Effect of Elevated pCO_2 on Metabolic Responses of Porcelain Crab (*Petrolisthes cinctipes*) Larvae Exposed to Subsequent Salinity Stress

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Abstract

Future climate change is predicted to alter the physical characteristics of oceans and estuaries, including pH, temperature, oxygen, and salinity. Investigating how species react to the influence of such multiple stressors is crucial for assessing how future environmental change will alter marine ecosystems. The timing of multiple stressors can also be important, since in some cases stressors arise simultaneously, while in others they occur in rapid succession. In this study, we investigated the effects of elevated pCO_2 on oxygen consumption by larvae of the intertidal porcelain crab *Petrolisthes cinctipes* when exposed to subsequent salinity stress. Such an exposure mimics how larvae under future acidified conditions will likely experience sudden runoff events such as those that occur seasonally along portions of the west coast of the U.S. and in other temperate systems, or how larvae encounter hypersaline waters when crossing density gradients via directed swimming. We raised larvae in the laboratory under ambient and predicted future pCO_2 levels (385 and 1000 µatm) for 10 days, and then moved them to seawater at ambient pCO_2 but with decreased, ambient, or elevated salinity, to monitor their respiration. While larvae raised under elevated pCO_2 or exposed to stressful salinity conditions alone did not exhibit higher respiration rates than larvae held in ambient conditions, larvae exposed to elevated pCO_2 followed by stressful salinity conditions consumed more oxygen. These results show that even when multiple stressors act sequentially rather than simultaneously, they can retain their capacity to detrimentally affect organisms.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Increasing atmospheric carbon dioxide (CO_2) is altering Earth's climate, creating multiple new stressors with which organisms must cope. Predicted changes include more intense and more frequent storms, shifts in the hydrologic cycle, higher air and seawater temperatures, and the ecological challenge of ocean acidification [1,2,3,4,5]. As the ocean absorbs CO_2 from the atmosphere, seawater pH decreases, which can make growth and survival more difficult for a wide range of marine and estuarine species [6,7,8,9,10]. Estuarine and coastal environments may be particularly sensitive to the effects of climate change, as these ecosystems not only experience both terrestrial and marine perturbations, but also encounter an extensive suite of anthropogenic stressors tied to nutrient loading, fishing, pollution, and habitat degradation [11].

Model predictions of future climate change for the northeastern Pacific Ocean include changes to precipitation and altered temperatures [12,13], in concert with the influence of ocean acidification. Changes in rainfall will affect not only average salinities, but also the frequency and intensity of the most acute runoff events. These events lead to low-salinity extremes, requiring coastal and estuarine organisms to cope with rapid decreases in

salinity. Adding complexity to these predicted changes is that areas that receive limited summer rainfall (e.g. Mediterranean climates) can experience hypersalinity and stratified water columns in estuaries and coastal areas; changes in thermal loading due to climate change could increase the frequency and duration of these stratification events [2,14,15].

Many emerging studies explore the effects of decreased pH in combination with simultaneous changes in another stressor [9], typically temperature but also salinity in a few studies [16,17,18]. By contrast, very little research exists to assess potential outcomes when multiple stressors are imposed sequentially instead of simultaneously; that is, when one stressor acts first by itself but is followed in rapid succession by another. Understanding how species respond to sequential stressors is particularly important in coastal and estuarine habitats. In these regions, organisms can experience sudden transitions from oceanic conditions to lower salinities due to episodic runoff events, or transitions to higher salinities due to vertical swimming through a stratified water column during mobile life stages. How future high-pCO_2 conditions could affect an organism's ability to cope with sudden changes in salinity has not yet been examined.

The porcelain crab *Petrolisthes cinctipes* serves as a useful model species for exploring such issues because it possesses life history

characteristics representative of a variety of nearshore inverte-brates that will experience changing hydrographic conditions in future decades. Although it occurs primarily in outer-coast intertidal mussel beds and cobble fields from Point Conception, California to the Queen Charlotte Islands, British Columbia [19], it also lives in estuaries where sufficient rocky habitat exists. Its congeners in the Atlantic are found most commonly in estuaries, where they associate with oyster beds and other biogenic habitat [20,21]. Its planktotrophic larval stage remains relatively close to shore during its moderately long pelagic larval duration [22], making it similar to the larvae of many other invertebrates, including ecologically and commercially important taxa such as barnacles, mussels, oysters, and many other species of crabs [23], so the results of this study may be representative of how many other larvae will respond to future climate change.

Adults of many species in the genus *Petrolisthes* are relatively resilient to stressors such as desiccation [24,25], high temperature [26,27,28], hypoxia [25,29], and hyposaline conditions [30], which is evolutionarily consistent with the routine exposure of these organisms to the variable and stressful conditions of the intertidal zone and estuarine habitats. Though larval stages of many taxa are often more susceptible to stress than adults [31], two previous studies investigated the effects of elevated pCO_2 in isolation on *P. cinctipes*, and both concluded that larvae were relatively resilient to pCO_2 stress [32,33]. These findings are consistent with the prediction that crustaceans will likely be one of the classes of organisms that are able to cope with future climate change [6,34].

In this study, we determined how respiration by newly hatched larvae of the porcelain crab *Petrolisthes cinctipes* cultured under current (385 µatm) and future predicted (1000 µatm) levels of atmospheric pCO_2 differed when exposed to low (22), normal (34), and high (40) salinity conditions. Though crustacean larvae are relatively good swimmers and can adjust their position in the water column to avoid unfavorable conditions, the mosaic of nearshore conditions results in larvae experiencing a range of salinities and pH. By culturing larvae under different pCO_2 conditions and then exposing them to subsequent changes in salinity, we mimicked how larvae might encounter a plume of hyposaline runoff during their pelagic development or might experience hypersaline conditions when migrating through a stratified coastal embay-ment.

Oxygen consumption through respiration is a commonly used metric of stress in marine organisms, with stressful conditions usually resulting in a more rapid depletion of oxygen from the water, though metabolism could be suppressed under some conditions of stress [35]. Our investigation of how oxygen consumption differs across pCO_2 and salinity treatments in this species therefore provides insights into how larval stages of nearshore invertebrates may respond to multiple, sequentially imposed stressors characteristic of future environmental conditions.

Materials and Methods

Fifty ovigerous *Petrolisthes cinctipes* were collected from Shell Beach (38° 25.033′ N, 123° 06.350′ W) and Twin Coves (38° 27.490′ N, 123° 08.621′ W), California, using permits obtained from the California Department of Fish and Wildlife and California State Parks (no protected species were collected). Crabs were held in individual mesh containers in flow-through seawater at Bodega Marine Laboratory for 2–14 days. Containers were checked daily for hatched larvae, and newly released individuals from multiple broods (n = 24 broods) were transferred in mixed

batches of 75 to replicate culture jars. These jars contained 0.45 um filtered seawater bubbled with prescribed pCO_2 levels to maintain carbonate chemistry and water movement, and were held at a constant temperature and normal salinity (approximately 34) [36,37]. At least five females contributed larvae to each culture jar, and broods were split evenly between pCO_2 treatments. Not all females released larvae on the same night, which allowed culture start dates to be staggered and respirometry trials to be run on sequential days using larvae of the same age. The use of larvae from multiple broods helped to minimize brood effects that have been seen in other studies [32,33], facilitating application of findings to the overall population.

Larvae were cultured at 14°C under two pCO_2 conditions: 385 µatm (ambient) and 1000 µatm (elevated). The elevated pCO_2 treatment, maintained by bubbling a premixed, NIST-traceable, air-CO_2 gas mixture through the culture water, was selected to replicate global-average conditions projected for 2100 by fossil-fuel intensive climate model projections (A1FI scenario) [15], but also mimics transient conditions encountered even today [36]. Larvae were held at a density of 75 larvae per 3 L, and were fed newly hatched *Artemia* sp. nauplii at a density of 1 nauplius/ml [38]. The water in each culture jar was changed every two days by siphoning approximately 95% of the water from the jar and replacing it with new filtered seawater that had been equilibrated to the appropriate temperature and pCO_2 treatment. During water changes, dead larvae and uneaten *Artemia* sp. were removed, and water exiting the jars was sampled for carbonate chemistry adhering to recommended practices (Table 1) [39]. We measured pH (total scale) [40] using a glass electrode (Accumet Excel XL60; Thermo Fisher Scientific, Waltham, Massachusetts, USA) and repeated analysis of certified TRIS reference material (measured in millivolts; batch no. 8; A. Dickson, Scripps Institute of Oceanography). Total alkalinity (TA) was measured by autotitration (Metrohm 809; Metrohm, Herisau, Switzerland), using the same certified reference material. Dissolved inorganic carbon (DIC) samples were processed using coulometric titration at the Monterey Bay Aquarium Research Institute (MBARI).

Respirometry trials were conducted in seawater at ambient pCO_2 using a dissolved oxygen meter (model 781b Strathkelvin Instruments Ltd., Glasgow, UK) with a Clark-type microcathode polarographic electrode with a 22 micron diameter platinum cathode and silver/silver chloride anode connected by a buffered potassium chloride electrolyte solution (model 1302). We used a low permeability polypropylene membrane so the electrode could be used in solutions with minimal stirring, though we also visually checked to ensure the movement of larvae was sufficient to maintain water flow over the surface of the membrane. Data were transmitted via a data interface unit to a computer and the oxygen consumption rates were monitored using the Strathkelvin 949 Oxygen System (Version 2.2, Strathkelvin Instruments Ltd., Glasgow, UK).

The electrode was placed in a PVC holder with double O rings to seal the chamber. The holder and electrode were inserted into a test chamber (26.34 mm×47.09 mm ID) holding 25 ml of test solution. To maintain the temperature at 14°C, this test chamber was jacketed in a dish (37.69 mm×75.30 mm ID) and kept in a constant temperature refrigeration unit. For each new trial, the electrode was calibrated using 2% sodium sulfite and air-saturated seawater to determine zero and saturated oxygen values, respectively. Before each series of experiments, a control chamber with no larvae was monitored to establish background microbial oxygen consumption rates, which were then subtracted from the values obtained during trial runs for our analyses. During each trial, eight first-stage larvae were removed from the culturing

Table 1. Culturing system carbonate conditions.

CO$_2$ Treatment	385 µatm	1000 µatm
pH (Total)	7.997±0.004	7.706±0.003
Ω Aragonite	2.033±0.019	1.127±0.007
Total Alkalinity (µmol/kg)	2243.5±5.1	2254.6±3.0
Dissolved Inorganic Carbon (µmol/kg)	2091.9±6.4	2178.3±4.0
pCO$_2$ (µatm)	450.1±6.1	949.8±7.5

Mean values (± S. E.) of carbonate system conditions in larval culturing jars under ambient (385 µatm) and elevated (1000 µatm) pCO$_2$ conditions. Larvae under ambient conditions experienced higher pH (p<0.0001) and lower DIC (p<0.0001) than those under elevated pCO$_2$ conditions.
doi:10.1371/journal.pone.0109167.t001

system and placed in the chamber for respirometry analysis, and visual analysis indicated that their activity was sufficient to maintain flow over the surface of the electrode. Seawater oxygen saturation state was measured every two minutes for one hour, though we only analyzed data from the start of the trial until the oxygen in the test chamber reached 5 mgL^{-1} to eliminate potential effects of hypoxia in the chamber on larval respiration (Figure 1). Trials with larvae from each pCO$_2$ treatment were run sequentially in pairs (i.e., one trial with larvae from ambient pCO$_2$ conditions was followed by one trial with larvae from elevated pCO$_2$ conditions), and 13 pairs of trials were run at salinities of 34 over a period of 14 days.

Distilled water was added to filtered, ambient-pCO$_2$ seawater (salinity 34) to make salinity 22 solution, and artificial seawater (Instant Ocean, Spectrum Brands, Inc.) was added to make salinity 40 solution. This procedure created two altered salinity treatments for testing effects of salinity stress on larvae drawn from each of our preceding pCO$_2$ treatments. We acknowledge that these manipulated solutions have slightly different alkalinities and aragonite saturation states than natural seawater, and may have
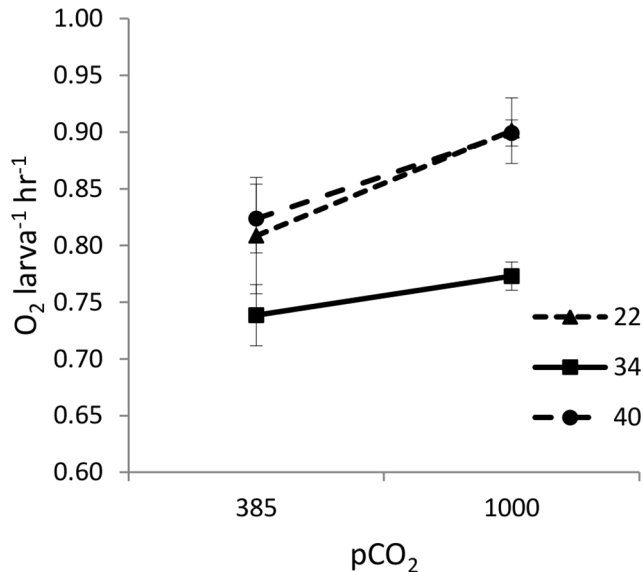

Figure 1. Larval oxygen consumption through time. Dissolved oxygen (mean total oxygen less background bacterial consumption rates, ± S.E.) during respirometry trials under ambient or dual-stressor conditions. Horizontal dashed line shows the lower limit of data analyzed in this study; data for oxygen levels under 5 mgL^{-1} are presented for completeness.
doi:10.1371/journal.pone.0109167.g001

minor differences in trace elements [18]. Though we were unable to analyze carbonate system parameters of our test solutions during the experiment due to low volumes, we created solutions using the same methods to determine pH and total alkalinity for post-hoc estimates of alkalinity and aragonite saturation state. We also calculated these carbonate system parameters for hypothetical ocean waters of the same salinities as our treatments, using data collected in local coastal and estuarine waters [41].

Larvae were allowed to acclimatize in treatment solutions for ten minutes prior to respirometry trials to attenuate a startle response, and the system was calibrated before each trial. Ten minutes was sufficient for larvae to begin active swimming following their transfer. Paired trials (4 pairs at salinity 22 and 3 pairs at salinity 40) with larvae from each pCO$_2$ treatment were run sequentially over a period of seven days.

We used ANOVAs to test for respiration rate differences between salinity treatments (ambient and stressful) and between pCO$_2$ treatments (ambient and elevated), and Levene tests revealed our data met the assumption of equal variances. We used a Tukey HSD test to analyze differences among all four treatments. We also used ANOVAs to verify that our pCO$_2$ treatments provided statistically different carbonate system conditions for developing larvae, and we used a Welch's ANOVA for the single parameter (Ω aragonite) that did not meet the assumption of equal variances. All statistical analyses were conducted in JMP 9 (SAS Institute, Cary, NC).

Results

Larvae experienced significantly different carbonate system conditions in our ambient and predicted future pCO$_2$ treatments in the culturing apparatus (Table 1), including pH (ANOVA, $F_{1,173} = 4451.7$, p<0.0001), Ω aragonite (Welch's ANOVA, $F_{1,87} = 1925.2$, p<0.0001), pCO$_2$ (ANOVA, $F_{1,87} = 2627.0$, p<0.0001), and dissolved inorganic carbon (ANOVA, $F_{1,59} = 137.0$, p<0.0001). Analysis of altered-salinity solutions revealed that alkalinity and aragonite saturation state ranged from 1479–2880 µmol/kg and 0.63–2.16, respectively (Table 2). These values are extremely close to those estimated for natural coastal and estuarine waters in our area at those salinities (Table 2).

There were no differences in oxygen consumption between larvae at ambient conditions and those exposed to a single stressor (Figure 2). Elevated pCO$_2$ alone had no effect on larval oxygen consumption (Figure 3; $F_{1,40} = 2.027$, p = 0.1626), and larvae consumed less oxygen at ambient salinities than at stressful salinities (Figure 3; $F_{1,40} = 9.529$, p = 0.0038). Crab larvae raised under elevated pCO$_2$ conditions that were subsequently exposed to high or low salinities consumed significantly more oxygen than

Table 2. Salinity treatment carbonate conditions.

		Treatment Solution	Predicted Natural Seawater
Salinity 22	pH	7.700	7.701
	pCO_2	703.9	689.3
	Total Alkalinity (µmol/kg)	1479	1453
	Ω Aragonite	0.63	0.62
	Dissolved Inorganic Carbon (µmol/kg)	1445	1419
Salinity 40	pH	7.805	7.804
	pCO_2	928.1	951.5
	Total Alkalinity (µmol/kg)	2880	2943
	Ω Aragonite	2.16	2.21
	Dissolved Inorganic Carbon (µmol/kg)	2707	2768

Calculated carbonate conditions of treatment solutions in which the salinity has been altered by adding distilled water (salinity 22) or Instant Ocean artificial seawater mix (salinity 40), compared to estimated carbonate conditions in natural coastal and estuarine waters of the same salinity [41].
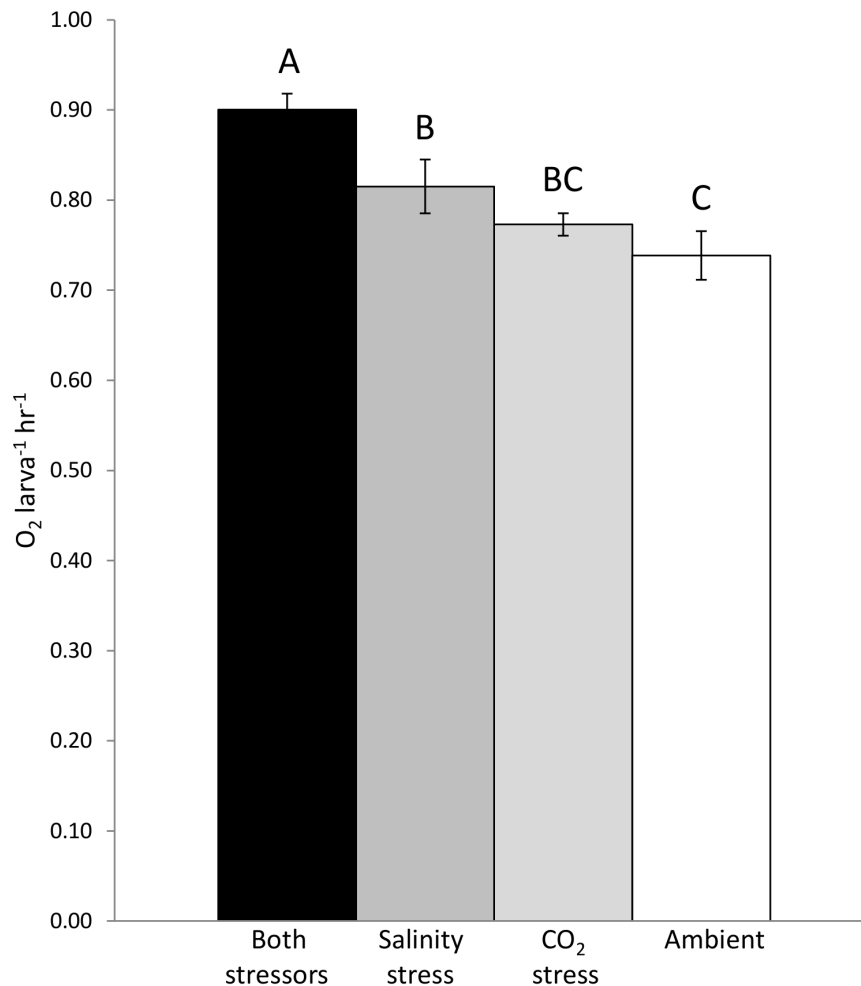doi:10.1371/journal.pone.0109167.t002

Figure 2. Multi-stressor effects on larvae. Oxygen consumption (mean ± S.E.) by larvae subjected to salinity stress, CO_2 stress, both stressors, and ambient conditions; bars connected by the same letter are not significantly different. Larvae subjected to ambient conditions consumed significantly less oxygen than larvae subjected to both salinity and CO_2 stress (p = 0.0149). There were no differences in oxygen consumption between larvae raised at ambient conditions and larvae subjected to a single stressor.
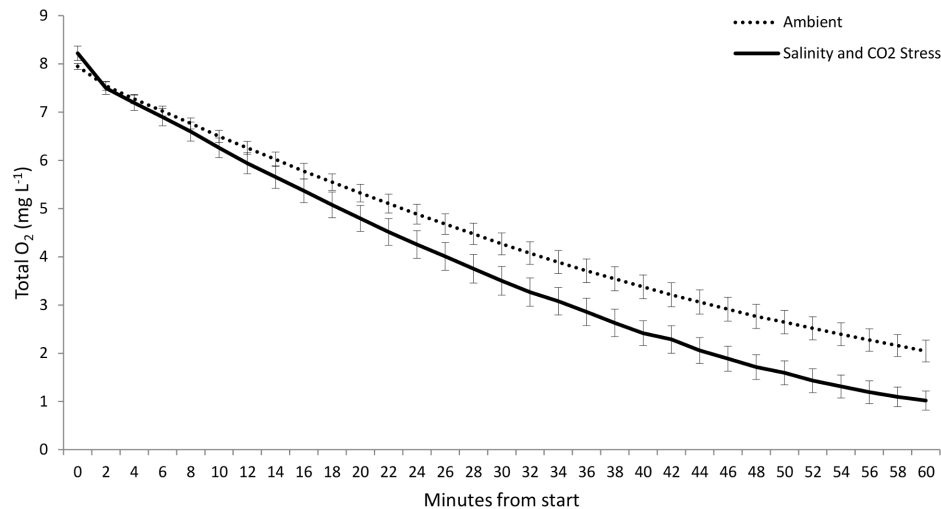doi:10.1371/journal.pone.0109167.g002

Figure 3. Respiration of larvae under stressful and ambient conditions. Oxygen consumption (mean $\pm$ S.E.) at stressful and ambient salinities by larvae raised under two pCO$_2$ treatments. Overall, larvae consumed significantly less oxygen at ambient (34) salinity (open squares) than at depressed or elevated (22 or 40) salinities (filled squares, p = 0.0038), though there was no difference in oxygen consumption between pCO$_2$ treatments (p = 0.1626).
doi:10.1371/journal.pone.0109167.g003

larvae raised under ambient conditions and tested subsequently under normal salinities (Figure 2; $F_{3,40}$ = 3.976, p = 0.0149).

Discussion

Throughout our experiment, differences in carbonate chemistry existed not only between our pCO$_2$ treatment conditions, but also among our salinity treatments used in the respirometry chamber. By moving larvae to solutions of altered salinities, alkalinities, and aragonite saturation states, we simulated the natural movement of larvae among water masses of varying physical characteristics in the field.

Crab larvae subjected to only a single stressor (salinity or CO$_2$) showed no difference in oxygen consumption compared to larvae raised under ambient conditions (Figure 2), demonstrating that larvae in our study were resilient to a single stressor. However, larvae subjected to both stressors consumed significantly more oxygen than larvae raised under ambient conditions (Figure 2). This finding indicates that these larvae were negatively affected by sequentially imposed stressors, even though they were largely unaffected by single stressors. This has important implications for understanding how species will respond to the multiple stressors expected with future climate change.

Our finding that the combined future increases in pCO$_2$ and changes to the hydrologic cycle could negatively affect crab larvae is somewhat surprising; most previous studies that have investigated the effect of future climate change on crustacean larvae have suggested that they will likely be largely unaffected [42,43,44,45,46,47], including two previous studies on the same species used in our study [32,33]. These studies, however, have primarily investigated single environmental stressors, or have imposed multiple stressors simultaneously. By applying multiple stressors sequentially in an environmentally relevant scenario that mimics realistic future conditions, we interpret that studies that investigate stressors individually might overestimate the resilience of crustacean larvae to future climate change. This investigation indicates that the timing of application of multiple stressors is an important factor to consider when studying the effects of future climate change. Not all stressors will act on the same timeframe,

and some stressors such as changes in salinity may be sudden but temporary events.

Stressful conditions appear to negatively impact these organisms by increasing metabolic rates, which would increase food demands. This effect has implications for performance and survival, particularly during the larval phase, when individuals require energy not only for their daily metabolic needs, but also for the energetically costly process of metamorphosis–a final step before recruiting into benthic populations. Larvae with reduced energetic reserves have been shown to select poorer settlement sites [48] and have lower reproductive output as adults [49] than larvae that settle with larger reserves.

Stressors associated with future climate change also have been shown to impair the sensory abilities of multiple taxa in tropical systems [50,51,52] and in intertidal crustaceans in the Atlantic [53,54]. Larvae of the genus *Petrolisthes* are gregarious settlers that rely on waterborne cues to find adult conspecifics that provide protection to newly settled postlarvae [55,56], so if their sensory abilities are impaired by the metabolic stress documented in our study, it would have a negative impact on their settlement success.

Finally, although adults of the genus *Petrolisthes* are highly resilient to abiotic stressors found in the intertidal zone [25,26,27,30], our work shows that larvae may be less resilient to stressors than previously believed, particularly when multiple stressors are imposed in succession, in a similar fashion to how they might be encountered by larvae swimming among water masses. This point indicates that even species that have evolved to live in abiotically stressful habitats such as the intertidal zone might be strongly affected by future climate change through susceptibilities manifesting during certain life stages.

Acknowledgments

Author Contributions

Conceived and designed the experiments: SHM SZ EHS BG TMH. Performed the experiments: SHM SZ EHS JDH. Analyzed the data: SHM JDH TMH. Wrote the paper: SHM SZ EHS BG JDH TMH.

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