# Effect of Seawater Acidity on the Initial Development of Kumamoto Oyster Larvae Crassostrea sikamea (Amemiya, 1928)

**Article** *in* Journal of Shellfish Research · April 2020

DOI: 10.2983/035.039.0103

CITATIONS
4

READS
152

**7 authors**, including:

Francisco Antonio Flores-Higuera
Autonomous University of Sinaloa
**11** PUBLICATIONS **30** CITATIONS
SEE PROFILE

Héctor Reyes-Bonilla
Universidad Autónoma de Baja California Sur
**376** PUBLICATIONS **6,971** CITATIONS
SEE PROFILE

Irasema Luis
Universidad Autónoma de Sinaloa, Mazatlán
**18** PUBLICATIONS **336** CITATIONS
SEE PROFILE

José Manuel Mazón-Suástegui
Centro de Investigaciones Biológicas del Noroeste S.C.
**142** PUBLICATIONS **1,745** CITATIONS
SEE PROFILE

# EFFECT OF SEAWATER ACIDITY ON THE INITIAL DEVELOPMENT OF KUMAMOTO OYSTER LARVAE CRASSOSTREA SIKAMEA (AMEMIYA, 1928)

**Article** *in* Journal of Shellfish Research · November 2020

DOI: 10.2983/035.039.0100

CITATIONS
0

READS
52

**7 authors**, including:

Francisco Antonio Flores-Higuera
Universidad Autónoma de Sinaloa
**10** PUBLICATIONS **10** CITATIONS
SEE PROFILE

Héctor Reyes-Bonilla
Autonomous University of Baja California Sur
**311** PUBLICATIONS **4,493** CITATIONS
SEE PROFILE

Irasema Luis
Universidad Autónoma de Sinaloa, Mazatlán
**18** PUBLICATIONS **186** CITATIONS
SEE PROFILE

José Manuel Mazón-Suástegui
Centro de Investigaciones Biológicas del Noroeste
**105** PUBLICATIONS **907** CITATIONS
SEE PROFILE

*Journal of Shellfish Research*, 39(1):21-30 (2020).

# EFFECT OF SEAWATER ACIDITY ON THE INITIAL DEVELOPMENT OF KUMAMOTO OYSTER LARVAE *CRASSOSTREA SIKAMEA* (AMEMIYA, 1928)

**FRANCISCO ANTONIO FLORES-HIGUERA,[1,3] HÉCTOR REYES-BONILLA,[2]* IRASEMA E. LUIS-VILLASEÑOR,[3] JOSÉ MANUEL MAZÓN-SUÁSTEGUI,[4] JOSÉ ANTONIO ESTRADA-GODINEZ,[3] PATRICIA HERNANDEZ-CORTÉS[4] AND JUAN MANUEL AUDELO-NARANJO[3]**

[1]*Doctorado en Ciencias en Recursos Acuáticos, Facultad de Ciencias del Mar Universidad Autónoma de Sinaloa, Paseo Claussen S/N, Col. Centro, Mazatlán 82000, México;* [2]*Departamento Académico de Biología Marina, Universidad Autónoma de Baja California Sur, 5.5 km Carretera al sur, Apartado Postal 19-B, Col. El Mezquitito, La Paz 23080, México;* [3]*Facultad de Ciencias del Mar, Universidad Autónoma de Sinaloa, Paseo Claussen S/N, Col. Centro, Mazatlán 82000, Sinaloa México;* [4]*Centro de Investigaciones Biológicas del Noroeste, Calle Instituto Politécnico Nacional 195, Col. Playa Palo de Santa Rita, La Paz 23090, México*

***ABSTRACT*** The oceans have absorbed more than 40% of the carbon dioxide ($CO_2$) generated by anthropogenic activities, causing a decrease in the average pH of 0.1 units in seawater since preindustrial times. This phenomenon has been called "ocean acidification." This change poses serious threats to the cultivation of oysters and especially to larval and spat production, activities carried out in coastal and estuarine areas, where pH levels are currently below the IPCC scenario for the year 2100 of pH = 7.8. The goal of the present work was to experimentally evaluate the effect of simulated acidification (pH 7.39 ± 0.04) on the culture in a short-term trial of Kumamoto oyster larvae *Crassostrea sikamea* taking the current ocean pH conditions (pH 8.116 ± 0.023) as a reference. The evaluation was carried out in an experimental system with continuous water flow and pH manipulation by $CO_2$ bubbling. Veliger larvae (6-day-old postspawn) were cultured at a density of six larvae $mL^{-1}$ and fed with a monoalgal diet based on *Isochrysis galbana* at 30,000 cells $mL^{-1}$. Mortality (%) and growth (shell length in μm) were evaluated, and damage to larval morphology (determined using scanning electron microscopy) and $Ca^{2+}$ contents in the shells (%) were quantified by X-ray fluorescence. The results show a high sensitivity of *C. sikamea* veliger larvae to low pH levels with negative impacts on growth and survival, decreases in the $Ca^{2+}$ concentrations of the shells, and the presence of morphological anomalies during the prodissoconch I stage, which were observed after the first 24 h of cultivation in experimental conditions and became progressively more evident, especially by the sixth day of culture. Acute exposure to a low pH and a saturation of aragonite (Ωar) <1 caused poor calcification in *C. sikamea* larvae, causing negative effects on larvae, such as shell lesions during development, smaller larvae, and higher mortality and relation to a control pH.

***KEY WORDS:*** *Crassostrea sikamea*, ocean acidification, Ostreidae, molluscs, calcification of shells, larviculture

## INTRODUCTION

Carbon dioxide ($CO_2$) is the most important greenhouse gases on the planet (Lacis et al. 2010), and its atmospheric concentration has increased by more than 140% from 280 ppm during the preindustrial era to above 400 ppm (NOAA 2016). The oceans, in turn, have absorbed approximately 40% of anthropogenic $CO_2$ emissions (Caldeira & Wickett 2003, Sabine et al. 2004) and have become the most important deposit of this greenhouse gas. The increased atmospheric $CO_2$, when mixed with seawater, forms carbonic acid ($H_2CO_3$). Depending on the pH of the seawater, carbonic acid will dissociate into bicarbonate ($HCO_3^-$) and carbonate ions ($CO_3^{2-}$) and release hydrogen ions (H+) (Sabine et al. 2004). Increase in hydrogen ions causes a decrease in sea pH, which is known as "ocean acidification (OA)." Along with the increase in the amount of $H^+$ ions and the consequent pH decrease in seawater, there have been marked decreases in two indicators of the saturation state of calcium carbonate ($CaCO_3$): Ω aragonite ($\Omega_{ar}$) and Ω calcite ($\Omega_{cal}$), which are two mineral forms of calcium carbonate that most bivalves use to construct shells (Feely et al. 2004, Sabine et al. 2004, Fabry et al. 2008, Barton et al. 2015). This reduction represents a great expenditure energetic necessary to build and maintain calcified structures formed mainly by calcium carbonate (Hofmann et al. 2010, Gazeau et al. 2011, Dauphin et al. 2013, Gazeau et al. 2013, Barton et al. 2015, Gaylord et al. 2015). This situation will pose a significant risk to coastal ecosystems because bivalves are of great ecological importance as ecosystem engineers that provide habitat, shelter, and food for numerous benthic, demersal, and nektonic species (Gutiérrez et al. 2003, Kurihara et al. 2007, Waldbusser et al. 2013, Waldbusser & Salisbury 2014, Barton et al. 2015). Likewise, these changes represent important risks for human welfare because of the economic value of wild-caught and aquaculture-raised bivalves (Barton et al. 2015).

The formation of the prodissoconch I (PDI) is a biological process that is highly energy demanding; for example, Pacific oyster larvae develop from an embryo (shell 0%) to oyster larvae with a hinge (~80%–100% shell formed) in a period of less than 24 h and probably in a time as short as 6 h (Barton et al. 2012), which is a feat that represents extensive energy drainage. Efforts have been made to characterize the possible effects of OA on commercially important bivalves, following scenarios of an expected change in aragonite saturation state, temperature, and pH (Barros et al. 2013, Ginger et al. 2013,

*Corresponding author. E-mail: hreyes@uabcs.mx
DOI: 10.2983/035.039.0103

Gazeau et al. 2014). The effects of the aragonite saturation state on the development of bivalve molluscs are supported by experimental studies where shellfish larvae were subjected to aragonite subsaturation ($\Omega_{ar} < 1$) conditions with generally harmful results, such as morphological abnormalities in the dorsal margin, irregular shape of the hinge, lesions in the PDI due to deficiencies during development, and incorporation of calcium carbonate (Barros et al. 2013, Waldbusser et al. 2015).

The Pacific oyster *Crassostrea gigas* is considered a biological model for the study of the effects of OA, mainly during larval development. Barton et al. (2012, 2015) reported negative effects of seawater with naturally low pH on hatchery-reared *C. gigas* larvae, citing poor shell integrity (hindered calcification) from drastic decreases in the seawater saturation state (driven by coastal upwelling) as the cause of severe loss of production in a commercial hatchery at the Whiskey Creek Shellfish Hatchery on the Oregon Coast.

The species *Crassostrea sikamea* (Amemiya, 1928) is an important aquaculture product that has a high market price because of its high meat-shell ratio for its size (Gordon et al. 2001), maintaining a high condition index even during the summer, when the Pacific oyster is in the spawning stage and has a low condition index (Robinson 1992), which allows its harvest throughout the year, increasing its commercial value (Nosho 1989). There is information about the reproductive aspects of *C. sikamea*, including the documentation of a reproductive precocity in this species with a start of gametogenesis within only 71 days of life since spawning (35 days after settlement) and an average shell height of 3.0 mm in the oysters of a commercial hatchery in Sinaloa, northwestern Mexico (Cáceres-Martínez et al. 2012). Likewise, Zhang et al. (2019) report an initial size of functional maturity of 9–12 mm in *C. sikamea* obtained in 90 days, in progeny obtained from organisms form the wild in Guangdong, China. In Yaquina Bay, OR, gametogenesis began in May, and the first mature ovules appeared in June–July (Robinson 1992); however, there is no information on the effect of acidification on the development of *C. sikamea* larvae.

In Baja California, Mexico, *Crassostrea gigas* and *Crassostrea sikamea* were introduced for commercial culture from the west coast of the United States around 1970 (Cáceres-Martínez et al. 2012). Oyster farming spread mainly through the Baja California Peninsula and Sonora, initiating activities such as oyster spat production in commercial hatcheries. Currently, in northwest Mexico, there are several hatcheries with technical and scientific capacity and sufficient infrastructure to produce oyster spat on a commercial scale; however, a decrease of –0.7 units (pH 7.4) in the pH has been reported (Fernando Garcia et al., personal communication) in hatcheries and the oyster growing areas. Cai et al. (2011) mention that these low pH levels currently occur in estuaries and in coastal lagoons that undergo natural and anthropogenic eutrophication. In Mexico, Rodríguez-Quiroz et al. (2016) reported an average pH of 7.82 ± 0.39, with lower levels of 7.1 in January, in an oyster farm located in Sinaloa. Likewise, Páez-Osuna et al. (2016) have reported pH variations in the lagoon system of Navachiste (Sinaloa), which range from 7.4 in an area under the influence of mangroves to 8.3 in areas near the ocean water. According to the RCP 8.5 emission scenario of $CO_2$ in the atmosphere, it is expected that these pH levels will be reached by the year 2300 (Caldeira & Wickett 2005, Hartin et al. 2016); however, these are already occurring in these hatcheries and oyster growth areas in northwestern Mexico, as well as in marshes where this species of oyster grows wild, with variations of 7.3–7.97 pH (Weng & Wang 2014).

In 2014, a production of 3,524.8 tons of oysters, with a value of 1.3 million dollars was registered by Mexican producers (CONAPESCA 2015). Therefore, *Crassostrea sikamea* is of great economic importance and is widely cultivated on the northwest coast of Mexico (Cáceres-Martínez & Vásquez-Yeomans 2013). It is important to analyze the effect of these alterations on the development of *C. sikamea* would provide information concerning the potential negative effects of OA in this species and provide baseline information to implement mitigation measures in larvae and spat hatchery production for aquaculture. The objective of this study was to determine the acute effect (over six culture days) of decreased pH (a proxy for OA) on the first stage of *C. sikamea* larval development. Effects of simulated acidified ocean levels projected for the year 2300 (pH ≈ 7.4) were experimentally evaluated against the current levels on the rate of growth, survival, and calcification of PDI, by quantifying the percentage of $Ca^{2+}$ content in the larval shell. To know the possible impact of these future conditions on the development of *C. sikamea*, test organisms were compared with a control group grown under conditions similar to those present along the western coast of Mexico (pH ≈ 8.1) (Caldeira & Wickett 2005, Álvarez-Borrego 2007).

## MATERIALS AND METHODS

### *Bioassay with Larvae of* Crassostrea sikamea

A short-term, 6-day experiment was carried out using veliger larvae with an average initial size of 54.00 ± 6.75 μm (6 days postfertilization) obtained from the Acuacultura Robles S.P.R. de R.I. commercial hatchery located in La Paz, Mexico. The culture was carried out in plastic containers with capacities of 4 L at a density of six larvae $mL^{-1}$. A treatment was established with pH 7.4 (low pH) and a control treatment with a pH of 8.1 (control pH), each with five replicates. To avoid a bias in the distribution of the larvae, these were taken from one single initial group of larvae that were homogenized before test organisms were taken. All the culture tanks were placed in a water bath to maintain constant temperature conditions. A random design with interdependent replicates was used according to Cornwall and Hurd (2015).

### *Acidified Seawater System and Distribution to Larval Tanks*

Manipulation of seawater pH was performed by bubbling $CO_2$ injection according to the model developed by Fangue et al. (2010). To reach the desired pH levels (low pH), a mixture of clean, dry air with $CO_2$ was made using the MicroTrak 101 and air Smart-Trak 100C (Sierra Instruments, Monterey, CA) mass flow controllers for $CO_2$ and air. The calibration value of the airflow was 2.63 L $min^{-1}$, whereas $CO_2$ was maintained at 5.00 mL $min^{-1}$ in the experiment with low pH levels.

The seawater for the experiments was taken from a plastic reservoir of 1,000 L after being filtered at 1 micron and irradiated with UV to be later mixed with the obtained gas mixture (air-$CO_2$) in a reserve bucket gas mixture instead of bubbling these gases directly into the larval culture tank. The gas-mixing reservoir bucket consisted of a 19-L food-grade plastic bucket and lid (high-density polyethylene) with a submersible aquarium pump, which in combination with a venturi injector mixes

each $CO_2$ treatment gas with seawater by directing the water through a small orifice, creating a negative pressure and extracting the gas mixture as a very fine stream of bubbles for efficient mixing. The submersible aquarium pump provided vigorous circulation inside the reservoir as well as the water pressure needed to pump $CO_2$-treated seawater to the larval culture tanks. The float valve was used to keep the reservoir constantly filled with FSW. In the control treatment, the water was pumped directly from the 1,000-L tank of seawater at 0.5 L $min^{-1}$ to allow two independent seawater flow-through systems.

### *Food and Water Quality*

Larvae were fed a diet of the haptophyte *Isochrysis galbana* at cell densities of 30,000 cells $mL^{-1}$. Sufficient concentrations of algal cells were fed to ensure food supply was not a limiting factor in survival, growth, and development. The pH was measured every 48 h with a Beckman 32 pH meter (precision ± 0.001) that was calibrated before each use (NBS buffers 4.00 ± 0.02, 7.00 ± 0.01, and 10.00 ± 0.02 at 25°C). Total alkalinity (TA) was estimated by acid titration, every 72 h, using the volumetric method with a phenolphthalein endpoint and methyl orange, and sulfuric acid ($H_2SO_4$) as the titrant (APHA 1995). To verify the precision and accuracy of the alkalinity measurements, a sample with a known TA (reference material for oceanic $CO_2$ measurement, lot 169, AT = 2,207.03 ± 0.57 μmol $kg^{-1}$, A. Dickson, Scripps Institution of Oceanography) was used.

Salinity and temperature were determined every 48 h from the outlet water of the culture tanks of both treatments, with YSI Model 85 multiparameter measuring equipment calibrated before each use. The chemical parameters of the carbonate system were calculated using $CO_2$SYS (Lewis & Wallace 1998) with the carbonic acid dissociation constants of Mehrbach et al. (1973) and refitted in different function forms by Dickson and Millero (1987). Information on the specific values of the elements of the carbonate system, salinity, and temperature in the experimental treatments is summarized in Table 1. T1

### *Mortality, Larval Growth, and Morphology of* Crassostrea sikamea

To evaluate larval growth, a 10-mL sample was taken daily from each culture tank of each treatment. From this sample, 30 larvae were taken randomly from each tank ($n = 30 \times 5$) for treatment with a digital camera (CoolSnap-Pro), adapted in a compound microscope (Nikon Olympus BX-41, 10×). Anteroposterior measurement (length) was determined using Image-Pro Plus software (v. 7.0, Media Cybernetics, Silver Spring, MD). The mortality percentage was determined by counting live and dead larvae in a random sample of 30 larvae for the tank of each treatment ($n = 30 \times 5$) (Fig. 1). F1

**TABLE 1.**

**Water chemistry parameters during the experiment.**

| Water chemistry | Control pH | Low pH |
|---|---|---|
| pH | 8.11 ± 02 | 7.39 ± 04 |
| AT μM/kgSW | 2,030.42 ± 154.04 | 2,008.49 ± 124.93 |
| $pCO_2$ (μatm) | 263.97 ± 24.1 | 1,864.46 ± 234.0 |
| $HCO_3$ (μmol/kgSW) | 1,432.45 ± 11.56 | 1,865.29 ± 12.87 |
| $CO_3$ (μmol/kgSW) | 231.86 ± 22.15 | 55.96 ± 5.42 |
| $\Omega_{cal}$ | 5.343 ± 0.52 | 1.29 ± 0.13 |
| $\Omega_{ar}$ | 3.536 ± 0.34 | 0.853 ± 0.09 |

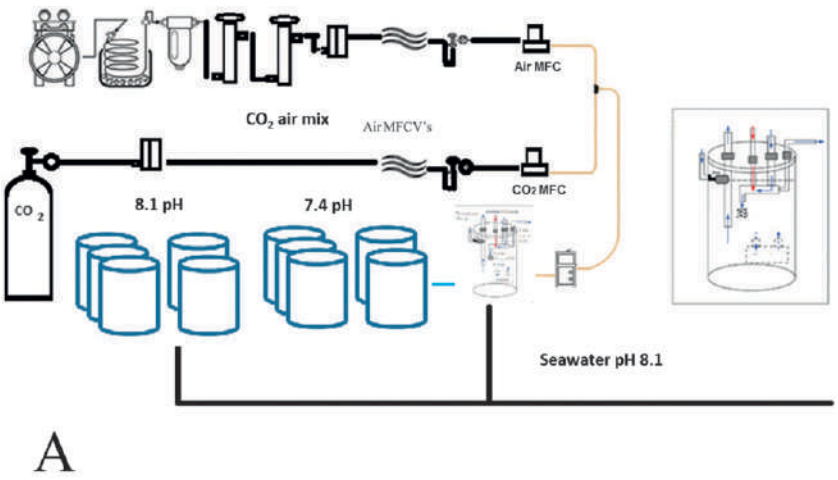
pH was determined in samples of seawater from the experimental units every 48 h. TA (AT μM/kgSW), $pCO_2$ (μatm), $HCO_3$ (μmol/kgSW), $CO_3$ (μmol/kgSW), $\Omega_{cal}$, and $\Omega_{ar}$ were calculated using $CO_2$SYS program, version 01.05. Data are means ± SD ($n = 20$).

### *Energy Dispersive X-ray Fluorescence Method and Scanning Electron Microscopy*

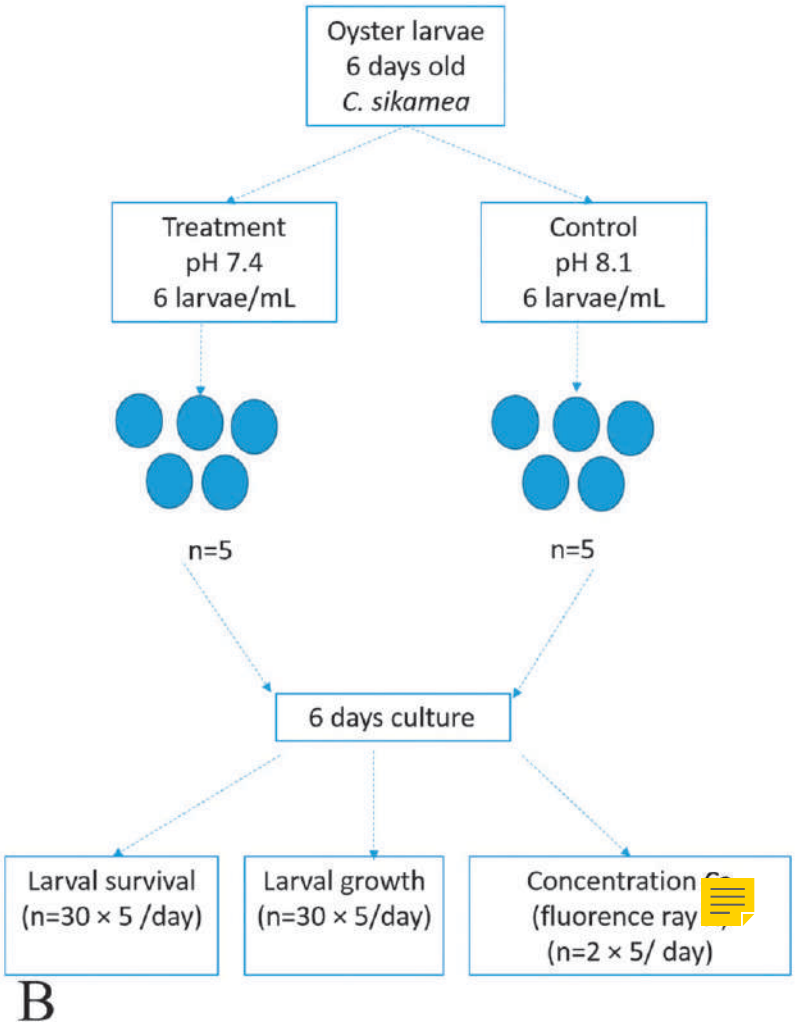
To determine the effect of low pH on the composition and morphology of the larval shell in a short-term trial, samples were taken from each culture tank at days 0, 1, 3, 5, and 6 and later fixed with a 2.5% solution of glutaraldehyde and stored at 4°C until processing. A standard method for the preparation of samples was used that consisted of dehydration in an alcohol series of 20%, 40%, 70%, 80%, and 100%; critical point drying; and mounting on metal plates. At this point, microanalysis of two larval shells per culture tank ($n = 10$ per treatment) for each treatment, prepared in this manner, was conducted using the energy-dispersive X-ray fluorescence method in a Hitachi S3000N scanning electron microscope, with an energy-dispersive X-ray spectroscopy detector (EDAX HIT S3000N) coupled to it. This analyzes the X-ray radiation generated by a sample to obtain its chemical composition (atomic) quickly and nondestructively, allowing any chemical element between $^{11}$Na and $^{92}$U to be distinguished (Mason & Nott 1980, Goldstein et al. 2003) to directly discern shell calcifications. Later, *Crassostrea sikamea* larvae were coated with gold (Goldstein et al. 2003), and the images were captured from two previously selected shells per culture tank (700×) ($n = 10$ per treatment). The prevalence of shell deformities was determined by visible damage to their morphology and basic structure, according to the criteria of Watson et al. (2009) and Barros et al. (2013).

### *Statistical Analysis*

To compare the percentage of $Ca^{2+}$ in the shells, these values were first transformed by a square root of arcsine transformation and the assumptions of normality (Shapiro–Wilk test) and homoscedasticity (Levene test). To evaluate the growth rates of *Crassostrea sikamea* larvae as a function of time (days elapsed in the experiment), linear regression models were applied to the growth curves, and the slope of the equation obtained was compared by covariance analysis. In addition, differences in larval growth and the percentage of $Ca^{2+}$ in the shells across pH treatments were assessed by repeated-measures ANOVA (RM ANOVA) using the growth and $Ca^{2+}$ in the shell per culture tank (pH: fixed factor, day: fixed repeated measurement, and tank (pH): random factor), followed by *post hoc* Tukey's tests for multiple comparisons using the appropriate mean square error. Estimates of components of variance in larval growth and the$Ca^{2+}$ in the shell were determined by restricted maximum likelihood estimation (REML) (Doncaster & Davey 2007). A logistic regression model was used to investigate the effect of two pH levels on mortality and the prevalence of shells with damage in *C. sikamea* larvae. The model predicts the probability of 1 for survival or shell with damage and 0 for mortality or normal shell, and the contrast will be through a chi-square test. Odds ratios were calculated from coefficients of variables in the final models. Statistical analyses were carried out using JMP 14 (SAS Institute Inc., Cary, NC).




A




B

**Figure 1. (A) Acidification system of seawater by bubbling $CO_2$. Modified from Fangue et al. (2010). (B) Design of the experiment, treatments, response parameters, and samples size.**

## RESULTS

### *Water Quality*

Salinity and temperature were low enough to consider the specimens in a homogeneous state during the test. Salinity during the experiment was 40 PSU, which can be considered high. La Ensenada de La Paz, BCS, Mexico, the area from where the seawater was taken for the experiment, has an anti-estuarine behavior, mainly in the summer months. The reduction of salinity with fresh water was avoided so as not to modify the chemistry of the carbonates in the experimental seawater. The results of the TA measurements of the reference material were AT = 1,942.1 ± 9.9 μmol $kg^{-1}$ (mean ± SD) ($n$ = 5 measurements), indicating an accuracy of 88.0% ± 0.4% compared with the reference material for the measurement of oceanic $CO_2$ (A. Dickson, Scripps Institution of Oceanography). The experimental conditions measured in both treatments are summarized in Table 1.

### *Larval Growth and Mortality*

ANCOVA tests showed manifest differences between the growth slopes of *Crassostrea sikamea* larvae grown at control pH and low pH ($F$ = 143.253, D.F. 1 $P < 0.001$; Fig. 2). The equations for both regressions were length (in micrometers) = 63.839 + (5.606 × elapsed experimental days) ($R^2$ = 0.693) for pH = 8.1 and length (in micrometers) = 61.166 + (5.405 × elapsed experimental days) ($R^2$ 0.733) for pH = 7.4.

A significant effect on the larval growth occurred for the factor day [RM ANOVA, $F$ (5) = 1,053.4 $P < 0.0001$], the pH treatment [pH, RM ANOVA, $F$ (5) = 255.25 $P < 0.0001$], and interactions between both (Day × pH) [RM ANOVA, $F$ (5) = 4.05 $P$ = 0.0012] (Table 2).

The differences between replicates were explored, and no significant effect on the growth of the larvae of *Crassostrea sikamea* was determined by estimating the components of the variance using REML (Table 3). The larvae of *C. sikamea* subjected to a low pH had higher mortality (62.33% ± 5.35%) than those grown at control pH (42.75% ± 3.13%). A logistic regression test applied to larval mortality showed that there was a significant effect of the pH treatment [Wald chi-square (1) = 41.957 $P \leq 0.0001$] and the day factor [Wald chi-square (6) = 183.289 $P < 0.0001$] (Table 4). The odds of larval mortality were 2.06 times higher at pH 7.4 than at pH 8.1. In addition, the odds of mortality increased over time mainly in the treatment of pH 7.4. This effect was particularly evident from days 5 and 6, for the low pH where the probabilities of larval mortality were significantly greater than the first days of culture (Table 6, Fig. 3).

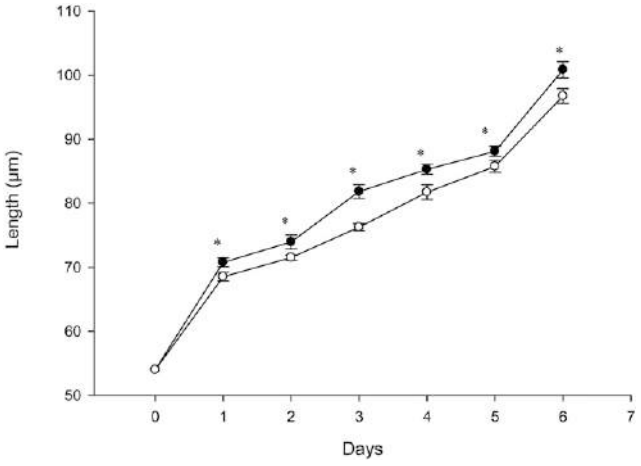



**Figure 2. Growth (mean ± SD) of *Crassostrea sikamea* larvae at two pH levels, 8.1 (•) and 7.4 (○). The significant differences between the larval lengths of both treatments are marked with an asterisk (*) α = 0.05.**

**TABLE 2.**

**Fixed effects test RM ANOVA for larval growth and $Ca^{2+}$ content in the larval shells of *Crassostrea sikamea*.**

| Source | *N* parameters | DF | DF D | *F* | *P* |
|---|---|---|---|---|---|
| **A. $Ca^{2+}$ in larval shell** | | | | | |
| pH | 1 | 1 | 8 | 124.729 | <0.0001* |
| Days | 2 | 2 | 16 | 9.5235 | 0.0019* |
| pH × days | 2 | 2 | 16 | 12.9232 | 0.0005* |
| **B. Larval growth** | | | | | |
| pH | 1 | 1 | 8 | 255.2598 | <0.0001* |
| Day | 5 | 5 | 1,780 | 1,053.41 | <0.0001* |
| Day × pH | 5 | 5 | 1,780 | 4.0521 | 0.0012* |

### *Scanning Electron Microscopy and XRF*

The differences between replicates were explored, and there was no significant effect on the variation of $Ca^{+2}$ (Table 3). At the onset of the experiment, normal development was observed in the larval shells of both treatments, with a prevalence of shells with 20% damage. During the first 24 h of culture, the images obtained from the treatment with low pH showed lesions in the ventral parts of the larval shells, with a prevalence of shells with a damage of 40% (Fig. 4B). Progressive damage occurred in the umbo on the third day of culture along with a 50% prevalence of shell damage (Fig. 4D). On days 5 and 6 of culture, the severity of the lesions increased, and the prevalence of damage in the shells was more intense, with values of 60% on both days (Fig. 4F, H). The larvae were observed without periostracum and with decalcification regions, which leave the larvae exposed and unprotected. By contrast, organisms cultured at the control pH (control) showed normally developed larvae characterized by a straight hinge and a smooth curvature along the edge of the valve. There was no evidence of deformations, fractures, or visible damage, with levels that can be considered within a normal range of 10%–30% (Fig. 5). The logistic regression test applied to the prevalence data showed that there was a significant effect of pH treatment on the levels of shells that showed damage [chi-square (1) = 4.3846 $P$ = 0.0363 $\alpha = 0.05$]; however, this effect was not observed through the culture days [chi-square (4) = 3.2002 $P$ = 0.5249 $\alpha = 0.05$] (Table 7).

The percentages of $Ca^{+2}$ content of the shells is shown in table 5. The data showed statistically significant differences in $Ca^{+2}$ content in the shells, between both pH treatments during the 6 days of culture (Table 2). Repeated measures ANOVA differences were obtained from both factors (pH and day) and their interactions on the $Ca^{2+}$ content in the larval shells of *Crassostrea sikamea* [day, RM ANOVA, $F$ (16) = 9.523

**TABLE 3.**

**Estimates of components of variance in larval growth and $Ca^{+2}$ in the larval shell by REML.**

| | Random effect | Reason for variance | Variance component | SE | Lower 95% CI | Upper 95% CI | *P* value of Wald | % |
|---|---|---|---|---|---|---|---|---|
| $Ca^{+2}$ in larval shell | Tank [pH] | –0.195 | –0.002 | 0.001 | –0.005 | 0.0007 | 0.15 | 0 |
| | Error | | 0.011 | 0.003 | 0.006 | 0.025 | | 100 |
| | Total | | 0.011 | 0.003 | 0.006 | 0.025 | | 100 |
| Larval growth | Tank [pH] | –0.002 | –0.063 | 0.056 | –0.173 | 0.046 | 0.256 | 0 |
| | Error | | 31.612 | 1.059 | 29.633 | 33.796 | | 100 |
| | Total | | 31.612 | 1.059 | 29.633 | 33.796 | | 100 |

$P < 0.0019$]; pH, RM ANOVA, $F(8) = 124.729$ $P < 0.0001$; and day × pH, RM ANOVA, $F(16) = 12.923$ $P = 0.0005$] (Table 3).

## DISCUSSION

The present work indicates that a low pH (7.4) significantly increased the percentage of mortality (compared with the control), as a consequence of a decrease in concentration of calcium in the shells, and the occurrence of physical defects in oyster larvae shells; these were revealed by scanning electron microscope images in which the larval shells were clearly corroded and deformed, particularly after the fifth day of the experiment (11 days postfertilization). Similar results were observed in *Saccrostrea glomerata* larvae exposed to low pH (7.6 and 7.8), where observed growth anomalies on the surface of the larval shell where there was a deficiency in shell deposition, delays in periostracum formation and increase in the dissolution of the shell, with a clear relationship between these effects with the low pH and the decrease of the saturation state of aragonite ($\Omega_{ar}$) (Watson et al. 2009). These same effects were observed in the present study in the Kumamoto oyster larvae subjected to a pH of 7.4, with respect to the deficiencies in the calcification of the shell beginning on the third day and the absence of the periostracum in several areas of the larval shell on day 5, which favors the dissolution effect of $CaCO_3$.

**TABLE 4.**

**A. The results from logistic regression describing the relationship between time and pH level on the likelihood of larval mortality; B. Wald test of the effects on the factors day and pH in an experiment where *Crassostrea sikamea* larvae were exposed to two pH levels (8.1 and 7.4) for 6 days.**

**A. Whole model test**

| Model | –Log: verisimilitude | DF | Chi-square | Probability > Chi-square |
|---|---|---|---|---|
| Difference | 226.272 | 7 | 452.5451 | <0.0001* |
| Full | 977.147 | | | |
| Reduced | 1,203.42 | | | |

**B. Wald effects test**

| | *N* parameters | DF | Wald Chi-square | Probability > Chi-square |
|---|---|---|---|---|
| Day | 6 | 6 | 183.289531 | <0.0001* |
| pH | 1 | 1 | 41.9577806 | <0.0001* |

The damage from low pH has also been observed in the species of the same genus by several researchers, who have shown the difficulties in the calcification process of *Crassostrea gigas* larvae after exposure to an increase in $pCO_2$ (Gazeau et al. 2011, Barton et al. 2012, Barros et al. 2013). At a low pH (7.4), the same to that used in the present work, reduced larval development and shell mineralization were observed in the oyster *C. gigas* (Kurihara et al. 2007). In Mexico, oyster culture activity is increasing, which drives the local production of larvae and spat (Chávez-Villalba 2014), the increasingly acidic conditions in the seawater compromise larval development in hatcheries, affecting the spat production of these species in commercial hatcheries and imparting negative consequences on their growth but still incipient oyster culture in Mexico (Fernando Garcia et al. Personal communication). The corrosive conditions of seawater were present throughout the experiment ($\Omega ar < 1$), in the low pH treatment, which favors the dissolution of the shells; this explains the decrease in the levels of $Ca^{+2}$ (Table 2), along with a lower growth in the shells at a low pH (Fig. 1). An increase in damage to the larval shell of *Crassostrea sikamea* suggests a lack of physiological capacity of the larvae to compensate acidosis and regulate physiological processes such

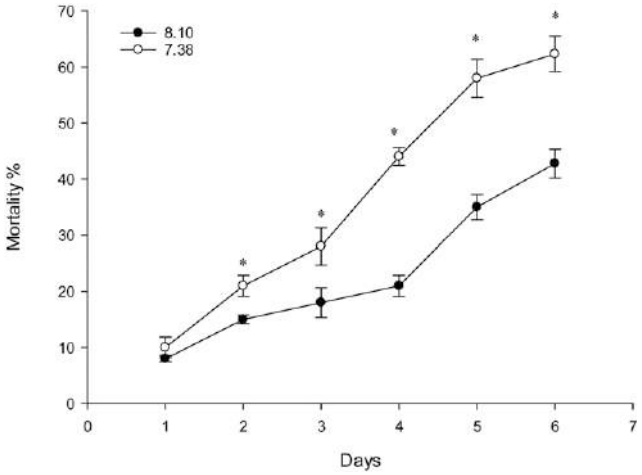



**Figure 3. Percentage of larval mortality (mean ± SD; *n* = 5) of *Crassostrea sikamea* cultivated at two pH levels, 8.1 (•) and 7. 4 (○). The significant differences between the larval lengths of both treatments are marked with an asterisk (*). Logistic regression model [day: Wald chi-square (6) = 183.289531; *P* < 0.001 and pH: Wald chi-square (1) = 41.957; *P* < 0.001].**

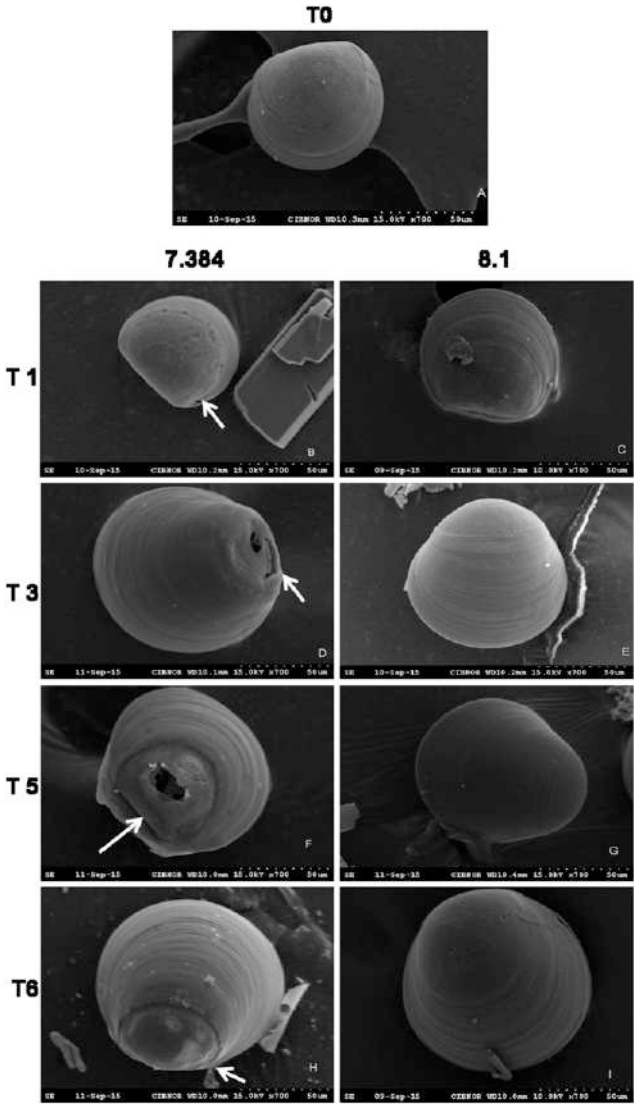



**Figure 4. Scanning electron microscope images of veliger *Crassostrea sikamea* larvae subjected to low and control pH conditions. T0–T6 indicate days of culture. The white arrows indicate damage to valves. Larvae cultured at pH 7.4 showed progressive lesions after 24 h (Fig. 3B) on the third day of culture lesions in the umbo region (Fig. 3D). On days 5 and 6 of the culture, the severity of the damage in the shells was more intense. The larvae showed decalcification regions at low pH (Fig. 3F, H). In the control treatment (pH 8.1), no evidence of deformation, fracture, or damage was observed (Fig. 3C, E, G).**

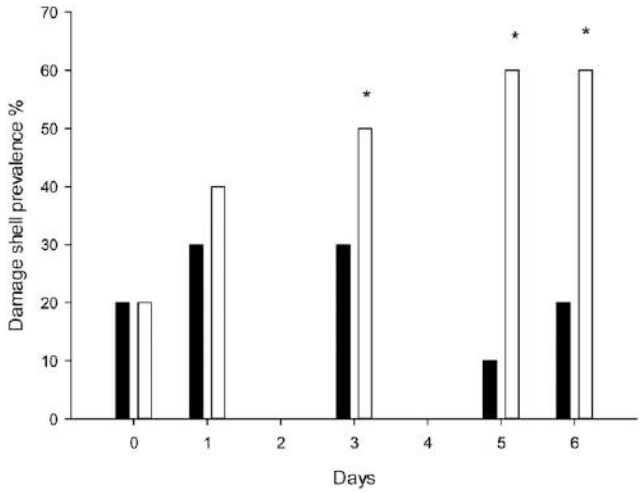



**Figure 5. Prevalence of shells with damage (percentage) of *Crassostrea sikamea* larvae subjected to two pH levels, 8.1 (•) and 7.4 (○) ($n = 10$ per treatment). * Significant difference. Logistic regression test: Chi-square (1) = 4.3846; $P = 0.0363$; $\alpha = 0.05$.**

as biomineralization; this has been evidenced in adult organisms of *C. gigas* exposed to low pH, with a negative effect in energy metabolism, stress responses, protein regulation, and calcium homeostasis (Lannig et al. 2010, Wei et al. 2015).

In this study, a decrease in the growth of organisms cultured at low pH (7.4) is reported (Fig. 1), which may reflect the increase in energy expenditure required for the mineralization of the shells. Among the negative effects that small size can have on the development of Kumamoto oyster larvae, their physical condition is reduced, thus reducing the competitive capacity and causing an increase in mortality after settlement (Gazeau et al. 2011). In addition, the efficiency in the competitiveness for food is reduced, which causes a disadvantage of larger larvae of the same cohort becoming more susceptible to starvation that compromises their survival (Kurihara et al. 2007).

The growth of the *Crassostrea sikamea* larvae subjected to low pH was delayed 1 day in relation to the larvae grown in the control pH treatment (Fig. 2). In addition, the deformities present in the larvae of *C. sikamea* were more evident in low pH treatment, showing areas with decalcifications and eroded zones which could have forced the larvae to invest more energy in the calcification process, having as consequences the smaller sizes increasing larval mortality. Barros et al. (2013) revealed that exposure to high-$CO_2$ seawater, particularly at more extreme levels (1.03872 μm × $d^{-1}$ for ΔpH = –0.4 and 2.2044 μm × $d^{-1}$ for ΔpH = –0.7), leads to increased frequencies of morphological abnormalities of the D-shaped *Crassostrea gigas* larvae. The reduction in the size of the larvae subjected to low pH also implies technical difficulties for the management of the larval culture because it delays the development process for a few days, which implies additional management of the larvae and extra expenditure of energy and resources for the maintenance of the larvae. This lack of capacity of biomineralization of shells, at this stage of development, is particularly critical because their energy budget depends, to a large extent, on the availability of food, and they do not have the capacity to store

**TABLE 5.**

**Percentage of $Ca^{2+}$ concentrations in the shells of *Crassostrea sikamea* larvae grown at two pH levels.**

| | Time (days) | | |
|---|---|---|---|
| **pH treatment** | **0** | **2** | **6** |
| Control pH | 1.22 ± 0.016[b] | 4,450 ± 1.479[a] | 4.70 ± 1.27[a] |
| Low pH | | 1,134 ± 0.477[b] | 1.04 ± 0.413[b] |

LS mean Tukey's HSD. Different letters are significantly different. $\alpha = 0.05$.

**TABLE 6.**

**Odds of *Crassostrea sikamea* larvae mortality as a function of the pH treatments (8.1 and 7.4) and the duration of the experiment (day: 0, 1, 2, 3, 4, 5, and 6).**

| | Levels | Odds ratio | *P* | CI 95% lower | CI 95% upper |
|---|---|---|---|---|---|
| pH | 8.1 vs. 7.4 | 0.485 | <0.0001 | 0.390073 | 0.604 |
| | 7.4 vs. 8.1 | 2.060 | <0.0001 | 1.6554109 | 2.563 |
| Day | 1 vs. 2 | 0.498 | 0.0058 | 0.303 | 0.817 |
| | 1 vs. 3 | 0.332 | <0.0001 | 0.207 | 0.535 |
| | 1 vs. 4 | 0.200 | <0.0001 | 0.126 | 0.317 |
| | 1 vs. 5 | 0.112 | <0.0001 | 0.071 | 0.177 |
| | 1 vs. 6 | 0.089 | <0.0001 | 0.056 | 0.140 |
| | 2 vs. 1 | 2.009 | 0.0058 | 1.224 | 3.296 |
| | 2 vs. 4 | 0.401 | <0.0001 | 0.272 | 0.592 |
| | 2 vs. 5 | 0.225 | <0.0001 | 0.154 | 0.330 |
| | 2 vs. 6 | 0.178 | <0.0001 | 0.122 | 0.261 |
| | 3 vs. 1 | 3.009 | <0.0001 | 1.870 | 4.842 |
| | 3 vs. 4 | 0.601 | 0.0061 | 0.417 | 0.865 |
| | 3 vs. 5 | 0.337 | <0.0001 | 0.236 | 0.482 |
| | 3 vs. 6 | 0.267 | <0.0001 | 0.187 | 0.381 |
| | 4 vs. 1 | 5.008 | <0.0001 | 3.158 | 7.941 |
| | 4 vs. 2 | 2 0.493 | <0.0001 | 1.689 | 3.681 |
| | 4 vs. 3 | 1.664 | 0.0061 | 1.156 | 2.395 |
| | 4 vs. 5 | 0.562 | 0.0007 | 0.401 | 0.785 |
| | 4 vs. 6 | 0.444 | <0.0001 | 0.318 | 0.621 |
| | 5 vs. 1 | 8.919 | <0.0001 | 5.660 | 14.056 |
| | 5 vs. 2 | 4.440 | <0.0001 | 3.030 | 6.506 |
| | 5 vs. 3 | 2.964 | <0.0001 | 2.076 | 4.231 |
| | 5 vs. 4 | 1.781 | 0.0007 | 1.273 | 2.491 |
| | 6 vs. 1 | 11.275 | <0.0001 | 7.154 | 17.772 |
| | 6 vs. 2 | 5.613 | <0.0001 | 3.830 | 8.227 |
| | 6 vs. 3 | 3.747 | <0.0001 | 2.624 | 5.349 |
| | 6 vs. 4 | 2.251 | <0.0001 | 1.610 | 3.149 |

CI, confidence interval. The table also contains the CI on the hazard ratio, and the resulting *P* value is displayed. Only significant values are shown ($P < 0.05$).

energy reserves (Sánchez-Lazo & Martínez-Pita 2012) to compensate for energy expenditure related to the assimilation of $CaCO_3$ in acidic conditions.

During normal development, the calcification rate of the larvae is rapid (Barton et al. 2012); however, when larvae are subjected to low pH conditions, they develop more inefficiently (reach a smaller size) than those grown at control pH levels (as observed in *Crassostrea gigas* by Waldbusser et al. 2015). In the present study, the larvae subjected to low pH showed a lower growth rate of 5,405 $\mu m \times d^{-1}$ compared with 5,606 $\mu m \times d^{-1}$ for the pH control, which denotes an unfavorable physiological condition.

These data indicate that bivalve molluscs are particularly sensitive to the effects of OA, and thus, in *Crassostrea sikamea*, climate change could cause inappropriate or even lethal future conditions in the estuarine environment where this species develops. In this work, the treatment of low pH increased the mortality of the larvae of *C. sikamea*, presenting final mortality of 62.33% ± 5.35% on day 6 for the culture in low pH, whereas those cultivated at control pH experienced 42.75% ± 3.13% mortality on day 6, representing a statistically significant difference. The cultures of oyster larvae present variability in survival in the D phase of the larvae, which can vary between 50% and 70% on average (Helm et al. 2004). Even if the mortality in the control pH treatment seems high, it is consistent with those obtained by Barros et al. (2013), who obtained similar results to those presented in this work, finding significant differences in mortality of larvae reared in seawater manipulated with $CO_2$ with variations of $\Delta pH = -0.4$ (83%) to $\Delta pH = -0.7$ (98%) in larvae of *Crassostrea gigas* grown for 144 h (6 days); thus, the significant difference found between both treatments in this study was not influenced by the mortality of the control treatment and is not necessarily the result of inadequate management or an adverse crop. Likewise, Watson et al. (2009) obtained variations determined in an experiment with larvae of *Saccrostrea glomerata*, with survival decreased by 43% at pH 7.8 and 72% at pH 7.6 compared with the control treatment (pH 8.1). Therefore, the future responses of *C. sikamea* aquaculture facilities cannot be ignored, and proper monitoring of the carbonate system in the hatchery can provide early detection of changes in the pH and chemistry of seawater, helping oyster farmers to adapt. The OA effects on the oyster farming industry are evident in intensely cultivated species, such as *C. sikamea*, but field studies show that reduced pH levels affect the increase in larval mortality that affects larval recruitment and juveniles, favoring the decrease of natural oyster populations (Cigliano et al. 2010) and causing damage to habitats, triggering cascading effects, including loss of biodiversity, reduction in biofiltration, and loss of coastal habitat protection (Rossoll et al. 2012).

**TABLE 7.**

**Odds of larval shell damage as a function of pH treatments (8.1 and 7.4).**

| | A. Odds ratio | | | | |
|---|---|---|---|---|---|
| | **Levels** | **Odds ratio** | ***P*** | **CI 95% lower** | **CI 95% upper** |
| pH | pH 8.1 vs. pH 7.4 | 0.3998 | 0.0363 | 0.1695 | 0.9430 |
| | pH 7.4 vs. pH 8.1 | 2.5012 | 0.0363 | 1.0604 | 5.8998 |

| | B. Wald tests of the effects | | | | |
|---|---|---|---|---|---|
| | **Source** | ***N* parameters** | **DF** | **Chi-square Wald** | **Probability > Chi-square** |
| | pH | 1 | 1 | 4.3846 | 0.0363 |
| | Day | 4 | 4 | 3.2003 | 0.5249 |

CI, confidence interval. The table also contains the CI on the hazard ratio, the Wald Chi-square statistic, and the resulting *P* value.

Parker et al. (2009) determined a lethal impact on the embryonic development of the straight hinge veliger larvae, of the *Saccostrea glomerata* oyster, if fertilization occurs at elevated levels of $pCO_2$ and elevated levels of temperature. Where they found D-veliger larvae with more deformities and smaller when they were subjected to high temperature and $pCO_2$ after fertilization to elevated $pCO_2$ and temperature compared to D-veliger larvae that developed after fertilization at $pCO_2$ and temperature "ambient." Abnormality of D-veligers was greatest at 1,000 ppm and 18 and 30 °C (90%) and least at 375 ppm and 26 °C (4%).

This study showed that decreases in pH and $\Omega_{ar}$ cause negative effects on several relevant parameters of larval development of Kumamoto oysters: less growth, higher mortality, lower calcium concentration in the shell, and more physical irregularities of the specimens. The development of veliger larvae of this species was compromised as acidification resulted in the absence of the periostracum and dissolution of the larval shell and probably caused an excessive energetic expenditure when larvae attempted to compensate for deficient biocalcification processes. By the end of this century, coastal areas may experience exposure to extreme conditions (low pH and high $CO_2$) (Clements & Chopin 2016). OA is not only a global effect of increasing $CO_2$ levels in the atmosphere but also exacerbated in eutrophic estuaries, such as most coastal bays where larvae and spat hatcheries are established in northwestern Mexico and where some episodes of larval mortality associated with low pH are already occurring (Phillipe et al. personal communication). This increase in the acidification of the oceans in estuarine environments, which are sources of seawater for hatcheries and areas of oyster growth, presents an uncertain outlook for the future trends of increasing $CO_2$ levels on the planet.

Marine bivalves appear to be sensitive to OA because of the limited degree to which they regulate the ionic balance and the pH of their hemolymph and their acute sensitivities in specific stages (Waldbusser et al. 2010), as well as their limited capacity to recover from exposure to OA during sensitive larval stages (Barton et al. 2012); however, in acute exposures of larvae to OA, whether compensatory growth is possible if the saturation state improves later during larval development (larval resilience) cannot be determined.

## ACKNOWLEDGMENTS

This research was funded by CONACYT 169809 and 183534, (Basic Science), 298 (Frontiers of Science) on behalf of H. R. B. F. A. F.-H. received doctoral fellowships from CONACYT (388861), and the results presented here are part of his doctoral thesis. We thank the Acuacultura Robles S.P.R. de R.I. company for the donation of the larvae for the experiments. We acknowledge Ariel Cruz-Villacorta for technical support for the SEM analysis. Pablo Monsalvo-Spenser and Pablo Ormart-Castro are thanked for logistical support during the experiments. Special thanks to Dr. José Martín Hernandez Ayon for providing the reference material for oceanic $CO_2$ measurement, lot 169 (A. Dickson, Scripps Institution of Oceanography).

## LITERATURE CITED

Álvarez-Borrego, S. 2007. Principios generales del ciclo del carbón en el océano. In: Hernández de la Torre, B. & G. Gaxiola-Castro, editors. Carbono en ecosistemas acuáticos de México. México, D.F.: INECC. pp. 11–28.

APHA. 1995. Method 2310 "Acidity," Standard methods for the examination of water and wastewater, 19th edition. Washington, D.C.: American Public Health Association-American Water Works Association. pp. 23–25.

Barros, P., P. Sobral, P. Range, L. Chícharo & D. Matias. 2013. Effects of seawater acidification on fertilization and larval development of the oyster *Crassostrea gigas*. *J. Exp. Mar. Biol. Ecol*. 440:200–206.

Barton, A., B. Hales, G. G. Waldbusser, C. Langdon & R. A. Feely. 2012. The Pacific oyster *Crassostrea gigas* shows negative correlation to elevated carbon dioxide levels naturally: implications for near-term effects ocean acidification. *Limnol. Oceanogr*. 57:698–710.

Barton, A., G. G. Waldbusser, R. A. Feely, S. B. Weisberg, J. A. Newton, B. Hales, S. Cudd, B. Eudeline, C. J. Langdon, I. Jefferds, T. King, A. Suhrbier & K. McLaughlin. 2015. Impacts of acidification on the coastal Pacific northwest shellfish industry and adaptation strategies in response implemented. *Oceanography* 28:146–159.

Cáceres-Martínez, J. & R. Vásquez-Yeomans. 2013. Diseases, parasites and oyster mortality episodes of commercial importance in Mexico and its implications for production. *Fish. Sci*. 21:5–48.

Cáceres-Martínez, J., R. Vásquez-Yeomans & Y. Guerrero-Rentería. 2012. Early gametogenesis of Kumamoto oyster (*Crassostrea sikamea*). *Hidrobiologica* 22:181–184.

Cai, W. J., X. Hu, W. J. Huang, M. C. Murrell, J. C. Lehrter, S. E. Lohrenz, W. C. Chou, W. Zhai, J. T. Hollibaugh, P. Zhao, X. Guo, K. Gundersen, M. Dai, Y. Wang & G. C. Gong. 2011. Acidification of subsurface coastal waters enhanced by eutrophication. *Nat. Geosci*. 4:766–770.

Caldeira, K. & M. E. Wickett. 2003. Oceanography: anthropogenic coal and ocean pH. *Nature* 425:365.

Caldeira, K. & M. E. Wickett. 2005. Ocean chemistry model predictions of changes from carbon dioxide emissions to the atmosphere and ocean. *J. Geophys. Res*. 110:12.

Chávez-Villalba, J. 2014. Culture of the oyster *Crassostrea gigas*: analysis of 40 years of activities in Mexico. *Hidrobiologica* 24:175–190.

Cigliano, M., M. C. Gambi, R. Rodolfo-Metalpa, F. P. Patti & J. M. Hall-Spencer. 2010. Effects of ocean acidification on invertebrate settlement at volcanic $CO_2$ vents. *Mar. Biol*. 157:2489–2502.

Clements, J. C. & T. Chopin. 2016. Ocean acidification and marine aquaculture in North America: potential impacts and mitigation strategies. *Rev. Aquacult*. 0:1–16.

CONAPESCA. 2015. Anuario estadístico de Pesca. Base de Datos Preliminary. Accessed July 5, 2016. Available at: http://www.conapesca.sagarpa.gob.mx/wb/cona/cona_anuario_estadistico_de_pesca.

Cornwall, C. E. & C. L. Hurd. 2015. Experimental design in ocean acidification research: problems and solutions. *ICES J. Mar. Sci*. 73:572–581.

Dauphin, Y., A. D. Ball, H. Castillo-Michel, C. Chevallard, J. P. Cuif, B. Farre, S. Pouvreau & M. Salome. 2013. *In situ* characterization of the distribution and organic content of the oyster *Crassostrea gigas* shell (Mollusca, Bivalvia). *Micron* 44:373–383.

Dickson, A. G. & F. J. Millero. 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep Sea Res*. 34:1733–1743.

Doncaster, C. P. & A. J. H. Davey. 2007. Analysis of variance and covariance: how to choose and construct models for the life sciences. Cambridge, United Kingdom: Cambridge University Press. 288 pp.

Fabry, V. J., B. A. Seibel, R. A. Feely & J. C. Orr. 2008. Impacts of ocean acidification on marine wildlife and ecosystem processes. *ICES J. Mar. Sci*. 65:414–432.

Fangue, N. A., M. J. O'Donnell, M. A. Sewell, P. G. Matson, A. C. MacPherson & G. E. Hofmann. 2010. A laboratory-based experimental system for the study of ocean acidification effects on marine invertebrate larvae. *Limnol. Oceanogr. Methods* 8:441–452.

Feely, R. A., C. L. Sabine, K. Lee, W. Berelson, J. Kleypas, V. J. Fabry & F. J. Millero. 2004. Impact of anthropogenic $CO_2$ on the $CaCO_3$ system in the oceans. *Science* 305:362–366.

Gaylord, B., K. J. Kroeker, J. M. Sunday, K. M. Anderson, J. P. Barry, N. E. Brown, S. D. Connell, S. Dupont, K. E. Fabricius, J. M. Hall-Spencer, T. Klinger, M. Milazzo, P. L. Munday, B. D. Russell, E. Sanford, S. J. Schreiber, V. Thiyagarajan, M. L. H. Vaughan, S. Widdicombe & D. G. Harley. 2015. Ocean acidification through the lens of ecological theory. *Ecology* 96:3–15.

Gazeau, F., S. Alliouane, C. Bock, L. Bramanti, M. López-Correa, M. Gentile, T. Hirse, H.-O. Pörtner & P. Ziveri. 2014. Impact of ocean acidification and warming on the Mediterranean mussel (*Mytilus galloprovincialis*). *Front. Mar. Sci.* 1:64.

Gazeau, F., J.-P. Gattuso, M. Greaves, H. Elderfield, J. Peene, C. H. R. Heip & J. J. Middelburg. 2011. Effect of alteration on the carbonate chemistry early embryonic development of the Pacific oyster (*Crassostrea gigas*). *PLoS One* 6:e23010.

Gazeau, F., L. M. Parker, S. Comeau, J.-P. Gattuso, W. A. O'Connor, S. Martin, H.-O. Pörtner & P. M. Ross. 2013. Impacts of ocean acidification on marine shelled mollusks. *Mar. Biol.* 160:2207–2245.

Ginger, K. W. K., C. B. S. Vera, R. Dineshram, C. K. S. Deniss, L. J. Adela, Z. Yu & V. Thiyagarajan. 2013. Larval and post-larval stages of Pacific oyster (*Crassostrea gigas*) are resistant to elevated CO2. *PLoS One* 8:e64147.

Goldstein, J., D. Newbury, D. Joy, C. Lyman, P. Echlin, E. Lifshin, L. Sawyer & J. Michael. 2003. Scanning electron microscopy and X-ray microanalysis, 3rd edition. New York, NY: Springer Science+ Business Media. 690 pp.

Gordon, D. G., N. E. Blanton & T. Y. Nosho. 2001. Heaven on the half shell. Portland, OR: West Winds Press. 119 pp.

Gutiérrez, J. L., C. G. Jones, D. L. Strayer & O. O. Iribarne. 2003. Mollusks as ecosystem engineers: the role of shell production in aquatic habitats. *Oikos* 101:79–90.

Hartin, C. A., B. Bond-Lamberty, P. Patel & A. Mundra. 2016. Ocean acidification over the next three centuries using a simple global climate carbon-cycle model: projections and sensitivities. *Biogeosciences* 13:4329–4342.

Helm, M. M., N. Bourne & A. Lovatelli. 2004. Hatchery culture of bivalves. A practical manual. Rome, Italy: FAO. 177 pp.

Hofmann, G. E., J. P. Barry, P. J. Edmunds, R. D. Gates, D. A. Hutchins, T. Klinger & M. A. Sewell. 2010. The effect of ocean acidification on calcifying organisms in marine ecosystems: an organism-to-ecosystem perspective. *Annu. Rev. Ecol. Syst.* 41:127–147.

Kurihara, H., S. Kato & A. Ishimatsu. 2007. Effects of increased seawater $pCO_2$ on early development of the oyster *Crassostrea gigas*. *Aquat. Biol.* 1:91–98.

Lacis, A. A., G. A. Schmidt, D. Rind & R. A. Ruedy. 2010. Atmospheric $CO_2$: principal control knob governing earth's temperature. *Science* 330:356–359.

Lannig, G., S. Eilers, H. O. Pörtner, I. M. Sokolova & C. Bock. 2010. Impact of ocean acidification on energy metabolism of oyster, *Crassostrea gigas*—changes in metabolic pathways and thermal response. *Mar. Drugs* 8:2318–2339.

Lewis, E. & D. W. R. Wallace. 1998. Program developed for $CO_2$ system calculations, ORNL/CDIAC-105. Oak Ridge, TN: Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy. 21 pp.

Mason, A. Z. & J. A. Nott. 1980. A rapid, routine technique for the X-ray microanalysis of micro-incinerated cryosections: an SEM study of inorganic deposits in tissues of the marine gastropod *Littorina littorea* (L.). *J. Histochem. Cytochem.* 28:1301–1311.

Mehrbach, C., C. H. Culberson, J. E. Hawley & R. M. Pytkowicz. 1973. Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol. Oceanogr.* 18:897–907.

NOAA. 2016. NOAA. Accessed April 29, 2016. Available at: http://www.esrl.noaa.gov/gmd/ccgg/trends/global.html.

Nosho, T. Y. 1989. Small-scale oyster farming for pleasure and profit. Aquaculture series. Seattle, WA: Washington Sea Grant Program, Services University of Washington. 12 pp.

Páez-Osuna, F., J. A. Sanchez-Cabeza, A. C. Ruiz-Fernández, A. C. R. Alonso-Rodríguez, J. G. Cardoso-Mohedano, Flores-Verdugo, J. L. Carballo, M. A. Cisneros-Mata, A. Piñón-Gimate & S. Álvarez-Borrego. 2016. Environmental status of the Gulf of California: a review of responses to climate change and climate variability. *Earth Sci. Rev.* 162:253–268.

Parker, L. M., P. M. Ross & W. A. O'Connor. 2009. The effect of ocean acidification and temperature on the fertilization and embryonic development of the Sydney rock oyster *Saccostrea glomerata* (Gould 1850). *Glob. Change Biol.* 15:2123–2136.

Robinson, A. 1992. Gonadal cycle of *Crassostrea gigas kumamoto* (Thunberg) in Yaquina Bay, Oregon and optimum conditions for broodstock oysters and larval culture. *Aquaculture* 106:89–97.

Rodríguez-Quiroz, G., M. García-Ulloa, A. L. Domínguez-Orozco, T. N. Valenzuela-Hernández, E. Nava-Pérez & A. M. Góngora-Gómez. 2016. Relación del crecimiento, condición y supervivencia del ostión del Pacífico *Crassostrea gigas* y las variables ambientales, cultivado en suspensión en el sistema lagunar Navachiste-Macapule, Sinaloa, México. *Rev. Biol. Mar. Oceanogr.* 51:541–551.

Rossoll, D., R. Bermudez, H. Hauss, K. G. Schulz, U. Riebesell, U. Sommer & M. Winder. 2012. Ocean acidification-induced food quality deterioration constrains trophic transfer. *PLoS One* 7:e34737.

Sabine, C. L., R. A. Feely, N. Gruber, R. M. Key & K. Lee. 2004. The oceanic sink for anthropogenic $CO_2$. *Science* 305:367–371.

Sánchez-Lazo, C. & I. Martínez-Pita. 2012. Biochemical and energy dynamics during development of the larval mussel *Mytilus galloprovincialis* (Lamarck, 1819). *Aquaculture* 358–359:71–78.

Waldbusser, G. G., H. Bergschneider & M. A. Green. 2010. Size-dependent pH effect on calcification in post-larval hard clam *Mercenaria* spp. *Mar. Ecol. Prog. Ser.* 417:171–182.

Waldbusser, G. G., E. L. Brunner, B. A. Haley, B. Hales, C. J. Langdon & F. G. Prahl. 2013. A developmental and energetic shell oyster larval basis linking formation sensitivity to acidification. *Geophys. Res. Lett.* 40:2.171–2.176.

Waldbusser, G. G. & J. E. Salisbury. 2014. Ocean acidification in the coastal zone from an organism's perspective: multiple system parameters, frequency domains, and habitats. *Annu. Rev. Mar. Sci.* 6:221–247.

Waldbusser, G. G., B. Hales, C. J. Langdon, B. A. Haley, P. Schrader, E. L. Brunner, M. W. Gray, C. A. Miller & I. Giménez. 2015. Saturation-state sensitivity of marine bivalve larvae to ocean acidification. *Nat. Clim. Chang.* 5:273–280.

Watson, S. A., P. C. Southgate, P. A. Tyler & L. S. Peck. 2009. Early larval development of the Sydney rock oyster *Saccostrea glomerata* under near-future predictions of $CO_2$-driven ocean acidification. *J. Shellfish Res.* 28:431–437.

Wei, G., Z. Wang, T. Ke, Y. Liu, W. Deng, X. Chen, J. Xu, T. Zeng & L. Xie. 2015. Decadal variability in seawater pH in the West Pacific: evidence from coral $\delta^{11}$ B records. *J. Geophys. Res. Oceans* 120:7166–7181.

Weng, N. & W.-X. Wang. 2014. Variations of trace metals in two estuarine environments with contrasting pollution histories. *Sci. Total Environ.* 485–486:604–614.

Zhang, Y., Y. Qin, L. Ma, Z. Zhou, S. Xiao, H. Ma, Y. Pan, J. Li & Z. Yu. 2019. Gametogenesis from the early history life stages of the Kumamoto oyster *Crassostrea sikamea* and their breeding potential evaluation. *Front. Physiol.* 10:524.