± SD), pH: seawater pH (mean ± SD), Sal: salinity (mean ± SD), pCO2: CO2 partial pressure, TA: Total alkalinity, Ωc: Saturation level of calcite, Ωa: Saturation level of aragonite.

Trial 1				
	Ambient Temperature		MHW Temperature	
	OA pH	Ambient pH	OA pH	Ambient pH
Temp	21.45 ± 0.31	21.45 ± 0.31	27.62 ± 0.27	27.62 ± 0.27
pH	7.56 ± 0.04	8.10 ± 0	7.54 ± 0.11	8.06 ± 0
Sal	30 ± 0	30 ± 0	30 ± 0	30 ± 0
pCO2	2581.21	703.36	3416.21	673.43
TA	3209.10	3086.80	3149.50	2858.90
Ωc	1.85	5.05	1.79	5.58
Ωa	1.20	3.26	1.17	3.66
Trial 2				
	Ambient Temperature		MHW Temperature	
	OA pH	Ambient pH	OA pH	Ambient pH
Temp	21.66 ± 0.20	21.66 ± 0.20	27.55 ± 0.33	27.55 ± 0.33
pH	7.57	8.07 ± 0.02	7.71 ± 0.02	8.18 ± 0.03
Sal	30 ± 0	30 ± 0	30 ± 0	30 ± 0
pCO2	2973.38	653.68	1378.51	557.50
TA	2897.50	2868.80	2056.43	3098.90
Ωc	1.36	4.73	2.77	7.50
Ωa	0.88	3.05	1.82	4.93

Table 2. ANOVAs of the effect of temperature (Temp) (2 levels, fixed: Ambient and MHW), pH (2 levels, fixed: Ambient and OA), and Trial (2 levels, random) at 2 hours post-fertilization on the percentage of embryos at 3 developmental stages: fertilized or greater level of development (F+), 2-cell or greater level of development (2+), and 4-cell or greater level of development (4+). **Bold** values are significant at $p \leq 0.05$. Percentage data were arcsine transformed prior to the analysis.

F+				
	df	MS	F	p
Trial	**1**	**0.307**	**5.32**	**0.042**
Temp	1	0.760	2.14	0.092
pH	**1**	**0.158**	**3.10**	**0.050**
Trial×Temp	**1**	**0.356**	**6.19**	**0.030**
Trial×pH	1	0.051	0.89	0.365
Temp×pH	1	0.031	0.53	0.481
Trial×Temp×pH	1	0.112	1.95	0.191
Error	11	0.058		
2C+				
	df	MS	F	p
Trial	**1**	**0.267**	**7.03**	**0.023**
Temp	**1**	**0.666**	**5.64**	**0.014**
pH	**1**	**0.130**	**6.84**	**0.008**
Trial×Temp	1	0.118	3.11	0.105
Trial×pH	1	0.019	0.512	0.489
Temp×pH	1	0.014	0.361	0.560
Trial×Temp×pH	1	0.003	0.074	0.791
Error	11	0.038		
4C+				
	df	MS	F	p
Trial	1	0.098	2.81	0.122
Temp	**1**	**1.138**	**8.56**	**0.004**
pH	**1**	**0.096**	**6.86**	**0.008**
Trial×Temp	1	0.133	3.81	0.077
Trial×pH	1	0.014	0.409	0.536
Temp×pH	1	0.007	0.204	0.660
Trial×Temp×pH	1	0.001	0.023	0.883
Error	11	0.035		

Table 3. Repeated measures ANOVAs of the effect of temperature (Temp) (2 levels, fixed: Ambient and MHW), pH (2 levels, fixed: Ambient and OA), and Hour (repeated measure: 1, 2, and 3 h) on the percentage of embryos at 3 developmental stages: fertilized or greater level of development (F+), 2-cell or greater level of development (2+), and 4-cell or greater level of development (4+). **Bold** values are significant at $p \leq 0.05$. Percentage data were arcsine transformed prior to the analysis.

F+				
	df	MS	F	p
Between-subject effects				
Temp	**1**	**1.59**	**29.8**	**0.012**
pH	**1**	**0.664**	**12.5**	**0.039**
Temp×pH	1	0.067	1.26	0.343
Error	3	0.053		
Within-subject effects				
Hour	2	0.267	3.21	0.112
Hour×Temp	2	0.056	0.676	0.544
Hour×pH	2	0.025	0.297	0.753
Hour×Temp×pH	2	0.024	0.290	0.758
Error	6	0.083		
2C+				
	df	MS	F	p
Between-subject effects				
Temp	**1**	**1.53**	**55.7**	**0.005**
pH	**1**	**0.304**	**11.1**	**0.045**
Temp×pH	1	0.009	0.337	0.603
Error	3	0.027		
Within-subject effects				
Hour	**2**	**0.312**	**9.87**	**0.013**
Hour×Temp	2	0.004	0.121	0.888
Hour×pH	2	0.005	0.149	0.864
Hour×Temp×pH	2	0.019	0.586	0.586
Error	6	0.032		
4C+				
	df	MS	F	p
Between-subject effects				
Temp	**1**	**1.91**	**167**	**0.001**
pH	**1**	**0.230**	**20.2**	**0.021**
Temp×pH	1	0.004	0.373	0.585
Error	3	0.011		
Within-subject effects				
Hour	**2**	**0.564**	**33.7**	**<0.001**
Hour×Temp	2	0.037	2.20	0.192
Hour×pH	2	0.003	0.166	0.851
Hour×Temp×pH	2	0.025	1.48	0.300
Error	6	0.017		

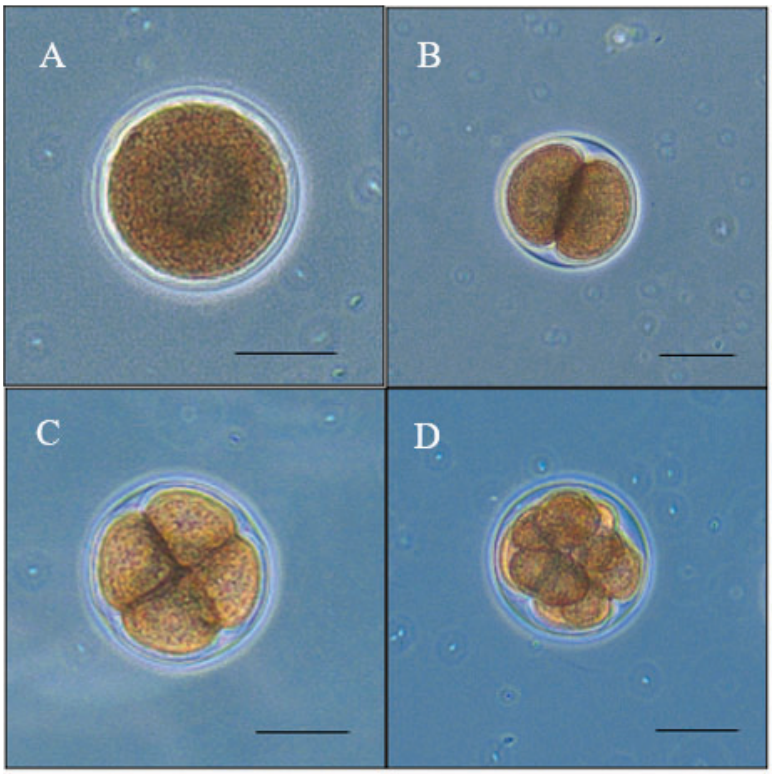

Figure 1. *Arbacia punctulata* embryonic development. Each photo is taken 2 hours post-fertilization showing (**A**) a fertilized egg, and embryos at the (**B**) 2-cell, (**C**) 4-cell, and (**D**) >4-cell stage of development. Scale bars = 40 μm.

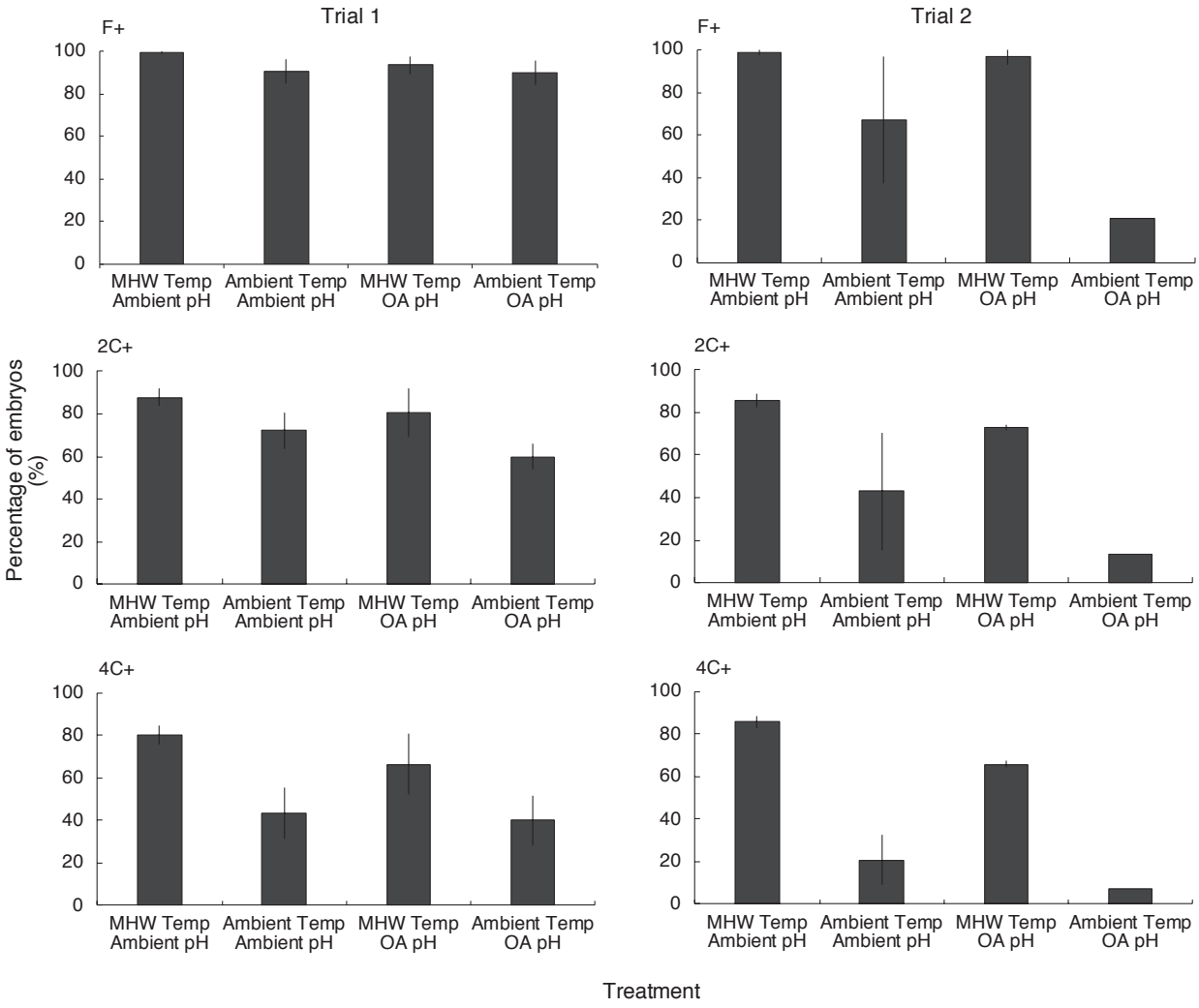

Figure 2. Percentage of embryos (%)in 4 treatment combinations of temperature (Temp) (ambient and MHW) and pH (ambient and OA) at 2 hours post-fertilization that have reached each of 3 developmental stages: top panel = fertilized or a greater level of development (F+); middle panel = 2-cell or a greater level of development (2C+); and bottom panel = 4-cell or a greater level of development (4C+). Trial 1 is shown in the left panel and Trial 2 in the right. Errors are standard errors.

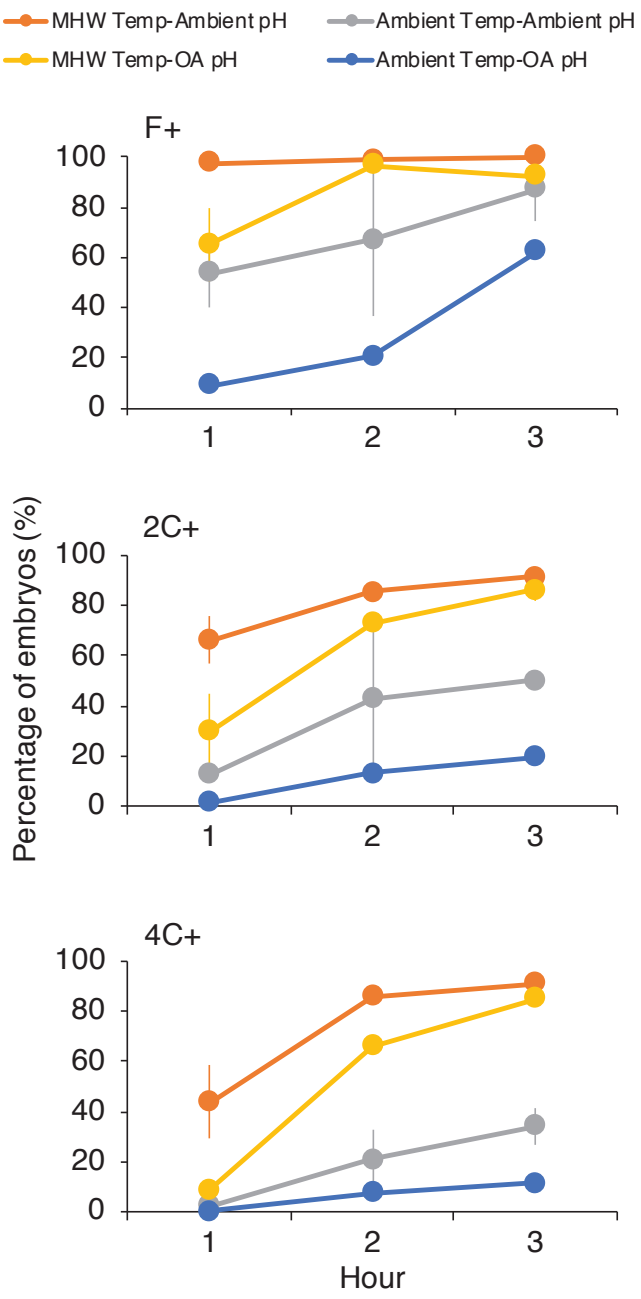

Figure 3. Percentage of embryos (%) at 1, 2 and 3 hours post-fertilization in 4 treatment combinations of temperature (Temp) (ambient and MHW) and pH (ambient and OA) in Trial 2 that have reached each of 3 developmental stages: top panel = fertilized or a greater level of development (F+); middle panel = 2-cell or a greater level of development (2C+); and bottom panel = 4-cell or a greater level of development (4C+). Errors are standard errors.

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