OAysters: Acidification effects on susceptibility of *Crassostrea gigas* larvae to infection by *Vibrio tubiashii*

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ABSTRACT

In recent years, the oyster *Crassostrea gigas* has been subject to recurrent outbreaks of vibriosis. The bacteria *Vibrio tubiashii* induces acute mortality in the veliger larval stage, with severe repercussions for aquaculture and recruitment. The disease is known to be correlated with environmental factors such as temperature and salinity. In our study, we sought to examine the relationship between a rapidly changing environmental factor, ocean acidification, and pathogenicity using a series of larval inoculations at two different carbon dioxide concentration, bacterial strains, larval ages, and bacterial dilutions. Our results of mortality and LD_{50} indicate that some strains of *V. tubiashii* may be less pathogenic under acidified conditions and these findings warrant more investigation.

BACKGROUND

Infection of the Pacific oyster, *Crassostrea gigas*, by the bacteria *Vibrio tubiashii* (referred to herein as Vt) is of economic and ecological concern. This species is the most commonly cultured shellfish in Puget Sound. In Washington alone, this industry contributes \$270 million $year^{-1}$ and 3200 jobs to the state economy. Ecologically, oysters are ecosystem engineers, creating complex reefs that serve as nursery, hunting, and refuge habitats for many fish and invertebrate species (Lennihan et al. 2001). Shellfish also act as indicator species

due to their sedentary nature and sensitivity to water quality, with disease outbreaks serving as a warning for big changes in ocean chemistry.

Outbreaks of Vt in *C. gigas* in the Pacific northwest (PNW) since 2006 have been associated with upwelling of cold, nutrient-rich (and *Vibrio* rich) seawater that mixes with unusually warm sea surface temperatures (Elston et al. 2008). These temperature changes, particularly warming, have been associated with larval losses as high as 59% in hatcheries (Elston et al. 2008). Bacterial blooms of Vt in coastal waters can be pumped into larval hatcheries in high concentrations, however the effects of these blooms on wild populations has not been examined. Other commercially important shellfish are also affected by Vt, such as Kumamoto oysters and geoduck clams (Elston et al. 2008).

Ocean acidification, the increase of CO_2 in the ocean due to increased emissions, is an issue of growing concern and investigation, especially in the PNW. Studies of the effects of acidic conditions on oysters suggest that there may be negative impacts on shell formation, particularly in the larval stage (Miller et al. 2009). Extra CO_2 from the atmosphere reacts with water to form carbonic acid, which increases H+ ion concentrations and lowers pH, shifting the ionic balance from carbonate to bicarbonate. Oysters must compensate by using more energy to extract carbonate to build calcium carbonate shells. Acidic conditions also influence the aragonite saturation ([?][?] Ar) and calcite saturation (**Ω** Ca), which determine whether these two calcium carbonate configurations are precipitated or dissolved. In a study of larval *C. gigas* at pH 7.4, shells of larvae that were transferred to acidic water dissolved completely under these conditions (Kurihara et al. 2007). One study that independently varied saturation states of Ca and Ar, pH, and carbonate ion availability found availability to be the only factor with that directly affected embryonic development (Gazeau et al. 2011),

suggesting that the findings of Kurihara et al. (2007) are the result of shifts in carbonate. This is particularly detrimental for larvae due to their thin aragonitic shells. Low pH conditions have also been correlated with larval failures outside the laboratory. An Oregon hatchery in a lagoon with natural daily pH fluctuations found that larvae spawned during a low pH event had much lower survival and growth (Barton et al. 2010).

Acidification may also impact other facets of oyster biology, such as immune response. Oysters subjected to mechanical stress are more susceptible to infection (Lacoste, 2001) and extreme salinity and temperature dampen the immune response by destroying hemocytes (Gagnaire et al. 2006). Preliminary results from a study by Friedman et al. presented in August (Friedman, 2012) show increased transcription of genes associated with stress in Olympia oysters (*Ostrea lurida*) kept in acidified water. Therefore, the physiological stress of living in acidified water may leave oysters more susceptible to infection and similar patterns may occur in other oyster species.

Immune response also varies through the ontogeny of *C. gigas*. (Tirape et al. 2007). For example, *Cg-timp*, an anti-metalloprotease gene, is expressed at higher levels after the D-hinge veliger stage. Metalloproteases are thought to be a virulence factor for Vt (Hasegawa and Häse, 2009), so differential expression of *Cg-timp* could influence the susceptibility of larvae at different stages of development.

As the impacts of ocean acidification and Vt outbreaks are better understood, it is important to realize how these factors may interact with each other. Recent research examining the effects of acidified water on growth of Vt found that several strains isolated from PNW hatcheries exhibited higher growth rates and higher total abundance under high $pC0_2$ than ambient conditions (Dorfmeier 2012). These findings are comparable to the

environmental conditions leading to blooms, with high CO_2 and high Vt concentrations coming to the surface (Timmons-Schiffman, pers. comm), suggesting an interactive relationship between environment and pathogen.

Dorfmeier's study of growth rates (2012) examined the RE 22 strain of Vt, and it is unknown whether this increased growth occurs in all strains. She also found that the bacteria raised under high CO_2 conditions were not more virulent than those raised under ambient conditions. As there are several pathogenic strains of Vt in the PNW with differing degrees of virulence plaguing the shellfish industry, determining how individual strains respond to increased CO_2 warrants further investigation.

OBJECTIVES

In our study, we wanted to find how ocean acidification may impact Vt infections in *Crassostrea gigas* by examining the following questions:

- Do CO_2 conditions influence pathogenicity of Vt?
- Does pathogenicity vary between different strains of Vt?
- Is there an interactive effect of CO_2 conditions and Vt strain?

METHODS

To determine the effects of CO_2 on Vt pathogenicity, we conducted oyster challenge experiments in the laboratory.

Oyster Larvae

All larvae were purchased from Taylor Shellfish Farms, Bow, WA, USA. Two age classes of *C. gigas* larvae were used: 18 day old diploids that had been maintained in the lab for 9 days prior to the experiment and 9 day old triploids that arrived the day prior to the experiment. Larvae were held in tanks with filtered seawater from the Ocean Acidification facility at UW Friday Harbor Laboratories, Friday Harbor, WA and fed Shellfish Diet in a 1:50 ml seawater dilution once a day. Water was changed every other day.

Vibrio Strains

Different strains of Vt were obtained from Dr. Ralph Elston of Aquatechnics, Sequim, WA, USA and provided to us by the Friedman Lab at the University of Washington. The strains selected for use in the current study were RE 22, RE 98, and RE 101; these strains were found to be highly pathogenic at different dilutions in preliminary trials conducted by the summer 2012 EIMD class at UW-FHL and by Estes et al. (2004).

The different Vt strains were revived by plating them on T1N2 agar plates. They were incubated at 35ºC for 24 hours. Two to three colonies from each strain were then added to 800 uL of 0.25% Tryptone media and vortexed. These bacterial suspensions were then used to inoculate two conical tubes with 30 mL of 0.25% Tryptone media, which were cultured for 24 h at room temperature under two CO_2 levels and used as bacterial stock.

From this bacterial stock, serial dilutions (10^{-1} to 10^{-7}) were prepared by adding 500 uL of bacterial suspension to 4.5 mL of sterile seawater with the corresponding CO_2 concentration. To estimate the initial concentration of Vt, spread plates were prepared for the 10^{-2} to 10^{-7}

dilutions of all strains and treatments. The dilutions that were used for the larval challenge were 10^{-1}, 10^{-2}, and 10^{-3}, which corresponded to ~10^{6}, 10^{5} and 10^{4} concentrations of Vt, respectively.

Environmental parameters

In order to assess the effect that pH has on larval mortality and Vt pathogenicity, two different CO_2 levels were used in the experiment: ambient and high CO_2. For the ambient treatment, air was bubbled into the sea water and bacterial inoculation; the resulting pCO_2 measured for this treatment was 603 μmol/atm. The high CO_2 treatment had 2000 μmol/atm of CO_2 bubbled into the sea water and bacteria. The pCO_2 obtained corresponded to 1973 μ mol/atm. The 0.25% Tryptone media in which the Vibrio strains were grown overnight also underwent these treatments for 24 h prior to inoculation.

Experimental Setup

Challenges were conducted in 12-well plates. Each plate contained 3 replicates of each larval age * Vt strain * CO_2 combination as well as 3 controls in ambient seawater. All the treatments used are presented in Table 1.

Table 1. All treatment combinations. Each treatment was applied to 1 plate.

Treatment	OA	Ploidy	Vt Strain
1	Ambient	Diploid	RE22
2	2000 ppm CO_2	Diploid	RE22
3	Ambient	Triploid	RE22
4	2000 ppm CO_2	Triploid	RE22
5	Ambient	Diploid	RE98
6	2000 ppm CO_2	Diploid	RE98
7	Ambient	Triploid	RE98
8	2000 ppm CO_2	Triploid	RE98
9	Ambient	Diploid	RE101
10	2000 ppm CO_2	Diploid	RE101
11	Ambient	Triploid	RE101
12	2000 ppm CO_2	Triploid	RE101

Approximately 40 larvae were added to each well. Sea water with the correct CO_2 level was added to all the wells to give a final volume of 3.9 mL. Finally, 100uL of the corresponding bacterial dilution was added to the wells; 100uL of sea water was added to the control wells to make sure all wells had a final volume of 4mL.

Lidded plates were stored in ambient air conditions and open plates were stored in a CO_2 chamber for 48 hours. Dead larvae in each well were counted and recorded at 24 and 48 h using an inverted microscope. Once 48 h larvae counts were conducted, a 10% bleach solution was added to each well, total larvae per well counts were performed, and proportion mortality determined. Proportion mortality at 24 and 48 hr was used to calculate LD_{50} (lethal dose to 50% mortality) for each treatment.

$$LD_{50} = 10^{a*b+c}$$

$$a = \frac{0.5 - d}{e - d}$$

$$b = \log\left(\frac{concentration\ that\ causes > 50\%\ mortality}{concentration\ that\ causes < 50\%\ mortality}\right)$$

$$c = \log(concentration\ that\ causes < 50\%\ mortality)$$

$d = proportion\ of\ larval\ mortality\ observed\ at\ concentrationed\ that\ caused < 50\%\ mortality\ (0 - 1\ value)$

$e = proportion\ of\ larval\ mortality\ observed\ at\ concentrationed\ that\ caused > 50\%\ mortality\ (0 - 1\ value)$

In addition, water samples were set up to mimic conditions in the treatments and used for water chemistry sampling after the experiment. Eight 250 ml beakers were filled with 100 ml of combinations of ambient or CO_2 seawater, 1000 diploid or triploid larvae, and 250 uL of RE 22 bacterial stock. Four beakers were placed in ambient conditions and four in the the CO_2 chamber with the CO_2 plates.

Water Chemistry

Total alkalinity (TA) and spectrophotometric pH were determined for ambient and CO_2 samples at the beginning and end of the experiment. A DL15 Titrator (Mettler Toledo) was used to find TA. Sample preservation and quality control (use of Certified Reference Material, CRM) followed protocols outlined by Dickson & Goyet (1994). However, we used an endpoint titration for TA measurements. Briefly, water samples from each treatment (as outlined above) were placed in a water bath of 25°C for 30 min prior to testing. The TA machine was first calibrated using CRM from the Dickson Lab to ensure proper functioning.

Then each water sample was titrated and the titrant volume provided. TA was calculated using the following equation:

$$Alkalinity = \frac{Volume\ of\ titrant * Titrant\ normality * Mass\ of\ CaCO_3 * 10^5}{Mass\ of\ sample\ (g)}$$

For spectrophotometric pH, water samples were put into glass cuvettes and warmed in a water bath to 25ºC. Samples were run through the spectrophotometer first without anything added and then with 70 mL of *m*-cresol purple dye. The wavelength readings were captured using Spectra Suite Software from Ocean Optics Inc. and calculations followed equations provided in Dickson & Goyet (DOE, 1994; Chap 7).

Temperature and salinity of initial and final water samples was found using a YSI EC300 conductivity meter. Once all variables (TA, pH, temperature, salinity) were found, CO_2Calc (Robbins et al., 2010) was used to calculate the pCO_2, pH, and **Ω** calcite and aragonite.

Statistical Analyses

A multiple-way ANOVA was used to test for interactive effects between CO_2 treatment, Vt strain, and time point. Pairwise comparisons were performed using Tukey's Test. Statistical results with $p \leq 0.05$ are presented as significant treatment effects. All analyses were conducted in JMP Pro 9.

RESULTS

Larval mortality and Vt pathogenicity

Due to high mortality of triploid larvae in all treatments, including controls, results presented below contain data on only diploid larvae. Additionally, high mortality occurred within 24 h in the 10^6 bacterial suspension. Therefore, the following results are those obtained for the diploid controls as well as diploids with the 10^5 and 10^4 bacterial suspensions added.

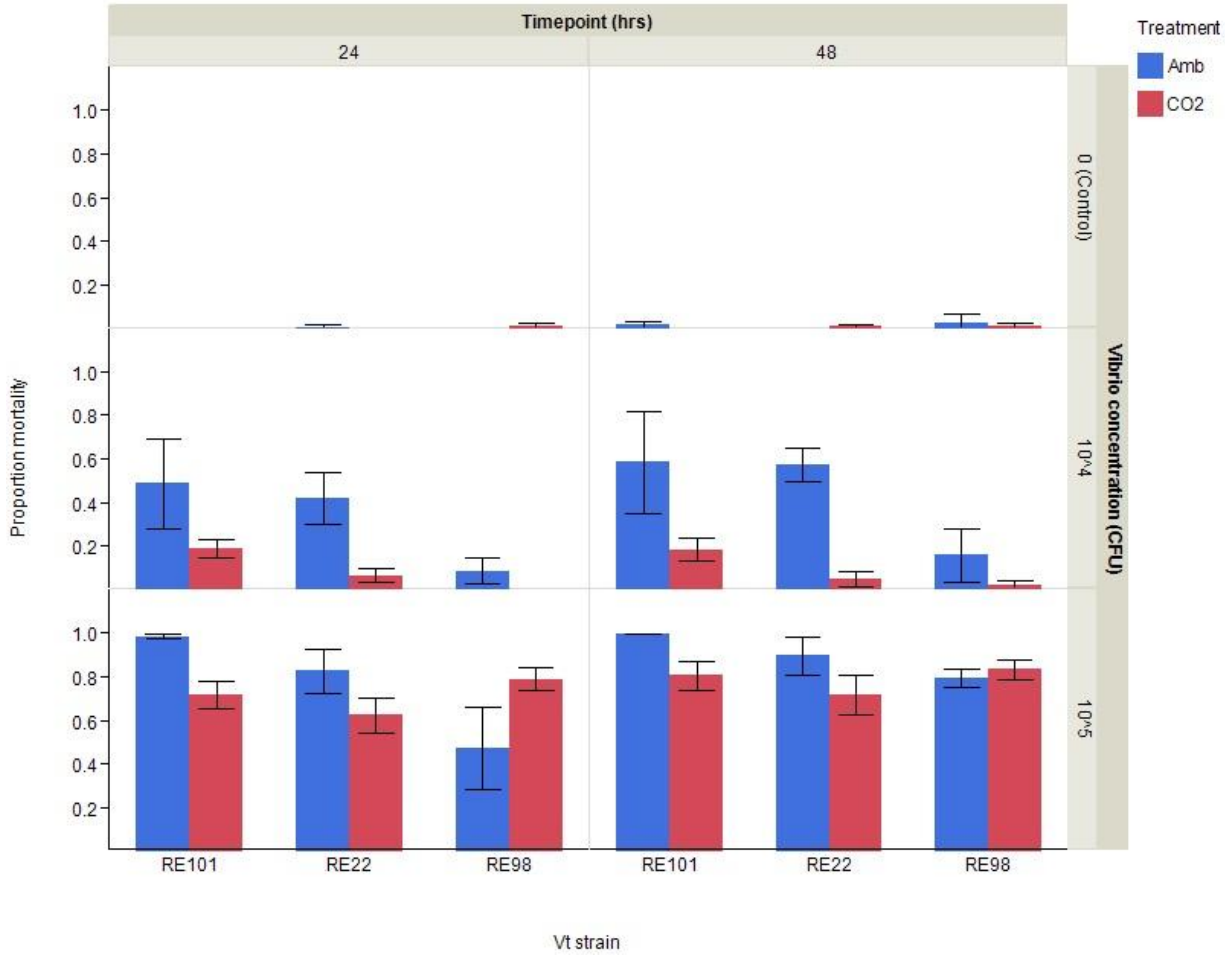

Figure 1. Larval mortality for diploid larvae under each treatment at 24 and 48 hours. Treatments include: Vt strain, bacterial concentration and CO_2 level.

For diploid larvae in the control treatment, mortality was not significantly different among treatments (below 5%) during the 48 h experiment. Mortality levels were different, however, among the larvae exposed to the different Vt and CO_2 treatments (Fig. 1). Sources of variation identified as statistically relevant include Vt strain, bacterial concentration, CO_2 treatment and timepoint (24 vs. 48 h) as shown in Table 2.

Table 2. Sources of variation identified with a four way analysis of variance.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Vt strain	2	2	0.344147	9.4606	0.0002
Treatment	1	1	0.374604	20.5957	<.0001
Vt strain*Treatment	2	2	0.288777	7.9385	0.0007
Timepoint (hrs)	1	1	0.082742	4.5492	0.0358
Vibrio concentration (CFU)	2	2	10.72002	294.6926	<.0001
Vt strain*Vibrio concentration (CFU)	4	4	0.243891	3.3523	0.0133
Treatment*Vibrio concentration (CFU)	2	2	0.430806	11.8428	<.0001

Mortality was significantly higher in the ambient treatment as opposed to the high CO_2 treatment for strains RE 101 and RE 22 strains (Fig. 2). Mortality was not significantly different for RE 98 between ambient and high CO_2 treatments and this strain was observed to be less pathogenic (Table 3).

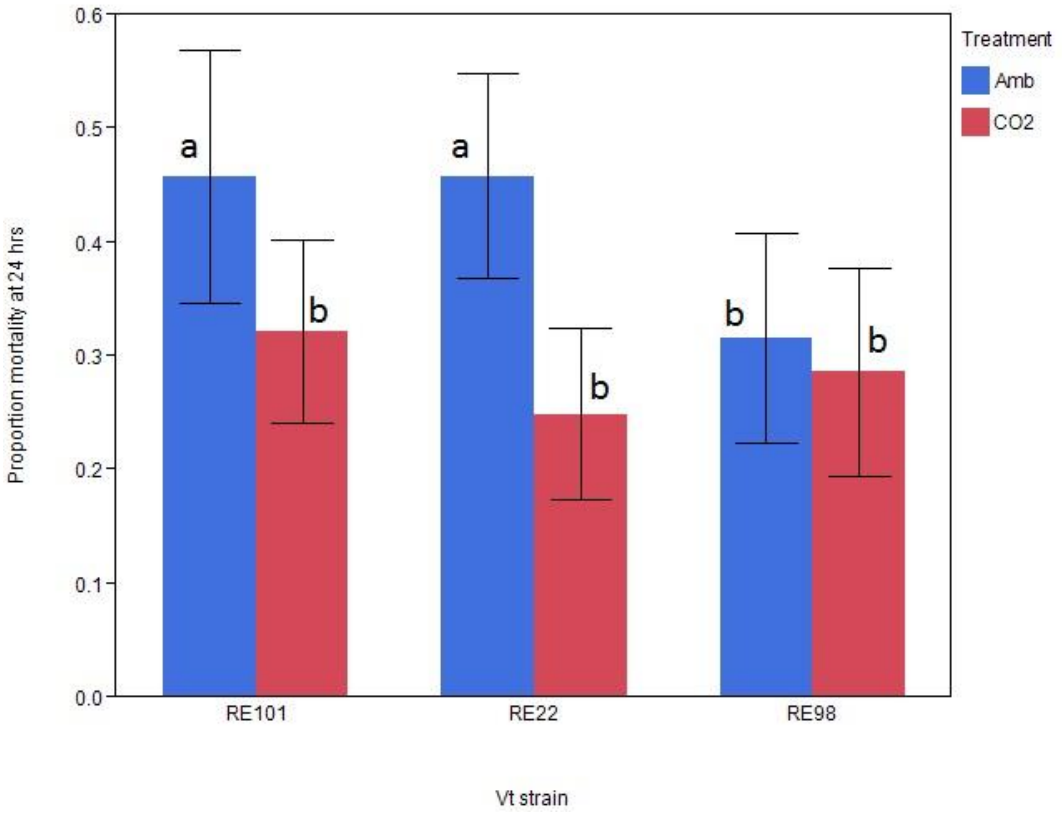

Figure 2. Mean proportion larval mortality for each Vt strain based on ambient and high CO2 conditions.

Table 3. LD_{50} for the different strains at 24 and 48 hours.

	24 hours		**48 hours**	
	Amb	CO2	Amb	CO2
RE101	1.24 x 104	5.20 x 104	N/A	4.34 x 104
RE22	1.60 x 104	9.71 x 104	N/A	7.67 x 104
RE98	1.09 x 105	5.29 x 104	4.05 x 104	4.76 x 104

Water Chemistry

Initial and final water samples of ambient and high CO_2 treatments showed the expected pattern of higher pH and lower pCO_2 under ambient conditions compared to high CO_2 conditions (Table 4). Both pH and pCO_2 decreased between initial and end samples with the addition of larvae and but then increased slightly when larvae and Vt were combined (Table 4), presumably due to increased biological processes. The saturation levels of calcite and aragonite were lower under high CO_2 conditions and in all samples, aragonite saturation was lower than calcite (Table 1).

Table 4. Water chemistry for initial and final samples.

Sample	Salinity	Temp (°C)	TA (µmol/kgSW)	pCO2 (µatm)	pH	Ω Ca	Ω Ar
Ambient Initial	25.2	25.3	1948.3	603.225	7.876	2.756	1.762
CO2 Initial Spec pH	24.8	24.7	1952.3	1917.018	7.414	1.014	0.646
Ambient w/Larvae Final	26.7	20.55	2043.4	741.957	7.803	2.161	1.373
CO2 w/Larvae Final	25.3	21.05	2030.9	1498.462	7.523	1.176	0.744
Ambient w/Larvae+Vt Final	26.75	20.4	2039.9	914.936	7.719	1.801	1.144
CO2 w/Larvae+Vt Final	25.9	20.65	1998	1645.129	7.475	1.037	0.657

DISCUSSION

In our study, we found that pCO_2 conditions influenced the pathogenicity of Vt, but not in the way that we expected. Vt incubated under high pCO_2 conditions grew to a higher abundance, as had been observed in prior work (Dorfmeier, 2012; Figure 3). We expected that the Vt strains cultivated under CO_2 conditions would cause greater mortality due to

faster growth under these conditions. However, we observed the opposite: mortality was significantly higher in the ambient treatments than the high CO_2 treatments for two of our three strains (Figure 2).

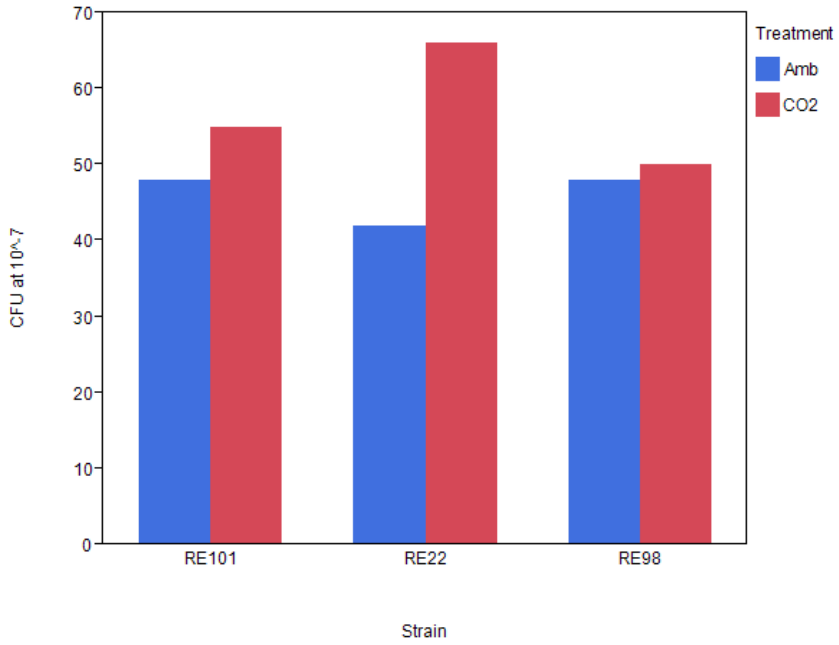

Figure 3. Colony forming units (CFU's) observed in the 10^{-7} dilutions of Vt.

There are several potential explanations for our observed results. First, Vt raised under high CO_2 conditions could have lower virulence, either due to phenotypic changes in the bacteria themselves or a decrease in the chemical effectiveness of their extracellular toxins. Also, it is possible that the stress of exposure to high CO_2 stimulated the larval immune system, preventing rapid infection that could be detected within 48 h.

Secondly, we found that pathogenicity varied significantly ($p=0.0002$, Table 2) between our three tested strains. Larval mortality was not significantly different between strains RE 101 and RE 22 under either CO_2 treatment, but RE 98 caused lower larval mortality under

ambient conditions than the other two strains (Fig. 2). Differences between the strains under ambient condition indicate that RE 98 may naturally have lower pathogenicity compared to the others. However, this study was run over a 48 h period and it's possible that given longer exposure to the host, RE 98 may demonstrate higher pathogenicity.

Lastly, we did see a significant interactive effect between CO_2 conditions and Vt strain ($p=0.0007$; Table 2). This relationship is demonstrated in RE 22 and RE 101 which both induced higher mortality under ambient compared to high CO_2 conditions, suggesting that high CO_2 conditions may reduce virulence in these strains. Similar larval mortality between CO_2 treatments in RE 98 (vs. lower mortality in the other strains) suggests that virulence in this strain may be less impacted by changes in CO_2.

Water chemistry is important in ecological processes in oceans, particularly with respect to bivalves, which rely on calcite and aragonite for shell construction differentially throughout their life stages. Larval oysters use mostly aragonite to build their shells (Miller et al. 2009). Our water chemistry results show that the saturation of aragonite under high CO_2 is below the supersaturation value of 1 (Table 1). Below this threshold, calcification can be reduced (Kelypas et al. 1999) and undersaturation of carbonate can lead to dissolution of already formed calcium carbonate structures. The large differences in aragonite saturation between our ambient and high CO_2 treatments suggest that projected levels of CO_2 increases (from IPCC) will lead to aragonite saturation levels below the supersaturation threshold. Such reductions in aragonite saturations will likely negatively impact the success of larval shellfish such as *C. gigas*.

Despite the potentially negative effects of altered seawater chemistry, our results suggest that some changes may be beneficial. We found that pathogenicity of some Vt strains was

lower under high CO_2, which might reduce lethal Vt infections in larval oysters. We tested three Vt strains that are known to affect larval survival in hatcheries in the PNW and two of these had lower pathogenicity under high CO_2 levels. To better understand the influence of increased CO_2 on Vt pathogenicity, more strains should be tested at varying levels of CO_2 that are more representative of projected increases.

Our results generated many new questions for future investigations. Two aspects of the experiment that were unsuccessful were the examinations of age and ploidy. We initially planned to compare pathogenicity between younger (triploid) and older (diploid) larvae. Although we knew we could not tease apart the different impacts of each, we had hoped to find if differences did occur. However, due to the high mortality in all triploids, including controls, the triploid data was not used. Determination of differences in susceptibility to Vt at different ages would benefit hatcheries and aquaculture facilities. Differential susceptibility based on age could lead to modifications in husbandry practices that alleviate Vt-induced mortality of cultured larvae. Additionally, differences between diploid and triploid susceptibility would be valuable information for hatcheries, as triploids are often used to increase weight and growth yields. Lower survival or performance of one ploidy over the other could aid in determination of which to use in culturing. Previous research has shown that in adult *C. gigas*, *Vibrio* sp. attacked the gonads but similar total mortality in diploids and triploids suggests no overall advantage of ploidy (De Decker et al. 2011), though such studies have not been conducted with larvae.

There were several sources of variation that may have influenced the results of our experiment. Variation in assessment of dead larvae during all timepoints led to large standard deviations between replicates. In such cases, we removed the replicate count that differed the

most from the other replicates. In future experiments, a single counter assessing all plates or calculating a correction factor for multiple counters that could be applied to final data would help alleviate this issue.

The highest dilution we used (10^6 CFU/mL) caused ~100% mortality within 24 h. This mortality occurred too rapidly to assess LD_{50}. This dilution was also not ecologically relevant, as reported bacterial concentrations in ocean water during the 2007 bloom only went as high as $1.6x10^5$ (Elston et al. 2008). In future experiments, lower dilutions should be used to assess more accurate LD_{50} values for this pathogen.

Overall, our study demonstrates that ocean acidification may have variable impacts on both the host and the pathogen. Due to the brevity of our study, further research is needed to validate our findings. It is important to understand how environmental factors influence host-pathogen interactions, as predicted environmental changes will likely have complex, large-scale impacts on these relationships and marine systems as a whole.

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