The effects of elevated CO2 and related stressors on megalope and juvenile stage Dungeness crab (*Cancer magister*)

**Methods**

Long term Exposure

*Crab collection*. Live Dungeness crab megalope were collected by biologist from the Lummi Nation using light traps at two locations in the Salish Sea (figure 1) over the night of August 16-17, 2018 and transported to the Lummi Tribal Center in Bellingham, Washington. The light traps, which are part of an ongoing Lummi Nation Dungeness megalope monitoring project, were constructed from square 20 L buckets with four inlet funnels and a battery powered LED light source (ref). Several hundred megalope from the two sites were separated from bycatch and transported in coolers with ice packs and air bubblers from the Lummi Tribal Center to the NOAA Northwest Fisheries Science Center Mukilteo Research Station in Mukilteo Washington (Figure 1).

*Megalope Rearing.* On April 17 equal numbers of megalope from each site were pooled, then distributed into the wells of 6-well plastic culture plates with one megalope per well. Well plates contain 222 megalope were distributed into six “CO2-chambers”, three of which were designated as “ambient CO2” (n = 99) and three as “high CO2” (n = 123). CO2-chambers are modified refrigerators that 1) regulate temperature using high-precision thermal sensors with a custom control system, 2) control atmospheric CO2 concentration using mass flow controllers, and 3) control the temporal pattern, color and intensity of light using 4-color LED strips (Figure 2). All chambers were at 12°C with the ambient CO2-chambers maintained at a CO2 concentration of 400 ppm and the high CO2-chambers at 2,800 ppm (Table 1). Water to fill the well plates (5ml per well) was taken from 2L jars of 1 μm filtered seawater from Puget Sound that had been equilibrated to each chambers CO2 concentration and temperature by bubbling mixed-gas from the chamber’s mass flow controllers inside the chamber for several hours. The megalope were checked for survival and molting every day except the first day after placing in CO2-chambers. Megalope were transferred to new well plates with a new batch of equilibrated seawater every three days using a small spoon. The megalope were fed newly hatched *Artemia* naupli (San Francisco Bay brand) *ad libitum* when initially placed into well plates and after each well plate transfer (i.e. every three days). As soon as a megalope was observed to have molted, it was transferred to the juvenile rearing system.

*Juvenile Rearing.* Juveniles were reared in individual 250 ml polypropylene screw-top mesh jars that had 5-10 mm of silica sand in the bottom (“crabitat cups”, Figure 3). The silica sand provided cover habitat and potentially helped keep the exoskeleton free of parasites (Pam Jensen, pers. comm.). The crabitat cups were placed in polycarbonate holders that maintained them just below the surface in a 4’ diameter, 700 L flow through seawater tanks. Seawater into the tanks was pumped from a Puget Sound intake 10m deep and ~30m from shore and passed through a sand filter. Tanks were maintained at ambient temperature (Table 1) and each tank had a flow rate ~## liters per minute. Half of the tanks (3) were maintained at ambient pH (mean = 7.77) and pH in the other three tanks was controlled at a mean pH of 7.15 using a Durafet® pH sensor feed-back system that regulated the injection of #second bursts of pure CO2 into the tank through air stones near the bottom of the tank. The exact frequency of CO2 injection depended on the pH and temperature of incoming water, but was approximately every ##. Both ambient pH and low pH tanks were constantly aerated by bubbling ambient air though air stones.

Juveniles from megalope in high CO2-chambers were placed randomly in one of the three low pH tanks and juveniles from megalope in the low CO2-chambers were placed in the ambient pH tanks. The juveniles where checked every day for survival and molting. The crabs were fed *ad libidum* small pieces of market squid (*Doryteuthis opalescens*), geoduck (*Panopea generosa*) or salmon (*Oncorhynchus sp.)* twice per week with any uneaten food removed before new food was added. Fresh sand was added as need to replace and that which was slowly lost through the filters. All of the crabs from one tank were transferred to another tank with the same treatment conditions once per week in an effort to reduce long-term tank effects. Thus, since the crabs were moved from tank to tank as a group, “tank group” is a random factor in the statistical analysis. On July 7, 2019, a total of 327 days after megalope were placed into CO2 chambers, all surviving juvenile crabs were flash frozen at -80°C for future genetic and chemical analysis.

*Seawater chemistry.* Mass flow controller output was recorded to provide a continuous measurement of the CO2 concentration input into each chamber. Spectrophotometric pH measurements were taken of the CO2-equilibrated water used to fill the wells in each chamber on each water-change day and spectrophotometric pH measurements of water in # wells were measured on # days. In the juvenile tanks, pH values from the Durafet probes were recorded daily and spectrophotometric pH measurement taken weekly. Spectrophotometric pH measurements followed the best practices SOP (ref) using an Ocean Optics USB 230 2000+. Salinity of water in the CO2-chambers was recorded at each water change using a hand held salinity meter (Orion Star A322) and salinity in the juvenile takes was recorded daily from a continuous conductivity sensor located in one of the tanks () The daily continuous probe salinity readings were validated with occasional measurements in all of the tanks with the handheld sensor. Temperature the CO2-chambers was recorded continuously using chamber temperature probe (Labjack EI-1034) and verified periodically using a Fluke reference thermometer (Fluke 1523 Reference Thermometer). Temperature measurements from the Durafet® pH probes in the juvenile tanks were recorded once per day, with occasional validation with the Fluke reference thermometer. Alkalinity in the chamber water and in the juvenile tanks was calculated using a Mukilteo-specific alkalinity/salinity relation as described in (Trigg et al. 2019). The Mukilteo relationship ((Alk = 50.345 \* salinity + 522.506) has a similar slope to the Washington-wide equation developed by (Fassbender et al. 2017), but with an offset intercept, likely reflecting the influence of local rivers.

*Juvenile size analysis.* One to two weeks after each molting, after the exoskeleton were assumed to have completely expanded (ref), the live juveniles were photographed under a dissecting microscope for size analysis. Live crabs were dabbed dry before being placed in six well-plate, then photographed using Infinity Analyze software combined with a Nikon SMZ 745T microscope. Magnification varied with the size of the crab so that all crabs could be photographed at near full frame. After photographing, juveniles were immediately returned to treatment tanks. Crab width (distance along the transverse axis of the carapace) was measured using the Infinity Analyze software at the time the photo was taken. Crab length (distance along the sagittal axis of the carapace) was measured from the photographs at a later date using the ImageJ software straight-line tool (ref). Discarded exoskeletons after molting were also collected for size analysis. The molted exoskeletons were air dried, then photographed #-# days after molting. The legs of the discarded exoskeletons were removed to obtain a clear view orthogonal to the plane of the carapace.

*Survival and molting statistics.* Analysis was conducted using the R statistical package (version 4.0.0). The effect of pH on overall survival was evaluated using a hazard model, with CO2-chamber and tank group considered random variables (i.e. clustered frailty terms) in a cluster randomized trial design.

The transition hazard function (i.e. instantaneous transition rate at time *t*, given that the transition has not happened prior to *t)* is:

Where λ*(t)* is the hazard at time *t*, λ*0(t)* is the baseline hazard, *zi* is a cluster-specific term modifying the baseline hazard in group *i*, β is a vector of the transition-specific treatment coefficients and *x* is the vector of covariates (i.e. treatment values). The hazard equation was fit using the frailtyEM R package (ref). The distribution of z was selected from options of gamma, positive stable, inverse gausian, and non-central gamma by comparing likelihood. The decision on whether to include the include the frailty term at all or opt for simpler cox proportional hazard model was made based on the Commenges-Andersen test for heterogeneity of frailty terms and by likelihood ratio test comparing the model with and without frailty.

To evaluate the effect of the effects of pH on molting rate and stage-specific survival we use a multi-state Markov hazards model. In the multi-state model, crabs at each molt stage could either molt to the next stage or die, resulting in 15 possible transitions (figure 3). The frailty hazard model is as described in equation 1, but with a separate hazard function λ*q(t)* and baseline hazard λ*q0(t)* for each *q* transition (i.e. separate strata for each transition).

The multi-state model cannot be fit using the frailtyEM package, so, after formatting the data into a form appropriate for a multi-state model using the mstate R package, the hazard coefficients were fit using the coxme package (ref). The coxme model assumes a log normal distribution for the mixed effects term, *zi*, and fits the coefficients using a proportional hazards partial likelihood approach. A permutation test was applied to evaluate whether the transition-specific β values significantly differ from zero. The permutation test was selected as more robust than a distribution-based test given the relatively low number of tank groups (3 per treatment) and the potential for type-1 errors in cluster randomized trials with few clusters. To further reduce the risk of type-1 errors, we applied a Holm-Bonferoni correction to account for testing 15 separate transitions. For comparison, a simple, cox proportional hazard model without the frailty term (i.e. CO2-chamber/tank group effect) was also evaluated. For visualization, probability plots of molt stage and survival for ambient and low pH treatments were generated using the mstate R package. The mstate package is not compatible with mixed effects models so the probability plots were generated with a model containing only pH treatment as a factor. The plot presents the marginal effect of CO2 treatment ignoring the CO2-chamber and tank group random variables.

*Size statistics.* Analysis was conducted using the R statistical package (version 4.0.0). Size response metrics included the carapace length and width measurements, the ratio of length to width (which provides a measure of “roundness”), the weights collected as part of the respirometry analysis, and the weight to width ratio (which provides a metric related to density). For the width, length and length/width metrics, the overall effect of CO2 treatment as evaluated as a repeated-measures (mixed-effects) model to account for measurements taken on the same individual at each life-stage. Mixed effects were analyzed using the lme4 R package. Considering potential CO2-by-stage interactions and random tank effects, the full model is:

*Size\_metric ~ CO2 + stage + CO2 \* stage + random(crab) + random(tank\_group).*

Likelihood ratio tests using the R stats and RLRsim packages were used to select among the potential models with all combinations of including or excluding the interaction and tank group terms. The weight measurements were taken only once per individual so the full was simply

*weight\_metric ~ CO2 + stage + CO2 \* stage.*

Weight measurements were taken from every tank group, but sample sizes were too small to evaluate any tank group effects. Likelihood ratio tests were used to select among weight models with and without the interaction terms. Although stage is an ordinal valued variable, it was treated as continuous in these overall models (ref). In addition to the overall analyses, treatment comparisons were made separately for each metric for each stage. Models with and without the random tank effect term were compared by likelihood ratio test.

**Results**

*Survival and molting rate.* Megalope had high survival (96.0 % survival in low CO2 and 97.6 % survival in high CO2) with no statistically significant effect of CO2 treatment (hazard model with inverse Gaussian frailty, p = 0.25). The megalope molted to the J1 stage relatively quickly after being placed in the CO2 chambers (mean = 4.3 d). Two megalope that had not molted after 10 d where removed from experiment and treated as right censored observations.

The overall survival of juveniles reared in high CO2 was higher than those reared in ambient CO2 (figure 4). The final fraction of survivors at the end of the experiment was #% (x/y) in ambient CO2 and #% (x/y) in high CO2. In the hazard model, the frailty term (tank group) was not significant (Commenges-Andersen p = 0.763, LRT p = 0.493), so the analysis was conducted as a cox proportional hazards model with only treatment as a fixed effect (p = 0.0005). The during the experiment the odds of dying were over twice as high in low CO2 as in high CO2 (exp(β) = 2.22). During the experiment, juveniles would occasionally escape when the containers were open during feeding or they escaped though small joints in the mesh jars. These escapees were treated as right censored observations (as were all crabs still alive at the end of the experiment). For comparison, the analysis was run both with and without the inclusion the right censored escapees in the dataset, which produced identical results with regards to treatment significance.

The probability of being in a particular stage as a function of time is shown in figure 5. In considering life-stage specific survival transitions (figure 6), only the J1 to J2 transition was found to be significant, with the odds of dying over four times greater in low CO2 than in high CO2 (coxme permutation model, p = 0.006, exp(β) = 4.79). Because none of the four crabs from the ambient CO2 treatment that made it to the J7 stage died before the end of the experiment, we cannot make any inference from the hazard model with respect to J7 survival. With a sample size of only four crabs in one of the treatments, we did not attempt any other sort of analysis of J7 survival and we consider it unknown.

In the molting transitions (figure 6), only the J5 to J6 transition was significantly different among treatments, with the odds of transitioning over three times great for crabs in high CO2 compared to crabs in low CO2 (coxme permutation model, p = <0.0001, exp(β) = 3.125). The mean duration of the J5 stage was 8.6 days shorter for crabs reared in high CO2 compared to those reared in ambient conditions.

*Size.* Juvenile Dungeness crab reared in ambient CO2 were overall larger and had a wider shape than crab reared in high CO2, though there was no difference in weight (Figure 8, Table 1). The width and length/width models include both a main treatment term and a treatment x stage interaction term, with only the interaction being significant. This is consistent with the stage-level analysis, which show some stages with substantial treatment differences in size by not others (Figure 8, Table 2). For the stage-level analysis, the selected model in nearly all cases included only the CO2 treatment term without the random tank group term, so tank group was dropped from the analysis. The greatest significant relative difference in size occur with length of the J6 stage, in which the crabs reared in ambient CO2 were nearly 14% longer (Table 2). Crabs reared in ambient CO2 had a wider, more oblong shape than crabs rear in high CO2. There was no effect of CO2 treatment on eight or on the quasi-density metric, weight/length. Sample sizes varied with crab abundance by stage and CO2 treatment, with relatively low power at the J7 stage in all of the size metrics and in J5 for the weight metric.

Overall, the live carapace width correlated tightly with discarded molt exoskeleton, with the discard being slightly smaller on average than the carapace on the live crab (Figure 9 upper panel; slope = 0.96, p << 0.001, R2 = 0.96). There was no significant difference in the discard to live width ratio with regard to CO2 treatment, but there was a significant difference among the stages (Figure 9 lower panel; for model discard/live = treatment + stage, ptreatment = 0.32, pstage < 0.01 for all pairwise comparisons).

**Tables**

**Table 1:** Selected model and p-value results from analysis of juvenile crabs reared in ambient and high CO2 treatments. Significant p-values in bold.

|  |  |  |  |
| --- | --- | --- | --- |
| Size metric | Selected Model | p-value (CO2) | p-value (CO2 x stage) |
| width | CO2 + stage + CO2 \* stage + random(crab) | 0.492 | **<0.0001** |
| length | CO2 + stage + random(crab) | **0.0201** | --- |
| length/width | CO2 + stage + CO2 \* stage + random(crab) + random(tank) | 0.4484 | **0.0008** |
| weight | CO2 + stage + CO2 \* stage | 0.8648 | 0.7192 |
| weight/width | CO2 + stage + CO2 \* stage | 0.3921 | 0.3437 |

**Table 2:** Sample sizes and p-values for analysis of the size of juvenile Dungeness crab reared in ambient and high CO2. Analysis compares the effect of CO2 within a single life-stage. The “Size Diff” is the average size of crabs rear in ambient CO2 minus the average size of crabs reared in high CO2. The “Percent Diff” is the percent difference between the ambient and high CO2 measurment Significant p-values in bold.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Size Metric | Life-Stage | Ambient CO2 Sample Size | High CO2 Sample Size | Total Sample Size | Ambient CO2 mean size | High CO2 mean size | Size Diff | Size Units | Percent Diff | p-value |
| width | J1 | 51 | 66 | 117 | 4.9 | 4.89 | 0.01 | mm | 0.2 | 0.7972 |
| width | J2 | 57 | 76 | 133 | 6.52 | 6.42 | 0.10 | mm | 1.6 | 0.1427 |
| width | J3 | 50 | 70 | 120 | 8.64 | 8.22 | 0.41 | mm | 5.0 | **0.0014** |
| width | J4 | 45 | 65 | 110 | 10.3 | 9.63 | 0.67 | mm | 7.0 | **<0.0001** |
| width | J5 | 39 | 56 | 95 | 12.84 | 12 | 0.84 | mm | 7.0 | **<0.0001** |
| width | J6 | 16 | 48 | 64 | 15.65 | 14.4 | 1.26 | mm | 8.7 | **0.0030** |
| width | J7 | 4 | 26 | 30 | 17.85 | 16.61 | 1.24 | mm | 7.4 | 0.1551 |
| length | J1 | 51 | 66 | 117 | 5.1 | 5.12 | -0.03 | mm | -0.5 | 0.5878 |
| length | J2 | 57 | 75 | 132 | 6.01 | 6 | 0.01 | mm | 0.2 | 0.8406 |
| length | J3 | 48 | 68 | 116 | 7.49 | 7.32 | 0.17 | mm | 2.4 | 0.1086 |
| length | J4 | 45 | 65 | 110 | 8.78 | 8.32 | 0.46 | mm | 5.5 | **<0.0001** |
| length | J5 | 37 | 56 | 93 | 10.6 | 10.14 | 0.46 | mm | 4.5 | **0.0077** |
| length | J6 | 14 | 46 | 60 | 12.74 | 11.87 | 0.87 | mm | 7.3 | **0.0097** |
| length | J7 | 4 | 21 | 25 | 13.93 | 13.62 | 0.30 | mm | 2.2 | 0.6366 |
| length/width | J1 | 51 | 66 | 117 | 1.04 | 1.05 | -0.01 | unitless | -0.7 | 0.3664 |
| length/width | J2 | 57 | 75 | 132 | 0.92 | 0.94 | -0.01 | unitless | -1.4 | **0.0288** |
| length/width | J3 | 48 | 68 | 116 | 0.87 | 0.89 | -0.02 | unitless | -1.9 | 0.0526 |
| length/width | J4 | 45 | 65 | 110 | 0.85 | 0.86 | -0.01 | unitless | -1.3 | **0.0344** |
| length/width | J5 | 37 | 55 | 92 | 0.82 | 0.85 | -0.02 | unitless | -2.9 | **<0.0001** |
| length/width | J6 | 14 | 46 | 60 | 0.81 | 0.83 | -0.01 | unitless | -1.5 | 0.0749 |
| length/width | J7 | 4 | 21 | 25 | 0.78 | 0.81 | -0.03 | unitless | -4 | **0.0206** |
| weight | J5 | 7 | 4 | 11 | 0.43 | 0.4 | 0.02 | g | 5.7 | 0.7336 |
| weight | J6 | 13 | 29 | 42 | 0.73 | 0.63 | 0.10 | g | 15.3 | 0.0745 |
| weight | J7 | 2 | 17 | 19 | 0.95 | 0.89 | 0.06 | g | 6.4 | 0.6964 |
| weight/width | J5 | 7 | 3 | 10 | 0.03 | 0.04 | -0.01 | g/mm | -13.1 | 0.4060 |
| weight/width | J6 | 13 | 28 | 41 | 0.05 | 0.04 | 0.0 | g/mm | 8.8 | 0.1901 |
| weight/width | J7 | 2 | 17 | 19 | 0.05 | 0.05 | 0.0 | g/mm | 1.9 | 0.8634 |

**Figure Legends**

**Figure 1:** Maps showing the location of Dungeness crab megalope collection sites and research station where experiments were conducted at the scale of the West Coast of North America (a) and at the scale of the Salish Sea (b). The two collection locations were Hale’s Pass (48.72923 -122.667) and Sandy Point (48.81622 -122.711), which are ~10km apart. The Hale’s Pass light trap site is ~150m from shore and the Sandy Point site is ~250m from shore.

**Figure 2:** Simplified schematic of CO2-chamber with door removed. The base unit is a small (311 L) refrigerator. The temperature, CO2 concentration and lighting are all controlled via a custom Labview® program running on a small Windows® platform computer. For temperature control, a high precision silicon temperature probe provides input with output vis USB relays controlling the refrigerator compressor for cooling and a PTC electric heater with fan for heating. The concentration of CO2 in the chamber is controlled with two mass flow controllers. One mass flow controller regulates the stream of CO2-free air from a CO2-adsorber. The second mass flow controller regulates the flow of CO2 from a compressed gas cylinder. To produce treatment water for initial distribution into well plates or to maintain the CO2 concentration in a chamber at target conditions, mixed-gas at the target CO2 concentration (e.g. 400 ppm or 2100 ppm) is bubbled into a beaker of seawater. Bubbling in water maintains high humidity in the chamber, which reduces evaporation in the well plates. Although most bubbling uses mixed gas at the target concentration, immediately after opening and closing the door, pure CO2 is briefly bubbled in the chamber to rapidly return the chamber from the ambient CO2 concentration experienced when the door opens (~400ppm) to the target concentration. To prevent adding too much CO2 to water being equilibrated for well plates, the temporary high CO2-stream is diverted by a 3-way valve to a second beaker of water. To control lighting, a DMX controller is used to regulate the intensity and color of four LED strips (two LED strips attached to the door are not shown in the schematic.) The LED strips are 4-color (rbgw), which allows production of any color frequency distribution in the visible spectrum. A light sensor in the chamber validates whether lights come on as programed. Sensor inputs to the computer are routed through a Labjack® DAQ. The labview program provides and interface for controlling set points, a mechanism for viewing and archiving chamber data and the ability to set alarm thresholds for text message chamber condition updates.

**Figure 3:** Transition diagram showing the 9 states and 15 transitions for multi-state hazard model.

**Figure 4:** Probability of survival for juvenile Dungeness crab reared in ambient CO2 water (pH = 7.77; blue line) or high CO2 (pH = 7.15; red line). The shaded areas show 95% confidence intervals.

**Figure 5:** Probability of Dungeness crab being in a given life-stage or dead as a function of time for ambient CO2 (upper panel) or high CO2 (lower panel).

**Figure 6:** Life-stage specific probability of survival as a function of time since the start of the experiment for Dungeness crab reared in ambient and high CO2 water. The only life-stage with a statistically significant difference in survival as a function of CO2 treatment (J1) is indicated with a thicker line.

**Figure 7:** Probability of molting between life-stages as a function of time since the start of the experiment for Dungeness crab reared in ambient and high CO2 water. The only transition with a statistically significant difference in molting probability as a function of CO2 treatment (J5 to J6) is indicated with a thicker line.

**Figure 8:** Size measurements of Dungeness crab juveniles reared in ambient CO2 (blue) and high CO2 (red). Points show measurements for individual crabs, with the bar of the box plot indicating the median, the box showing 25th and 75th percentiles and the whiskers 1.5 \* inter-quartile range. The black stars indicate juvenile stages with significant treatment differences. Width is a measure of carapace width, length/width is a ratio describing the shape of the crab, weight is live weight and weight/width is related to crab density.

**Figure 9:** The upper panel shows the relationship between the carapace width of live Dungeness crab at a particular juvenile stage and the width of the discarded carapace after the crab molted out of that stage into the next. Each point is an individual crab at a particular life-stage and the black inline is the linear regression through all points. The lower panel shows the discarded molt to live crab carapace ratio at each life-stage for crab reared in ambient CO2 (blue) and high CO2 (red). A molt-to-live ratio of one would indicate that the discarded carapace was exactly the same size as the carapace on the live crab before it was discarded. The points are individual crab at a particular life-stage with the bar indicating the median, the box encompassing the 25th and 75th percentiles and the whisker showing 1.5 times the inter-quartile range.