Effect of low pH on early life stages of the decapod crustacean, Dungeness crab (*Cancer* magister)

Jason James Miller

A thesis
submitted in partial fulfillment of the
requirements for the degree of

Master of Science

University of Washington
2015

Committee:
Carolyn Friedman
Steven Roberts
Julie Keister
Paul McElhany

Program Authorized to offer Degree:
School of Aquatic and Fishery Sciences

University of Washington

ABSTRACT

Effect of low pH on early life stages of the decapod crustacean, Dungeness crab (*Cancer* magister)

Jason James Miller

Chair of Supervisory Committee:
Professor Carolyn Friedman
School of Aquatic and Fishery Sciences

Dungeness crab (*Cancer magister*) eggs and larvae were exposed to laboratory-controlled, low-pH seawater in an effort to assess current and predicted-future impacts of Ocean Acidification (OA) on hatching success, survival and growth. Treatment levels of pH ~8.0, ~7.5 and ~7.1 represented the wide range of pH-levels relevant to current-open-ocean, current-upwelled and future-upwelled conditions associated with *C. magister* habitat in the northeast Pacific Ocean. For this study, pH ~8.0 represented the "control". *C. magister* eggs were exposed to treatment levels for 34 days. There was no effect of treatment on probability of hatching, however there was a delay in hatch-timing for eggs in pH 7.1. Newly hatched *C. magister* larvae were exposed to treatment levels for 45 days with 57.9%, 13.5%, and 21.1% surviving in pH 8.0, 7.5, and 7.1 respectively. Larvae in the low-pH treatments were 2.5-3 times less likely to survive than in the control. There was no effect of treatment on larval size at a particular larval stage, however, larvae in the low-pH treatments progressed through larval stages at a slower rate than the control. While some larvae survived the low-pH conditions to the end of the experiment, the lowest survivorship occurred in seawater reflective of pH-levels that can currently be experienced in estuaries and areas of upwelling. The results of this study indicate that low-pH seawater caused by OA can slow down progression through early life stages and that long-duration exposure can result in mortality.

Contents

Figures and Tables

Dedication

I dedicate this thesis to my wife, Letty, and to my daughters, Maya and Meli. It has not been lost on me that my efforts moving forward with the work described within these pages took my attention away from them; I hope to make up for that in the years to come. Their support, care, humor and smiles mean the world to me.

1 Literature Review

1.1 Introduction to ocean acidification (OA)

As nations continue to rely on fossil fuels for producing energy, global concentrations of atmospheric carbon dioxide (CO_2) are increasing at an alarming rate. Over the last 200 years the global average atmospheric CO_2 concentration increased from 280 ppm to 394 ppm and is expected to increase to above 1000 ppm by the year 2100 (IPCC 2013). Of the anthropogenic CO_2 released into the atmosphere, 26% is absorbed by oceans (Canadell et al. 2007; Le Quere et al 2009). The oceans have thus absorbed over 118 billion metric tons of anthropogenic CO_2 emissions over the past two centuries (Sabine 2004; Feely et al. 2010). Notably, an estimated 35% of those CO_2 emissions were absorbed within the last twenty-five years (Khatiwala et al. 2009). Although the ocean's ability to absorb CO_2 is a benefit to reducing atmospheric 'greenhouse gases', dissolved CO_2 reacts in seawater and reduces seawater pH. Since pre-industrial times the pH in global ocean surface water decreased 0.1 pH units and is projected to drop another 0.3 to 0.4 units by year 2100 (Orr et al. 2005; Royal Society 2005; IPCC 2013). The resultant drop in seawater pH due to the ocean's absorption of atmospheric, anthropogenic CO_2 has been termed, ocean acidification (Feely et al. 2004; Caldiera and Wicket 2005; Field et al. 2011). Ocean acidification (OA) will alter marine ecosystems and have deleterious impacts on certain marine organisms (Fabry et al. 2008).

1.2 The effect of low pH on decapod crustaceans

One way to decipher whether decapod crustaceans are susceptible to low pH is to expose them to OA-relevant pH levels. Reported effects from low pH on decapod crustaceans range from impacts on survival to non-lethal physiological and behavioral impacts to no impacts at all.

The effects may depend on whether the organism is predisposed to dynamic habitats (e.g. in the intertidal) where water chemistry might regularly fluctuate, or relatively stable habitats (e.g. deep water) where water chemistry is slower to change. The effects may also depend on life stage (e.g. planktonic, larval stage versus benthic, adult stage). For the intertidal Porcelain crab, *Petrolisthes cinctipes*, exposure to pH 7.6 had no effect on larval survival relative to pH 7.9, however continued exposure of juveniles (40 days) resulted in a 30% reduction in survival at the same pH levels (Ceballos-Osuna et al. 2013). When two life-stages of the deep-water Red King crab, *Paralithodes camtschaticus*, were exposed to low pH, survival was reduced in both larvae (pH 7.7, Long et al. 2013a) as well as juveniles (pH 7.5 and pH 7.7, Long et al. 2013b) relative to the control pH 8.0.

Exposure to low pH may change how a decapod crustacean forms its exoskeleton. The calcium content of Tanner crab, *Chionoecetes bairdi*, juveniles after a 200-day exposure pH 7.5 and pH 7.8 seawater resulted in reduced levels of calcium content relative to those in a pH 8.0 treatment (Long et al. 2013b). When exposed to low pH, larval European lobsters, *Homarus gammarus*, grew to have lighter dry weights and less mineral content in the exoskeleton (Arnold et al. 2009), while adult the blue crab, *Callinectes sapidus*, and lobster, *Homarus americanus*, created thicker shells in acidified treatments (Ries et al. 2009). Impacts from low pH on exoskeleton structure, whether from delaying the calcification process or from greater expenditure of energy to create it, could result in increased mortality rates from predation or general lack of fitness from reallocation of energetic reserves (Whitely 2011).

Effects of low pH have been reported on reproductive life-stages in decapod crustaceans. More work with *P. cinctipes* measured reduced embryonic metabolism during short-term exposure to low pH (Carter et al. 2013). Low pH 7.64 reduced reproductive capability for a

marine shrimp, Palaemon pacificus (Kurihara et al. 2008). The eggs of the northern shrimp (Pandalus borealis) showed no effect on hatch-timing when exposed to low pH seawater (Arnberg et al. 2013), while Long et al. (2013a) found a pH of 7.7 increased the hatching duration of *P. camtschaticus* relative to a control of pH 8.0.

Some low-pH exposure studies have shown little to no effect at all. The adult velvet crab, *Necora puber*, showed very little reaction to low pH over a 16-day trial other than increased bicarbonate in their hemolymph (Spicer et al., 2006); adult palaemonid prawns responded similarly (Dissanayake et al. 2010).

Given the high variability in response to low pH among decapod crustacean species, species-specific experimentation under ecologically relevant conditions remains necessary.

1.3 An overview of the decapod crustacean, *Cancer magister* Dana 1852[1]

1.3.1 Life Stages

The range of *C. magister* extends from south central California to the Gulf of Alaska (Fig.1) with adults inhabiting depths from the intertidal nearshore to 230 meters (Jensen 2014). *C. magister* adults mature after about 2 years (Pauley 1989, Armstrong et al. 2010) and females extrude eggs in the fall months typically beginning earliest in southern latitudes and progressing northward (Stone and O'Clair 2001). Mature females can produce up to two million eggs annually (Pauley et al. 1986). *C. magister* females brood their eggs and tend to stay in place while doing so at depths from 1 to 16 meters (Armstrong et al 1988; Stone and O'Clair 2002).

[1] Schweitzer and Feldmann (2000) proposed elevating the *Cancer* subgenera, *metacarcinus*, outlined by Nations (1975) to the generic level re-classifying the Dungeness crab as *Metacarcinus magister* based solely on the shape of carapace teeth. Due to a lack of molecular evidence (see Harrison and Crespi 1999) to support Nations' subdivisions, I elected to maintain the use of *Cancer magister*.

Hatch timing and larval duration are influenced by water temperature (Shirley et al. 1987; Sulkin and Mckeen 1989) with eggs hatching 90-160 days post-extrusion (Rasmuson 2013). Immediately after hatching, the larvae, referred to as 'zoeae', rise in the water column where they catch tidal currents moving them offshore, away from the nearshore environment, out of estuaries or bays, and into deeper offshore waters (Gaumer 1970; Pauley et al. 1989; Roegner et al. 2003). Based on *C. magister* fishery-harvests and *C. magister* life-history characteristics an estimated 26-78 trillion *C. magister* larvae enter the water column annually across their entire geographical range (see Appendix for calculations). The larval stage consists of five zoeal stages and the transitional megalops stage (Fig.1). In between each stage the crab molts its exoskeleton. Depending on ocean conditions, such as water temperature, *C. magister* larvae spend 2-5 months in the water column before settling into the nearshore benthos as juveniles (Moloney et al. 1994; Park and Shirley 2005).

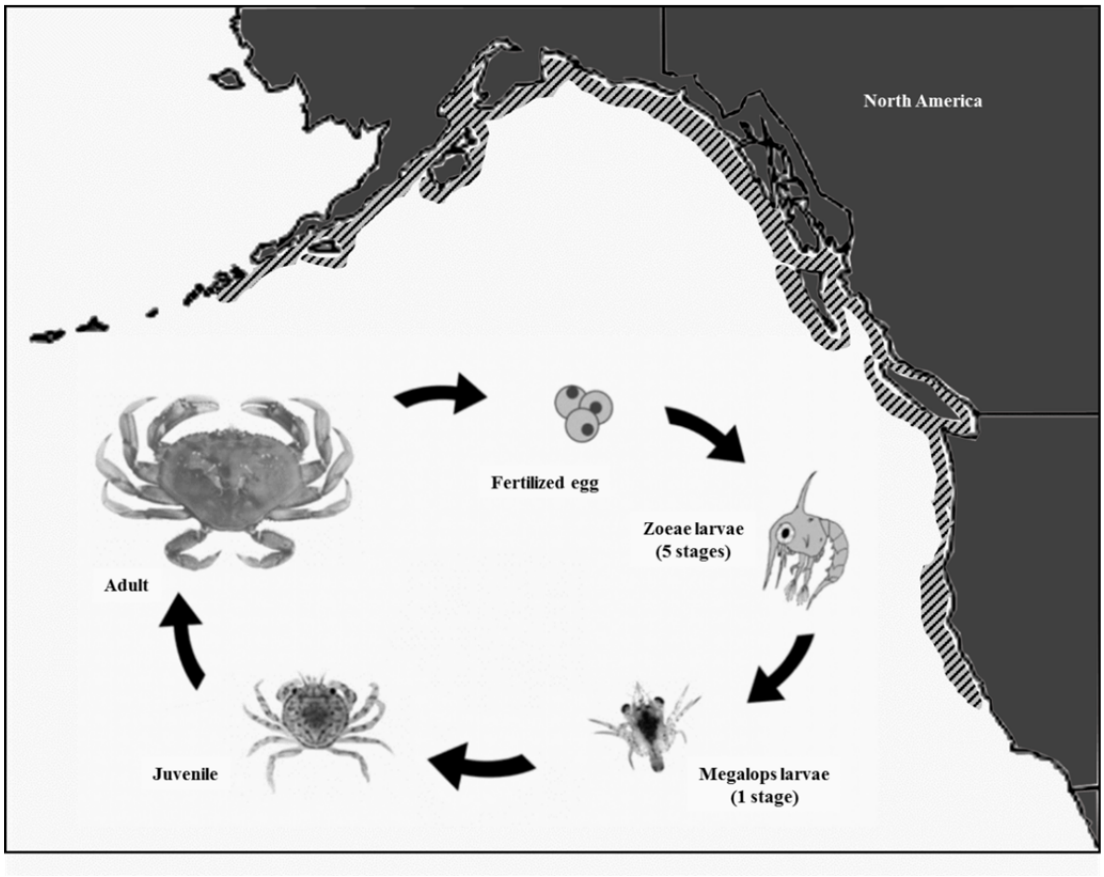

Fig 1. Map of C. magister distribution in northeast Pacific (gray shaded region along coast) with inset of life-history cycle (adapted from Fisher and Velasquez 2008). This study exposed 'fertilized egg' and planktonic 'zoeae larvae' life stages to low-pH seawater

1.3.2 Larval dispersal and migration

It is important to understand how *C. magister* larvae interact within the water column and water currents to assess their vulnerability to OA. *C. magister* larvae emerge in estuaries, emigrate to develop in coastal regions then return to the estuaries to settle into the benthos (Park and Shirley 2008). With initial zoeal swimming speeds estimated at 1-14mm/sec in lab-based observations (Gaumer 1970), movement against a current is unlikely. However in the megalops stage, larvae have exceeded 0.40m s^{-1} giving them greater chances of directing their dispersal (Fernandez et al. 1994).

Diel vertical migration

During the 2-5 months in the water column *C. magister* zoeae perform diel vertical migrations away from surface waters during daylight typically reaching depths of 20-30m with the ability to perform deeper migrations as they progress through later stages (Sulkin and McKeen 1989; Hobbs and Botsford 1992; Shanks 2013). Megalopae perform diel vertical migrations to depths of 60 to 70m (Hobbs et al. 1992; Shanks 2013) and have been recorded to exceed 160m depth in the Strait of Georgia, BC (Jamieson and Phillips 1993).

Offshore, longshore and within-estuary migration

Along the northern California, Oregon and Washington coasts, evidence suggests *C. magister* larvae spread horizontally with later stages being found further offshore (Lough 1976). Reilly (1983) found stage-1 zoeae 42km from shore with stage-3 and stage-5 zoeae as far as 185km offshore. This nearshore to offshore progression is also found in southeast Alaska (Park and Shirley 2008). As coastal *C. magister* make offshore progress they become entrained in the California Current System (CCS). There is evidence that *C. magister* larvae from CCS can disperse as far north as the Gulf of Alaska (Park et al. 2007) as well as into the Salish Sea (Dinnel et al. 1993). Larvae hatched in large, estuarine systems like Puget Sound, WA, follow a similar pattern of moving to deeper, offshore waters, however dispersal ranges are more greatly influenced by geological constraints (Armstrong et al. 1988).

Outer coast currents/California current

To understand the potential impacts of OA on the outer coast population of *C. magister* larvae it is important to have a sense of what the California Current System (CCS) is doing between the months of January through May when the larvae are there. In late winter months when zoea-stage larvae are present in the water column, currents are predominately northward. At some point in the spring the Spring Transition arrives, winter winds and storms die down, summer wind regimes take over and upwelling occurs along the coast (Strub et al. 1987). Typically at this time larvae are approaching their final zoeal stages and transitioning to the megalops stage when they will begin the journey back to estuaries to find suitable habitat to settle into the benthos and become juveniles. The upwelling waters along the Pacific coast are high in beneficial nutrients to the CCS ecosystem as well as naturally low in pH (Feely et al. 2008). Modeling of the resultant surface-water pH from the 'high emissions' atmospheric CO_2 loadings scenario (Nakicenovic et al. 2000) showed pH 7.82 in the nearshore 10 km environment of central CCS by 2050, a drop of over 0.13 pH units (over 30% increase in acidity) from 2005 (Gruber et al. 2012). If *C. magister* are susceptible to deleterious impacts caused by low-pH exposure, the combination of upwelled, low-pH seawater and acidified surface water in the CCS could increase *C. magister* vulnerability.

1.3.3 Value of Cancer magister

C. magister is an ecologically, economically and culturally important marine species. Decapod crustaceans like *C. magister* fill ecologically important roles as prey items for marine-based food webs (Field et al. 2006, Harvey et al 2010). For the 2 – 5 months that they are in the water column, *C. magister* larvae provide valuable prey items for forage fish and high-profile

species like salmon, rockfish and herring (Rielly 1983; Pauley et al. 1989; Bollens et al. 2010). Once settled into the benthic community *C. magister* are preyed upon by a suite of new predators including sea otters, fish, octopuses and humans (Pauley et al. 1989; Fisher and Velasquez 2008). Adult *C. magister* males are heavily harvested and culturally important throughout their coastal range (Fig.1). The annual combined commercial and tribal harvest averaged over 2003 to 2012, inclusive was 35.7 thousand tonnes (FAO.org) With ex-vessel commercial and tribal landings reaching over $176 million in 2012, more than $343 million income was generated sustaining over 7200 jobs (IO-PAC model, Leonard and Watson 2011). OA effects on commercially important decapod crustaceans could have large socio-economic impacts.

1.4 Suquamish Tribe in Washington State concerned, *C. magister* and OA

Even though catch records in Puget Sound, WA remain stable overall (Fisher and Velasquez 2008), harvested numbers out of Hood Canal, a fjord-like arm of Puget Sound, experienced a three-year decrease beginning in 2005 (Williams et al. 2009). With consistent fishing effort, the harvested numbers dropped from 700,000 pounds in 2004 to 150,000 pounds in 2008 (Williams et al. 2009). While harvest levels are known to be erratic, the observed decrease from 2005 to 2008 was unprecedented. This reduction in harvest affected Coastal Native American tribes such as the Suquamish tribe who rely on marine crustaceans for subsistence and economic well-being. The hardship motivated state and tribal biologists to start looking for causes of the decline. One of the potential stressors is believed to be OA. Seawater pH levels in Hood Canal have already been recorded below the levels projected for 2100 (pH 7.6-7.7, IPCC 2013) and were estimated to range from 7.3 to 7.9 (Feely et al. 2010).

References

Armstrong, DA, Armstrong, J, Dinnel P (1988) Distribution, abundance and habitat association of Dungeness crab, Cancer magister, in Guemes Channel, San Juan Islands, Washington. J. Shellfish Res. 7:147-148

Armstrong, D.A., P.S. McDonald, G.H. Kruse, A.H. Hines, and J.M. Orensanz (2010) A Crab for all Seasons: The Confluence of Fisheries and Climate as Drivers of Crab Abundance and Distribution. In: G.H. Kruse, G.L. Eckert, R.J. Foy, R.N. Lipcius, B. Sainte-Marie, D.L. Stram, and D. Woodby (eds.), Biology and Management of Exploited Crab Populations under Climate Change. Alaska Sea Grant, University of Alaska Fairbanks. doi:10.4027/bmecpcc.2010.05

Arnberg M, Calosi P, Spicer JI, Tandberg AHS, Nilsen M, Westerlund S, and Bechmann RK (2013) Elevated temperature elicits greater effects than decreased pH on the development, feeding and metabolism of northern shrimp (Pandalus borealis) larvae. Mar Biol 160(8):2037-2048 DOI 10.1007/s00227-012-2072-9

Arnold, K.E., Findlay, H.S., Spicer, J.I., Daniels, C.L., and Boothroyd, D (2009) Effect of CO2-related acidification on aspects of the larval development of the European lobster, Homarus gammarus (L.) Biogeosciences 6:1747-1754

Bollens, SM, vanden Hoof, R, Cordell, JR, Frost, BW (2010) Feeding ecology of juvenile Pacific salmon (Oncorhynchus spp.) in a northeast Pacific fjord: diet, availability of zooplankton, selectivity for prey, and potential competition for prey resources. Fish. Bull. 108(4): 393-407.

Carter, HA, Ceballos-Osuna, L, Miller, NA, Stillman, JH (2013) Impact of ocean acidification on the metabolism and energetics of early life stages in the intertidal porcelain crab Petrolisthes cinctipes. J. Exp. Biol. 216:1412-1422.

Ceballos-Osuna, L, Carter, HA, Miller, NA, and Stillman, JH (2013) Effects of ocean acidification on early life-history stages of the intertidal porcelain crab Petrolisthes cinctipes. J. Exp. Biol. 216:1405-1411.

Dinnel P.A., Armstrong D.A., and McMillan, R.O. 1993. Evidence for multiple recruitment-cohorts of Puget Sound Dungeness crab, *Cancer magister*. Marine Biology 115:53-63

Dissanayake, A, Clough, R, Spicer, JI, and Jones, MB (2010) Effects of hypercapnia on acid-base balance and osmo-/iono-regulation in prawns (Decapoda: Palaemonidae). Aquatic Biology 11:27-36.

Dunn, P., Shanks, A., 2012. Mating success of female Dungeness crabs (Cancer magister)in Oregon coastal waters. J. Shellfish Res. 31, 835–839.

Ebert, E. E., R. O. Kelly, and A. W. Haseltine. 1975. Observations on the larval hatching success of Dungeness crab, Cancer magister, from the San Francisco and Eureka-Crescent City regions. California Department of Fish and Game. Marine Resources Technical Report No. 29

Fabry, VJ, Seibel, BA, Feely, RA, Orr, JC (2008) Impacts of ocean acidification on marine fauna and ecosystem processes. ICES Journal of Marine Science, 65: 414-432.

Feely, RA, Alin, SR, Newton, J, Sabine, CL, Warner, M, Devol, A, Krembs, C, Maloy, C (2010) The Combined Effects of Ocean Acidification, Mixing, and Respiration on pH and Carbonate Saturation in an Urbanized Estuary. Estuarine. Coastal and Shelf Science 88:442-449.

Feely, R. A., C. L. Sabine, J. M. Hernandez-Ayon, D. Ianson, and B. Hales. 2008. Evidence for upwelling of corrosive "acidified" water onto the continental shelf. Science Express, 22 May 2008, doi: 10.1126/science.1155676.

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

Fernandez, M., O. Iribarne, and D. Armstrong. 1994. Swimming behavior of Dungeness crab, Cancer magister dana, megalopae in still and moving water. Estuaries 17:271–275

Fisher, W., and D. Velasquez. 2008. Management recommendations for Washington's priority habitats and species, Dungeness crab (Cancer magister). Washington Department of Fish and Wildlife. 17pp. http://wdfw.wa.gov/publications/00028/wdfw00028.pdf

Forward, R. B. Jr., R. A. Tankersley, and D. Rittschof. 2001. Cues for metamorphosis of Brachyuran crabs: An overview. Amer. Zool. 41:1108-1122

Gaumer, T. F. 1970. Controlled rearing of Dungeness crab larvae and the influence of environmental conditions on their survival. Commercial Fisheries Research and Development Act Progress Report, July 1, 1969 to June 30, 1970. Fish Commission of Oregon Processed Report. 13p.

Gruber, N. et al. 2012. "Rapid progression of ocean acidification in the California Current System." Science 337.6091: 220–223.

Hankin, D. G. 1985. Proposed explanations for fluctuations in abundance of Dungeness crabs: a review and critique. In: Metleff, B. R., editor. Proceedings of the Symposium on Dungeness Crab Biology and Management. Alaska Sea Grant report no. 85-3. Fairbanks, AK: Alaska Sea Grant Program. pp. 305–326.

Hankin DG, Butler TH, Wild PW, Xue Q (1997) Does intense fishing on males impair mating success of female Dungeness crabs? Can J Aquat Sci 54(3):655-669

Hauri C.C., Gruber N., Vogt M.,Doney S.C., Feely R.A., Lachkar Z., Leinweber A., McDonnell A.M.P., Munnich M., Plattner G.-K. 2013. "Spatiotemporal variability and long-term trends of ocean acidification in the California Current System." Biogeosciences 10(1): 193-216.

Hobbs, RC, and Botsford, LW (1992) Diel vertical migration and timing of metamorphosis of larvae of the Dungeness crab Cancer magister. Mar Biol 112:417-428.

Hobbs R. C., L.W. Botsford, and A. Thomas. 1992. Influence of hydrographic conditions and wind forcing on the distribution and abundance of Dungeness crab, Cancer magister, larvae. Can J Fish Aquat Sci 49:1379–1388

Jamieson, GS, and Phillips, A (1993) Megalopal spatial distribution and stock separation in Dungeness crab (Cancer magister). Can J Fish Aquat Sci 50:416-429.

Jensen, GC (2014) Crabs and Shrimps of the Pacific Coast: A guide to shallow-water decapods from Southeaster Alaska to the Mexican border. ISBN 978-0-9898391-0-5 MolaMarine, Bremerton, WA 240pp

Kemp, IM, Beauchamp, DA, Sweeting, R, Cooper, C (2013) Potential for competition among herring and juvenile salmon species in Puget Sound, Washington. North Pacific Anadromous Fish Commission Technical Report No. 9: 139-143.

Kurihara, H, Matsui, M, Furukawa, H, Hayashi, M and Ishimatsu, A (2008) Long-term effects of predicted future seawater CO2 conditions on the survival and growth of the marine shrimp Palemon pacificus. J. Exp. Mar. Biol. Ecol. 367:41-46.

Leonard, J and Watson, P (2011) Description of the input-output model for Pacific Coast fisheries. U.S. Dept. Commer., NOAA Tech. Memo. NMFS-NWFSC-111, 64 p.

Long, WC, Swiney, KM, Foy, RJ (2013a) Effects of ocean acidification on the embryos and larvae of red king crab, Paralithodes camtschaticus. Marine Polution Bulletin 69: 38-47

Long, WC, Swiney, KM, Harris, C, Page, HN, Foy, RJ (2013b) Effects of ocean acidification on juvenile Red King crab (Paralithodes camtschaticus) and Tanner crab (Chionecetes bairdi) growth, condition, calcification and survival. PlosOne 8(4):1-10

Lough RG (1974) Dynamics of crab larvae (Anomura, Brachyura) off the central Oregon coast, 1969–1971. Ph.D. Dissertation, Oreg. State Univ., Corvallis. 299 p

Methot RD Jr, Botsford LW (1982) Estimated preseason abundance in the California Dungeness crab (Cancer magister) fisheries. Canadian Journal of Fisheries and Aquatic Sciences 39: 1077-1083

Moloney, C. L., L. W. Botsford, and J. L. Largier. 1994. Development, survival, and timing of metamorphosis of planktonic larvae in a variable environment, the Dungeness crab as an example. Marine Ecology Progress Series 113:61-79

Nakicenovic et al., Special Report on Emissions Scenarios: A Special Report of Working Group III of the Intergovernmental Panel on Climate Change (Cambridge Univ. Press, New York, 2000).

Nations JD (1975) The genus Cancer (Crustacea: Brachyura): systematics, biogeography, and fossil record. Natural History Museum of Los Angeles County Science Bulletin 23: 1-104.

Oh, S. J. & D. G. Hankin. 2004. The sperm plug is a reliable indicator of mating success in female Dungeness crabs, Cancer magister. J. Crustac. Biol. 24:314–326.

Park, W, and Shirley, TC (2005) Diel vertical migration and seasonal timing of the larvae of three sympatric Cancrid crabs, Cancer spp., in Southeastern Alaska. Estuaries 28(2):266-273.

Park, W., D. C. Douglass, and T. C. Shirley. 2007. North to Alaska: evidence for conveyor belt transport of Dungeness crab larvae along the west coast of the United States and Canada. Limnol. Oceanogr. 52:248-256

Park, W. and T. C. Shirley. 2008. Development and distribution of Dungeness crab larvae in Glacier Bay and neighboring straits in southeastern Alaska: Implications for Larval Advection and Retention. Animal Cells and Systems 12:279-286

Pauley, GB, Armstrong, DA, Van Citter, R, and Thomas, GL (1989) Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (Pacific Southwest) - Dungeness Crab. U.S. Fish and Wildlife Service Biological. Report 82(11.121).

Rasmuson LK (2013) The Biology, Ecology and Fishery of the Dungeness crab, Cancer magister in Lesser, M. 2013. Advances in Marine Biology 65

Reilly, P. N. 1983. Dynamics of the Dungeness crab, Cancer magister, larvae off central and northern California. In: Wild and Tasto (eds), Life history, environment, and mariculture studies of the Dungeness crab, *Cancer magister*, with emphasis on the central California fishery resource. Cal Fish Game Bull 172:57-84

Ries, JB, Cohen, A, and McCorkle, DC (2009) Marine calcifiers exhibit mixed responses to CO2-induced ocean acidification. Geology 27(12):1131-1134

Roegner, G. C., D. A. Armstrong, B. M. Hickey, and A. L. Shanks. 2003. Ocean Distribution of Dungeness Crab Megalopae and Recruitment Patterns to Estuaries in Southern Washington State. Part B: The Pacific Northwest Coastal Ecosystems Regional Study Estuaries 26(4):1058-1070

Shanks, A. L. 2013. Atmospheric forcing drives recruitment variation in the Dungeness crab (Cancer magister), revisited. Fisheries Oceanography. doi:10.1111/fog.12020 pp. 1-10

Shanks AL, Roegner GC (2007) Recruitment limitation in Dungeness crab populations is driven by variation in atmospheric forcing. Ecology 88(7):1726-1737

Shirley S, Shirley T, and Rice S (1987) Latitudinal variation in the Dungeness crab, Cancer magister, zoeal morphology explained by incubation temperature. Mar. Biol. 95:371-376

Smith, B. D. and G. S. Jamieson. 1989. Exploitation and mortality of male Dungeness crabs (Cancer magister) near Tofino, British Columbia. Can. J. Fish. Aquat. Sci. 46:1609–1614.

Spicer, JI, Raffo, A, and Widdicombe, S (2007) Influence of CO2-related seawater acidification on extracellular acid-base balance in the velvet swimming crab. Necora puber Mar. Biol. 151:1117–1125

Stone RP, and O'Clair CE (2001) Seasonal movements and distribution of Dungeness crabs Cancer magister in a glacial southeastern Alaska estuary. Mar. Ecol. Prog. Ser. 214:167-176. doi:10.3354/meps214167

Stone RP and O'Clair CE (2002) Behavior of female Dungeness crabs, Cancer magister, in a glacial southeast Alaska estuary: homing, brooding-site fidelity, seasonal movements, and habitat use. J. Crustacean Biol. 22:481-492

Strub, P.T., Allen, J.S., Huyer, A., Smith, R.L. 1987. Large-scale structure of the spring transition in the coastal ocean off western north America. Journal of Geophysical Research: Oceans 92:1527-1544

Sulkin, SD and McKeen, GI (1989) Laboratory study of survival and duration of individual zoeal stages as a function of temperature in the brachyuran crab Cancer magister. Marine Biology 103: 31-37.

Whiteley NM (2011) Physiological and ecological responses of crustaceans to ocean acidification. Marine Ecology Progress Series. 430:257-271

2 Exposure to low pH reduces survival and delays development in early life stages of Dungeness crab (Cancer magister)

Abstract

Dungeness crab (*Cancer magister*) eggs and larvae were exposed to laboratory-controlled, low-pH seawater in an effort to assess current and predicted-future impacts of Ocean Acidification (OA) on hatching success, survival and growth. Treatment levels of pH ~8.0, ~7.5 and ~7.1 represented a wide range of pH-levels relevant to current-open-ocean, current-upwelled and future-upwelled conditions associated with *C. magister* habitat in the northeast Pacific Ocean. For this study, pH ~8.0 represented the "control". *C. magister* eggs were exposed to treatment levels for 34 days. Although the probability of hatching was unaffected by reduced pH, hatch-timing for eggs was delayed in those held at pH 7.1. Significantly more *C. magister* larvae survived after 45 days when held under control relative to reduced pH condition: 57.9%, 13.5%, and 21.1% survived in pH 8.0, 7.5, and 7.1, respectively ($p < 0.05$). Larvae held under low-pH treatments were 2.5-3 times less likely to survive than those held under control conditions. Larval size at each larval stage was unaffected by treatment ($p > 0.05$), however, larvae in the low-pH treatments progressed through larval stages at a slower rate than those in the control treatment. The results of this study indicate that low-pH seawater caused by OA can slow developmental progression through early life stages and that long-duration exposure can result in mortality. The lowest survivorship occurred in seawater at pH levels reflective of current and future conditions in estuaries and areas of upwelling.

Introduction

Ocean acidification (OA), the decrease in seawater pH due to an increase in dissolved CO_2, has the potential to substantially change the abundance and distribution of individual marine species and alter entire ecosystems (Fabry et al. 2008). Laboratory experiments rearing organisms in seawater with control and low-pH are a key way of estimating how individual species may respond to ocean acidification. These experiments have shown a variety of positive, negative and no-effect responses (Kroeker et al. 2013). The response to low pH can be species-specific within a genus (Dupont and Thorndyke 2009) and can vary among populations or strains within a single species (Dupont et al. 2010). This level of variability presents a real challenge to understanding the ecological and economic consequences of OA because it is difficult to extrapolate results from experiments on one species to other species. To understand potential OA effects on a species of importance on the North East Pacific Coast, we conducted a series of pH lab experiments on the eggs and larvae of Dungeness crab, *Cancer magister*[2] Dana 1852.

C. magister is an ecologically, economically and culturally important marine species. A mature female crab can produce over two million offspring (Pauley et al. 1986); these offspring provide valuable prey items for forage fish and high-profile species like salmon, rockfish and herring (Reilly 1983; Bollens et al. 2010; Kemp et al. 2013). Based on *C. magister* fishery-harvests and *C. magister* life-history characteristics an estimated 26-78 trillion *C. magister* larvae

[2] Schweitzer and Feldmann (2000) proposed elevating the *Cancer* subgenera, *metacarcinus*, outlined by Nations (1975) to the generic level re-classifying the Dungeness crab as *Metacarcinus magister* based solely on the shape of carapace teeth. Due to a lack of molecular evidence (see Harrison and Crespi 1999) to support Nations' subdivisions, the lead author elected to maintain the use of *Cancer magister*.

annually enter the water column across their geographic range (Appendix A). Adult *C. magister* males are heavily harvested and culturally important throughout their coastal range (Fig.1) from south central California to the Gulf of Alaska (Jensen 2014). The annual combined commercial and tribal harvest averaged over 2003 to 2012 was 35.7 e^3 tonnes (FAO.org) With ex-vessel commercial and tribal landings reaching over $176 million in 2012, more than $343 million income was generated sustaining over 7200 jobs (IO-PAC model, Leonard and Watson 2011).

Female *C. magister* extrude and brood eggs beginning in the fall months (Stone and O'Clair 2001). Although water temperature is known to influence hatch timing and larval duration (Shirley et al. 1987; Sulkin and Mckeen 1989), the effect of pH on these parameters are unknown. Eggs hatch 90-160 days post-extrusion (Rasmuson 2013). Immediately after hatching the zoeae larvae rise in the water column where they catch tidal currents moving them offshore. Larvae progress through five zoeal stages and the transitional megalops stage, (Fig.1).

While adult *C. magister* inhabit depths from the intertidal nearshore to 230 meters (Jensen 2014), *C. magister* planktonic zoeae perform diel vertical migrations away from surface waters during daylight typically reaching depths of 20-30m with the ability to perform deeper migrations as they progress through later stages (Sulkin and McKeen 1989; Hobbs and Botsford 1992). Megalopae perform diel vertical migrations to depths of 60 to 70m (Hobbs et al. 1992; Shanks 2013) and have been recorded to exceed 160m depth in the Strait of Georgia, BC (Jamieson and Phillips 1993). *C. magister* larvae spend 2-5 months in the water column before settling into the nearshore benthos as juveniles (Moloney et al. 1994; Park and Shirley 2005). Due to their diverse geographical, meroplanktonic and benthic life history, *C. magister* are likely exposed to a variety of pH levels during development.

Puget Sound (Washington, USA), where the adult crabs for this study were collected, is characterized by relatively low pH values and highly variable conditions (Feely et al. 2010; Reum et al. 2014). An analysis of carbonate chemistry data in this region shows summer pH levels in the top 50m of the water column ranging from ~8.1 pH to ~7.6 (360 to 1270µatm CO_2, Reum et al. 2014). Laboratory pH treatments should be established based on the carbonate chemistry environment experienced in the natural habitat (Reum et al. 2014). It is important to choose treatment levels that encompass current conditions as well as the worst-case future scenarios to get an idea of worst-case impacts to *C. magister* populations and to understand limits of physiological tolerance.

Low pH (elevated CO_2) exposure studies with other decapod crustaceans have shown a variety of effects ranging from reduced survival (e.g. Red King crab, *Paralithodes camtschaticus*, Long et al. 2013b; intertidal Porcelain crab, *Petrolisthes cinctipes*, juveniles, Ceballos-Osuna et al. 2013) to sub-lethal, physiological impacts (e.g. slowed metabolic rate in *P. cinctipes* embryos, Carter et al. 2013), to no noticeable effects (e.g. *P. cinctipes* larvae, Ceballos-Osuna et al. 2013; Northern shrimp, *Pandalus borealis*, Arnberg et al. 2013). Given the high variability in response to low pH among decapod crustacean species, species-specific experimentation under ecologically relevant conditions remains necessary.

In this laboratory study we exposed *C. magister* eggs and newly-hatched zoeae to pH levels of 8.0, 7.5, and 7.1 (corresponding to CO_2 concentrations of ~ 466, 1781 and 3920µatm respectively) to encompass the wide range of pH levels currently experienced by *C. magister* larvae as well as any potential future decreases. We hypothesized that measured metrics of hatching success, hatch rate, survival, growth and rate of development would decrease relative to the reference condition pH 8.0 with each incremental pH reduction tested.

Materials and Methods

Animal collection

Non-gravid, adult *C. magister* females were collected on several dates in September and October of 2012[3] and held in individual aquaria at NOAA's Mukilteo Research Station, WA at ambient temperature conditions (~9° C). Aquaria were filled with sand (approx. 10cm depth) found on site to provide burrowing habitat and help with formation of the egg mass during egg deposition. *C. magister* females were fed live mussels (*Mytilus sp.*) collected off nearby piers. Eggs and freshly hatched zoeae were collected from the gravid females in February and March of 2013 as described in the following sections.

Laboratory system

All experiments were conducted at NOAA's NWFSC ocean acidification laboratory in Seattle, WA. The laboratory houses a 20,000L recirculating seawater system consisting of six individually controlled units that delivered filtered (1μm), UV sterilized (Emperor Aquatics, Pottstown, PA), degassed (membrane contactors, Liqui-Cel, Charlotte, NC) seawater to experiments at target temperature and pH levels. An automated feedback loop algorithm using Labview Software (National Instruments, Austin, TX) maintained target pH levels with inputs from pH probes (Honeywell Durafet III) and computer-controlled gas solenoid valves bubbling on-site generated CO_2-free gas (Twin Tower Engineering, Broomfield, CO) and CO_2 gas. The pH probes, calibrated at 12°C with pH-certified Tris buffer (Dickson Laboratory, Scripps Institution of Oceanography, San Diego, CA), continuously measured system pH and temperature. De-ionized water was added to the system to reduce the effects of evaporation.

[3] Washington Department of Fish and Wildlife permit #13-204

Salinity was measured from treatment-tank conductivity probes (Honeywell model 4905) as well as from discrete water samples (ThermoScientific Orion Star A322).

To validate the readings from the system pH probes, discrete water samples were drawn weekly from each of the six treatment tanks and were analyzed for dissolved inorganic carbon (DIC) and total alkalinity (TA) using the methods outlined in the Guide to Best Practices for Ocean CO_2 Measurements (Dickson et al. 2007). In addition to continuous readings from pH probes and discrete water samples, spectrophotometric pH (spec-pH) measurements (Yamazaki et al. 1992, Ocean Optics USB 230 2000+ Fiber Optic Spectrometer) were taken from each system at minimum, twice per week. Spec-pH and DIC were then used to calculate TA and pCO_2 using the Seacarb package (Lavigne and Gattuso 2013) in R (R Core Team. 2013), with K1 and K2 constants from Lueker et al. (2000) and B_T constant from Lee et al. (2010). All pH values were reported on the Total pH scale.

The three target pH levels were randomly assigned to the six tanks providing a replicate for each treatment. The pH levels were set at 8.0, 7.5 and 7.1 to span current open-ocean conditions, a low, but not uncommon level in Puget Sound and an extreme future low pH respectively. The 8.0 pH level was considered the "control" or reference condition.

Test subjects were held in customized 250ml polypropylene jars designed for equal distribution of treatment water (Fig 2). Flow was gravity controlled at a rate of approximately 40ml/min. To help maintain treatment temperature, the jars were placed in a system-delivered water bath. On each sampling day, spec-pH measurements were collected from within two randomly selected 250ml jars in each of the six treatment tanks and compared with pre-jar system-treatment water to ascertain whether the pH of the seawater in the jar was representative

of the treatment. Water bath tanks were enclosed in blackout curtains to isolate eggs and zoeae from light within the laboratory.

Egg exposure experiment

Multiple egg strands were removed with forceps from the egg mass of a single, live, gravid *C. magister* female. Wet-weights of the egg strands were recorded in an effort to place equal numbers of eggs into each jar. Egg strands were divided into 18 jars previously filled with pH 8.0 treatment water. Three jars were placed into each of the six tanks. Eggs from each 250ml jar were inspected daily for atypical coloration, fungal outbreaks, and hatching before being transferred to a clean jar. No fungicides or antibiotics were used. Hatched larvae were enumerated, assessed for survival and removed from the jar. Unhatched eggs remaining at the end of the exposure experiment were counted under a dissecting microscope. Counts of hatched larvae were added to the unhatched-egg counts to obtain the initial number of eggs per jar. The exposure study lasted 34 days and ended when hatching had ceased.

Zoeal exposure experiment

Eggs from each of three adult *C. magister* females hatched 151 days post egg-deposition. Approximately 500 newly hatched zoeae (< 12h after hatch) were collected from each of the three broods and transported to the laboratory research facility at NWFSC, Seattle, WA. After a 1 h acclimation to the system-water temperature of 12°C, zoeae were individually placed into jars, one zoea per jar, and fed *Artemia salina* (San Francisco brand) nauplii at a target concentration of 1 nauplii ml^{-1} of seawater. Seven zoeae from each brood were held in each of

the six treatment tanks (n = 21 jars per tank). Jars were then allowed to reach target pH as treatment water flowed into the jars at ~ 40ml min^{-1}. Every third day each zoea was assessed for survival, placed into a clean jar and fed fresh *A. salina* nauplii. *A. salina* were hatched in pH 8.0 seawater and utilized within a day of hatching. Mortalities were preserved individually in ETOH. The exposure study lasted 45 days. Zoeae surviving to the final day were euthanized and preserved in ETOH. Of the 126 zoeae introduced into the experiment, 13 were lost in the sampling process leaving 113 to enter into survival analysis. Individuals preserved in ETOH were digitally photographed then assessed for zoeal stage (1-5) according to documented morphological traits (Poole 1966: Lough 1974). Damaged or unmeasurable zoeae were removed from subsequent size analysis.

Analysis

All analysis were run in the statistical program R, ver. 2.13.1 (R Dev. Core Team 2013) unless otherwise noted. Mixed-effects models were used when possible to accommodate for the potential variation from random 'jar' or 'tank' effects. Significance was assessed at the 0.05 level.

Eggs

An egg-hatch ratio was calculated for each jar by dividing the total number of individuals hatched by the total number of eggs. To test for differences among treatments for probability of hatching, or 'hatching success', a generalized linear mixed model was run with a binomial distribution using the 'Lme4' package (Bates et al. 2013). To test for differences in 'time-to-hatch' among treatments an accelerated failure time (AFT) model was constructed with the binomial, Weibull distribution (Kleinbaum and Klein 2005, chapter 7) and run with the

'Survival' package (Therneau 2013). 'Time-to-hatch' was defined as the period of time beginning with the day hatching commenced among all jars to the day each egg hatched. Daily counts of hatched eggs were divided by the total number of hatched eggs to produce hatch-proportions within each individual jar. These daily proportions entered the AFT model as the dependent variable with 'jar' and 'tank' as random variables.

Zoeae

To test for differences in survival probability among tanks, broods and treatments Kaplan-Meier (KM) survival analyses were employed using the logrank chi square test statistic in the 'Survival' package (Therneau 2013). Multiple pairwise comparisons among KM plots were performed using the Holm-Sidak method (Sigma Plot, Systat Software, Inc). An AFT model (Kleinbaum and Klein 2005, chap 7), run within the 'Survival' package (Therneau 2013) provided a parametric method to produce odds-ratios of mortality among treatments while incorporating the potential variation from the random effect of 'tank'. For AFT analysis the binomial, loglogistic distribution was used with the 'time to mortality' set as the dependent variable.

In order to assess any differences in 'size-at-stage' among treatments, preserved zoeae were measured for carapace length (CL) following the method from Hirota and Fukuda (1985) using digital images and imaging software (Nikon NIS-Elements). Dry weight (DW) of individual zoeae was estimated using the equation from Hirota and Fukuda (1985).

$$Log10\ \boldsymbol{DW}\ (\mu g)\ =\ -7.772\ +\ (3.239\ *\ Log10\ (\boldsymbol{CL}\ (\mu m))$$

Lengths and weights were assessed for normality using the Shapiro-Wilks test. Averages of the measurements were tested against the null assumption of equality among treatments with one-way ANOVA.

To assess any impacts of treatment on the zoeal stage reached by the end of the experiment (day 45), a-priori contrasts were set up (Crawley 2007) within a generalized linear mixed model (Lme4 package, Bates et al. 2013) to test for a difference between the stage reached by zoeae in the control pH treatment (pH 8.0) versus the low-pH treatments (pH 7.5, 7.1).

Results

Chemistry[4]

The similarity of within-jar and treatment-tank spec-pH samples verified that the target pH was maintained inside the 250ml jars (Table 1). The overall mean of measured TA was 2219.98 ± 86.29 µmol/kg SW. The standard deviation of measured TA across all 6 treatment tanks on any one of the eleven individual sample days was < 8 µmol/kg SW. Calculated TA averaged 2190.95 ± 72.73 µmol/kg SW. Mean DIC for treatments pH 8.0, pH 7.5, and pH 7.1 measured 2058.25 ± 77.43, 2207.91 ± 86.11, and 2305.57 ± 87.20 µmol/kg SW, respectively. Accuracy and precision for DIC measurements were 1.74 and 3.39 µmol/kg SW (= 0.084 and 0.167 %) respectively. Salinity averaged 31.21 ± 1.07 PSU. Calculated mean pCO_2 for treatments pH 8.0, pH 7.5, and pH 7.1 were 465.86 ± 24.12, 1781 ± 106.17, and 3920.37 ± 306.02 µatm CO_2, respectively. Calculated aragonite saturation (Ω_a) for treatments pH 8.0, pH 7.5, and pH 7.1 were 1.74 ± 0.11, 0.54 ± 0.04, and 0.25 ± 0.03 respectively. Evaporation

[4] Summaries are presented as mean ± standard deviation for all measurements and calculations.

exceeded freshwater replacement over the course of the experiment; TA, DIC, salinity and Ω_a all followed a similar temporal trend (Fig.2). Average temperatures in the treatment tanks ranged from 12.05-12.13°C with a mean of 12.11 ± 0.03. Additional tables on treatment chemistry are provided in Appendix B.

Egg exposure study

The mean ± standard deviation for numbers of eggs within in each jar was 593 ± 98. Total eggs in pH 8.0, pH 7.5, and pH 7.1 treatments were 3671, 3220, and 3794 respectively. Eggs incubated in treatment water for 22 days before hatching commenced. Eggs within each treatment started hatching on the same day and daily counts of hatch numbers peaked for all jars 3-4 days after hatching commenced (Fig 3a). The proportion of eggs hatching in pH 8.0, pH 7.5, and pH 7.1 treatments were 0.77, 0.59, and 0.72, respectively. The probability of hatching was similar in all three treatments with the 95% CI for pH 8.0, 7.5, and 7.1 at 0.62-0.97, 0.26-0.80, 0.48-0.90 respectively (Fig 3). The AFT model results showed that 'time-to-hatch' for eggs in the pH 7.5 treatment was 0.93 (95% CI: 0.91-0.94) times the 'time-to-hatch' pH 8.0 treatment, while the eggs in the pH 7.1 treatment took 1.24 (95% CI 1.22, 1.26) times as long to hatch relative to those in the pH 8.0 treatment level (Fig. 4b). No fungus or developmental abnormalities were observed during the experiment.

Zoeal exposure study

Survival was highest in the pH 8.0 treatment with an average survival by the last day of the experiment of 57.9%; average survival in the pH 7.5 and the pH 7.1 were 13.5% and 21.1% respectively (Table 2).

Survivorship did not vary between experimental tanks within a single treatment, nor were there significant differences in survival probabilities among the broods used within each treatment (Table 3). All tanks and broods within each treatment were therefor pooled; the initial numbers zoeae used in the analyses for treatment pH 8.0, pH 7.5, and pH 7.1 were 38, 37, and 38 respectively. Survivorship differed among treatments (Table 4, Fig. 5, $\chi^2 = 13.8$, 2 df, $p = 0.001$) Survival for zoeae held at pH 8.0 was significantly higher than those held at pH 7.5 and pH 7.1, which were similar to one another (table 4). The odds of mortality (AFT model) for zoeae held in pH 7.5 and pH 7.1 treatments, relative to the reference condition of pH 8.0, were 3.08:1 (95%CI = 1.28,7.44) and 2.42:1 (95%CI = 1.003,5.89), respectively.

There were no differences in CL measurements among treatments within a zoeal stage, however, a greater proportion of larvae progressed to zoeal stage 4 in the control versus the two low-pH treatments. Carapace degradation or breakage from handling reduced the numbers of preserved zoeae for zoeal stage identification or CL measurements. Of the 113 zoeae entering survival analysis, 91 were identified to zoeal stage and 61 were measureable for CL. The highest zoeal stage reached by any zoea was stage 4. ANOVA indicated no significant differences in CL among the treatments for zoeal stage 2, 3, and 4: $F_{2,5} = 0.52$ ($p=0.5$), $F_{2,27} = 0.22$ ($p = 0.8$), $F_{2,17} = 0.80$ ($p = 0.5$), respectively. Similarly, dry weight for zoeal stage 2, 3, and 4 were similar among treatments: $F_{2,5} = 0.80$ ($p = 0.5$), $F_{2,27} = 0.09$ ($p = 0.9$), $F_{2,17} = 0.58$ ($p = 0.6$), respectively. The proportion of stage 4 to stage 3 zoeae (Fig.5) in a generalized linear mixed model with 'tank' as a random factor, was significantly higher in the control than in the low-pH treatments (Chisq = 7.00, df = 2, p = 0.03). The odds ratio of being a stage 4 zoeae in the control verses the low-pH treatments was 1.77:1.

Discussion

In this study, egg and larval stages of *C. magister* were exposed to pH levels encompassing present as well as future predicted levels associated with OA conditions at the end of the century. While hatching success, measured by the probability of hatching, was similar in all three pH treatments, the egg hatching rate in pH 7.1 was delayed relative to those held in pH 7.5 and pH 8.0. At the larval stage there was reduced survival as well as a developmental delay in low-pH treatments relative to those held at pH 8.0. These results indicate that low pH seawater does affect *C. magister* early life history stages suggesting future effects of acidified seawater chemistry from OA could have deleterious impacts on the population scale.

Egg hatching and hatch duration

Contrary to our hypothesis of decreased hatching success in low-pH seawater, our results show that hatching success was not significantly affected by low pH. Our non-significant result is similar to a low-pH exposure study where the hatching success of the intertidal crab, *P. cinctipes*, was not different between pH 7.6 and pH 7.9 (Ceballos-Osuna et al. 2013). Ceballos-Osuna et al. (2013) used multiple broods and posited that hatching success may be brood-specific relative to low-pH; some broods had greater and some lower success in pH 7.6 relative to pH 7.9. The present egg development study used a single brood and the hatching success, or the proportions of hatched eggs, varied widely among the jars within a treatment (Fig. 7). There is evidence that multiple male parentage can occur in a single *C. magister* brood; 40% of *C. magister* broods within a parentage-assignment study had multiple male parents (Jensen and Bentzen 2012). If the effect of low pH on hatching success is brood specific in *C. magister* as it

may be in *P. cinctipes*, the potential multiple male parentage within the single brood in this study may account for some of the variation within jars in a single tank. It will be important to include multiple broods in future studies.

It is possible that *C. magister* eggs may be predisposed to low pH as adult females tend to brood their eggs while buried in the benthic sediment for weeks at a time (Jensen 2014) where interstitial pore waters could have a relatively low pH (Murray and Gill 1978). In this case, a more suitable "control" pH treatment for eggs might be lower than pH 8.0. It is also possible that the 21-day treatment exposure period, prior to the eggs hatching, might have been too short to see an effect. Given the 151-day incubation period from time of extrusion for *C. magister* egg masses in the zoeal study, a 22-day treatment exposure represents < 15% of the embryonic development period.

While hatching success was not affected by low pH, it is interesting that pH had a significant effect on the time-to-hatch. Eggs required 24% longer to hatch in the pH 7.1 treatment than those held in the pH 8.0 treatment. In contrast, eggs held in pH 7.5 hatched 7% faster than in the pH 8.0 treatment suggesting possible benefit of pH 7.5 (eg. hormesis, Miller et al. 2013) and a potential threshold for negative effects between pHs 7.5 and 7.1. Eggs from *P. camtchaticus*, a deep-water crab, experienced a 33% longer hatch-duration, the period of time when hatching commenced to when hatching ended, in seawater at a pH 7.7 versus an ambient pH 8.0 (Long et al. 2013a). Embryos from *P. cinctipes* exposed for 7-10 days to acidified seawater at pH 7.6 were found to have an 11% lower metabolism than those held in pH 7.9 (Carter et al. 2013). The 21-day exposure of *C. magister* embryos to low pH in the present study may have reduced their metabolic rate. A reduction in metabolism could decrease developmental rates and illicit a longer hatch duration.

A prolonged hatch duration may be advantageous for *C. magister* by diversifying the chances of encountering favorable ocean conditions for dispersal. On the other hand, a prolonged hatch duration could cause newly hatched zoeae to become vulnerable to trophic mismatch (Edwards and Richardson 2004; Byrne 2011). Any change in time-to-hatch or hatch duration brought about by water chemistry could alter the natural rhythm that *C. magister* larvae rely upon to keep them in sync with surface tidal currents used to assist their transport to their target offshore habitat. Similarly, changes in zooplankton timing and presence in the water column have been attributed to changes in atmospheric conditions with implications of altering dispersal (Shanks 2013) as well as affecting ecological relationships with commercial fish species who depend on them for prey (Hays et al. 2005, Schindler et al. 2005).

Reduced Zoeal Survival and prolonged stage duration

Zoeal survival was significantly reduced upon exposure to low pH demonstrating negative effects of ocean acidification on crab larvae. Three to four times more zoea survived in the pH 8.0 treatment (55.0 - 66.7%) than in those held at pHs of 7.1 (21.1%) and 7.5 (13.5%). Survival of pH 8.0 larvae was similar to previous studies in which 50-76% of larvae survived through zoeal stage 4, the highest stage reached by zoeae in the present study (Sulkin and McKeen 1989; Gaumer 1973). pH 7.5 has recently been measured in the top 50m in Puget Sound (Feely et al. 2010; Reum et al. 2014) and pH 7.1 is 0.2 pH units lower than seasonal pH drops observed in deep waters of Hood Canal, WA (Feely et al. 2010) indicating that current and near future ocean chemistry may impact *C. magister* life history. Additional research is needed to discern if the observed effects carry-over into subsequent life history stages and generations and if the observed effects are due to direct, indirect or combined mechanisms.

Whether the observed reduced survival was due to direct effects on crab larvae or indirectly through *A. Salinas* prey becoming less nutritious to crab larvae in low pH conditions is not known. As *A. Salinas* were fed within a day of hatching it is unlikely that biochemical changes altered their nutritional value. While feeding efficiencies were not calculated in this study, it is apparent that the zoeae were receiving nutritional value from the feed; unfed *C. magister* zoeae survive in the laboratory no longer than 15 days (Mayer 1973, Sulkin et al. 1988, Miller unpub. results).

It is important to recognize that a shorter experiment might not have captured the significant differences in survival between treatments. It took 27 days before a notable divergence in the probability of survival between treatment-levels occurred. It is unknown whether the initial exposure to low-pH took three weeks to produce a survival response or if the reduction in survival was brought about by continuous exposure. There is also the possibility that larvae became susceptible to low pH at ~27 days post hatch. The pattern of a delayed response of reduced survival in low pH relative to controls has been seen in other decapod crustacean species (Kurihara et al. 2008; Long et al. 2013b). The delay in response could be due the ability to regulate internal acid-base equilibrium (Henry and Wheatly 1992). Adult *C. magister* have the ability to recover from a 24-hour exposure to pH 7.1 (Pane and Barry 2007). Regardless of the ability of *C. magister* larvae to maintain internal acid-base equilibrium, continued regulation likely comes with increased energetic costs and less energy prioritized for other physiological functions (Whiteley 2011).

Although we found no significant differences in size-at-stage among the treatments, development was delayed in zoeae held in low pH waters. More time was required for a zoea in low pH (pH 7.5, 7.1) to reach stage 4; however, once a zoea reached each stage its size was

indistinguishable from those in the control (pH 8.0) treatment. Zoeae held in low-pH seawater took longer to progress through their zoeal stages than did those held at the reference condition. This developmental delay from low-pH exposure has occurred in other species at the larval stage as well: Northern shrimp, *Pandalus borealis*, at pH 7.6 relative to those in pH 8.1 (Arnberg et al. 2011); sea urchin, *Strongylocentrotus purpuratus*, at pH 7.7 relative to those in pH 8.1 (Stumpp et al. 2011); spider crab, *Hyas araneus*, at pH 7.3 relative to those in pH 8.1 (Walther et al. 2010). The mechanism for this delay could be a decrease in metabolism. Exposure to low pH seawater can create changes in the extra- and intracellular acid-base balance in crustaceans leading to lower metabolic rates (Whiteley 2011). If *C. magister* metabolism is being affected by low pH as observed in the embryonic stages of the intertidal porcelain crab (Carter et al. 2013), the resultant developmental delay at the larval stage could have negative implications for *C. magister* at the population level. An increase in the duration of larval stages could increase the risk of predation in the water column and/or put settling larvae out of sync with other food or habitat resources (Dupont and Thorndyke 2009).

As surface waters continue to acidify from absorption of rising levels of atmospheric CO_2, the likelihood of long-term, low-pH exposure of *C. magister* larvae increases. Locations of greatest vulnerability will likely be areas where deep waters, naturally low in pH, meet with acidified surface waters. This exchange often occurs in areas of coastal upwelling as well as estuarine systems (Hoffman et al. 2011). Predictions for annual-average surface pH in the eastern Pacific along the continental United States reach pH 7.8 by the year 2050 with implications for even lower pH during times of coastal upwelling (Gruber et al. 2012). Estuarine systems such as in Hood Canal, WA have been measured as low as pH 7.6 in the top 50m of the water column (Feely et al. 2010). These current as well as predicted low-pH values stress the

importance of future coastal pH-level monitoring as well as research efforts to explore potential survival or physiological effects within the pH range of 8.0 and 7.5, where the greatest reduction in survival occurred in this study.

This study shows that early life stages of *C. magister* are susceptible to low levels of pH relevant to those predicted for the effects of OA. Lowered survival combined with slower development would likely have population scale impacts. Either by an increase in predation exposure, reduction in surviving numbers, or by a mismatch with tidal or ocean currents necessary for dispersal both off- and onshore, long-term exposure to low pH could be very influential concerning the number of *C. magister* recruits to the nearshore and ultimately into the fishery.

Acknowledgements:

Mukilteo staff: Casimir Rice, Kathleen Neely and Mark Tagal; NWFSC Dive team, Dan Bascom, Paul Williams, Jeff Leonard, Shallin Busch

Funding:

Research was funded by NOAA Ocean Acidification program, the Suquamish Tribe, NOAA Northwest Fisheries Science Center, and Washington Sea Grant.

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

Arnberg M, Calosi P, Spicer JI, Tandberg AHS, Nilsen M, Westerlund S, and Bechmann RK (2013) Elevated temperature elicits greater effects than decreased pH on the development, feeding and metabolism of northern shrimp (Pandalus borealis) larvae. Mar Biol 160(8):2037-2048 DOI 10.1007/s00227-012-2072-9

Bernatis, J, Gerstenberger, SL, McGaw, IJ (2007) Behavioural responses of the Dungeness crab, *Cancer magister*, during feeding and digestion in hypoxic conditions. Marine Biology 150:941-951.

Bollens, SM, vanden Hoof, R, Cordell, JR, Frost, BW (2010) Feeding ecology of juvenile Pacific salmon (*Oncorhynchus spp.*) in a northeast Pacific fjord: diet, availability of zooplankton, selectivity for prey, and potential competition for prey resources. Fish. Bull. 108(4): 393-407.

Byrne, M (2011) Impact of ocean warming and ocean acidification on marine invertebrate life history: Vulnerabilities and potential for persistence in a changing ocean. Oceanography and Marine Biology 49: 1-42

Carter, HA, Ceballos-Osuna, L, Miller, NA, Stillman, JH (2013) Impact of ocean acidification on the metabolism and energetics of early life stages in the intertidal porcelain crab Petrolisthes cinctipes. J. Exp. Biol. 216:1412-1422.

Ceballos-Osuna, L, Carter, HA, Miller, NA, and Stillman, JH (2013) Effects of ocean acidification on early life-history stages of the intertidal porcelain crab Petrolisthes cinctipes. J. Exp. Biol. 216:1405-1411.

Crawley M (2007) The R Book. 2nd ed. John Wiley & Sons. West Sussex, United Kingdom 1076pp

de la Haye, KL, Spicer, JI, Widdicombe, S, Briffa, M (2012) Reduced pH sea water disrupts chemo-responsive behavior in an intertidal crustacean. J. Exp. Mar. Biol and Ecol 412: 134-140

Dupont, S. and M. C. Thorndyke. 2009. "Impact of CO2-driven ocean acidification on invertebrates early life-history – What we know, what we need to know and what we can do." *Biogeosciences Discussions* 6: 3109-3131.

Dupont S, Ortega-Martinez O, Thorndyke M (2010) Impact of near-future ocean acidification on echinoderms. Ecotoxicology 19: 449–462

Edwards M, Richardson AJ (2004) Impact of climate change on marine pelagic phenology and trophic mismatch. Nature 430: 881-884

Fabry, VJ, Seibel, BA, Feely, RA, Orr, JC (2008) Impacts of ocean acidification on marine fauna and ecosystem processes. ICES Journal of Marine Science, 65: 414-432.

Feely, RA, Alin, SR, Newton, J, Sabine, CL, Warner, M, Devol, A, Krembs, C, Maloy, C (2010) The Combined Effects of Ocean Acidification, Mixing, and Respiration on pH and Carbonate Saturation in an Urbanized Estuary. Estuarine. Coastal and Shelf Science 88:442-449.

Fisher, W. and Velasquez, D. (2008) Management recommendations for Washington's priority habitats and species, Dungeness crab (Cancer magister). Washington Department of Fish and Wildlife. 17pp. http://wdfw.wa.gov/publications/00028/wdfw00028.pdf

Gaumer, TF (1973) Controlled rearing of Dungeness crab larvae and the influence of environmental conditions to their survival. Oreg. Fish Comm., Portland, NOAA 73082801, NMFS 1 5 R, 53 p.

Gruber N, Hauri C, Lachkar Z, Loher D, Frölicher TL, Plattner G-K (2012) Rapid progression of ocean acidification in the California Current System. Science 337:220-223

Hays GC, Richardson AJ, Robinson C (2005) Climate change and marine plankton. Trends in Ecology and Evolution 20(6):337-344

Henry RP, Wheatly MG (1992) Interaction of Respiration, ion regulation, and acid-base balance in everyday life of aquatic crustaceans. American Zoology 32:407-416

Hirota, R, and Fukuda, Y (1985) Dry weight and chemical composition of the larval forms of crabs (Decapoda : Brachyura). Bull. Plankton Society of Japan. 32(2): 149-153.

Hobbs, RC, and Botsford, LW (1992) Diel vertical migration and timing of metamorphosis of larvae of the Dungeness crab *Cancer magister*. Mar Biol 112:417-428.

Hobbs R C, Botsford LW, Thomas A (1992) Influence of hydrographic conditions and wind forcing on the distribution and abundance of Dungeness crab, Cancer magister, larvae. Can J Fish Aquat Sci 49:1379–1388

Hoffman GE, Smith J, Johnson KS, Send U, Levin LA, Micheli F, Paytan A, Price NN, Petersen B, Takeshita Y, Matson PG, Crook ED, Kroeker KJ, Gambi MC, Rivest EB, Frieder CA, Yu PC, Martz TR (2011) High-frequency dynamics of ocean pH: a multi-ecosystem comparison PLoS ONE 6(12):1-11

Jamieson, GS, and Phillips, A (1993) Megalopal spatial distribution and stock separation in Dungeness crab (*Cancer magister*). Can J Fish Aquat Sci 50:416-429.

Jensen CJ and Bentzen P (2012) A molecular dissection of the mating system of the Dungeness crab *Metacarcinus magister* (Brachyura: cancridae) J Crustacean Bio 32(3): 443-456

Jensen, GC (2014) Crabs and Shrimps of the Pacific Coast: A guide to shallow-water decapods from Southeaster Alaska to the Mexican border. ISBN 978-0-9898391-0-5 MolaMarine, Bremerton, WA 240pp

Kemp, IM, Beauchamp, DA, Sweeting, R, Cooper, C (2013) Potential for competition among herring and juvenile salmon species in Puget Sound, Washington. North Pacific Anadromous Fish Commission Technical Report No. 9 pp139-143.

Kleinbaum DG, Klein M (2005) Survival Analysis. A self-learning text. 2nd ed. Springer Science, New York, NY pp 257-327

Kroeker, KJ, Kordas, RL, Crim, RN, Hendricks, IE, Ramajo, L, Singh, GG, Duartes, CM, Gattuso, J (2013) Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. Global Change Biology 19: 1884-1896.

Kurihara, H, Matsui, M, Furukawa, H, Hayashi, M and Ishimatsu, A (2008) Long-term effects of predicted future seawater CO2 conditions on the survival and growth of the marine shrimp Palemon pacificus. J. Exp. Mar. Biol. Ecol. 367:41-46.

Lee K, Kim T-W, Byrne RH, Millero FJ, Feely RA, Liu Y-M (2010) The universal ratio of boron to chlorinity for the North Pacific and North Atlantic oceans. Geochimica et cosmochimica acta 74(6):1801-1811

Leonard, J and Watson, P (2011) Description of the input-output model for Pacific Coast fisheries. U.S. Dept. Commer., NOAA Tech. Memo. NMFS-NWFSC-111, 64 p.

Long, WC, Swiney, KM, Foy, RJ (2013a) Effects of ocean acidification on the embryos and larvae of red king crab, Paralithodes camtschaticus. Marine Polution Bulletin 69: 38-47

Long, WC, Swiney, KM, Harris, C, Page, HN, Foy, RJ (2013b) Effects of ocean acidification on juvenile Red King crab (*Paralithodes camtschaticus*) and Tanner crab (*Chionecetes bairdi*) growth, condition, calcification and survival. PlosOne 8(4):1-10

Lough RG (1974) Dynamics of crab larvae (Anomura, Brachyura) off the central Oregon coast, 1969–1971. Ph.D. Dissertation, Oreg. State Univ., Corvallis. 299 p

Mayer, DL (1973) The ecology and thermal sensitivity of the Dungeness crab, Cancer magister, and related species of its benthic community in Similk Bay, Washington. Dissertation, University of Washington

McGaw IJ (2004) Ventilatory and cardiovascular modulation associated with burying in two sympatric crabs *Cancer magister* and *Cancer productus*. J Exp Mar Biol Ecol 303:47-63

Melzner F, Thomsen J, Koeve W, Oschlies A, Gutowska A, Bange HW, Hansen HP, Körtzinger A (2013) Future ocean acidification will be amplified by hypoxia in coastal habitats. Mar Biol 160:1875-1954

Moloney, CL, Botsford, LW, Largier, JL (1994) Development, survival and timing of metamorphosis of planktonic larvae in a variable environment, the Dungeness crab as an example. Marine Ecology Progress Series 113:61-79.

Miller GM, Watson S-A, McCormick MI, Munday PL (2013) Increased CO_2 stimulates reproduction. Global Change Biology 19(10):3037-3045

Murray JW and Gill G (1978) The geochemistry of iron in Puget Sound. Geochimica et Cosmochimica Acta. 42:9-19

Nations JD (1975) The genus *Cancer* (Crustacea: Brachyura): systematics, biogeography, and fossil record. *Natural History Museum of Los Angeles County Science Bulletin* 23: 1-104.

Park, W, and Shirley, TC (2005) Diel vertical migration and seasonal timing of the larvae of three sympatric Cancrid crabs, *Cancer* spp., in Southeastern Alaska. Estuaries 28(2):266-273.

Pauley, GB, Armstrong, DA, and Heun, TW (1986) Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (Pacific Northwest)--Dungeness crab. U.S. Fish Wildl. Serv. Biol . Rep. 82(11.63). U. S. Army Corps of Engineers, TREL-82-4. 20 pp.

Poole, RL (1966) A description of laboratory-reared zoeae of *Cancer magister* Dana, and megalopae taken under natural conditions (*Decapoda Brachyura*). Crustaceana 11(1):83-97.

R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Rasmuson LK (2013) The Biology, Ecology and Fishery of the Dungeness crab, *Cancer magister* in Lesser, M. 2013. Advances in Marine Biology 65

Reilly PN (1983) "Predation on Dungeness crabs, *Cancer magister*, in central California. in: "Life history, environment, and mariculture studies of the Dungeness crab, *Cancer magister*, with emphasis on the central California fishery resource." P. W. Wild and R. N. Tasto, eds. *California Department of Fish and Game Fish Bulletin* 172: 155-164.

Ries, JB, Cohen, A, and McCorkle, DC (2009) Marine calcifiers exhibit mixed responses to CO2-induced ocean acidification. Geology 27(12):1131-1134

Reum JCP, Alin SR, Feely RA, Newton J, Warner M, et al. (2014) Seasonal Carbonate Chemistry Covariation with Temperature, Oxygen, and Salinity in a Fjord Estuary: Implications

for the Design of Ocean Acidification Experiments. PLoS ONE 9(2): e89619. doi:10.1371/journal.pone.0089619

Schindler DE, Rogers DE, Scheuerell MD, Abrey CA (2005) Effects of changing climate on zooplankton and juvenile Sockeye salmon growth in southwestern Alaska. Ecology 86(1):198-209

Shanks AL (2013) Atmospheric forcing drives recruitment variation in the Dungeness crab (Cancer magister), revisited. Fisheries Oceanography. doi:10.1111/fog.12020 pp. 1-10

Shirley S, Shirley T, and Rice S (1987) Latitudinal variation in the Dungeness crab, *Cancer magister*, zoeal morphology explained by incubation temperature. Mar. Biol. 95:371-376

Spicer, JI, Raffo, A, and Widdicombe, S (2007) Influence of CO2-related seawater acidification on extracellular acid-base balance in the velvet swimming crab. Necora puber Mar. Biol. 151:1117–1125

Stone RP, and O'Clair CE (2001) Seasonal movements and distribution of Dungeness crabs Cancer magister in a glacial southeastern Alaska estuary. Mar. Ecol. Prog. Ser. 214:167-176. doi:10.3354/meps214167

Stumpp M, Wren J, Melzner F, Thorndyke MC, Dupont ST (2011) CO_2 induced seawater acidification impacts sea urchin larval development I: Elevated metabolic rates decrease scope for growth and induce developmental delay. Comparative Biochem Physiol, Part A 160:331-340

Sulkin S, Blanco A, Chang J, Bryant M (1998) Effects of limiting access to prey on development of first zoeal stage of the brachyuran crabs Cancer magister and Hemigrapsus oregonensis. Mar. Biol. 131, 515–521.

Sulkin, SD and McKeen, GI (1989) Laboratory study of survival and duration of individual zoeal stages as a function of temperature in the brachyuran crab *Cancer magister*. Marine Biology 103: 31-37.

Walther K, Anger K, Pörtner HO (2010) Effects of ocean acidification and warming on the larval development of the spider crab *Hyas araneus* from different latitudes (54° vs. 79°). Mar Ecol Prog Ser 417:159-170

Whiteley NM (2011) Physiological and ecological responses of crustaceans to ocean acidification. Marine Ecology Progress Series. 430:257-271

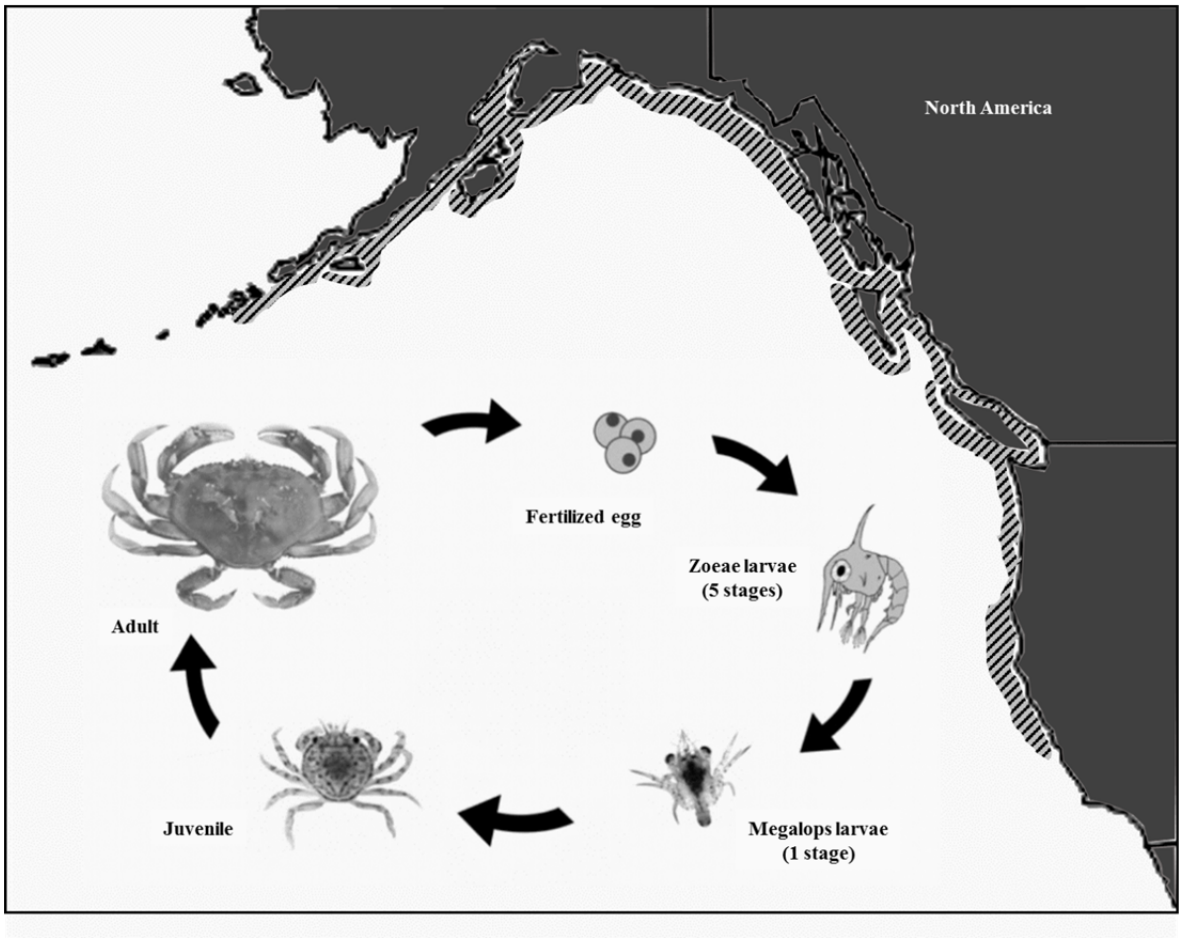

Fig. 1 Map of C. magister distribution in northeast Pacific (gray shaded region along coast) with inset of life-history cycle (adapted from Fisher and Velasquez 2008). This study exposed 'fertilized egg' and planktonic 'zoeae larvae' life stages to low-pH seawater

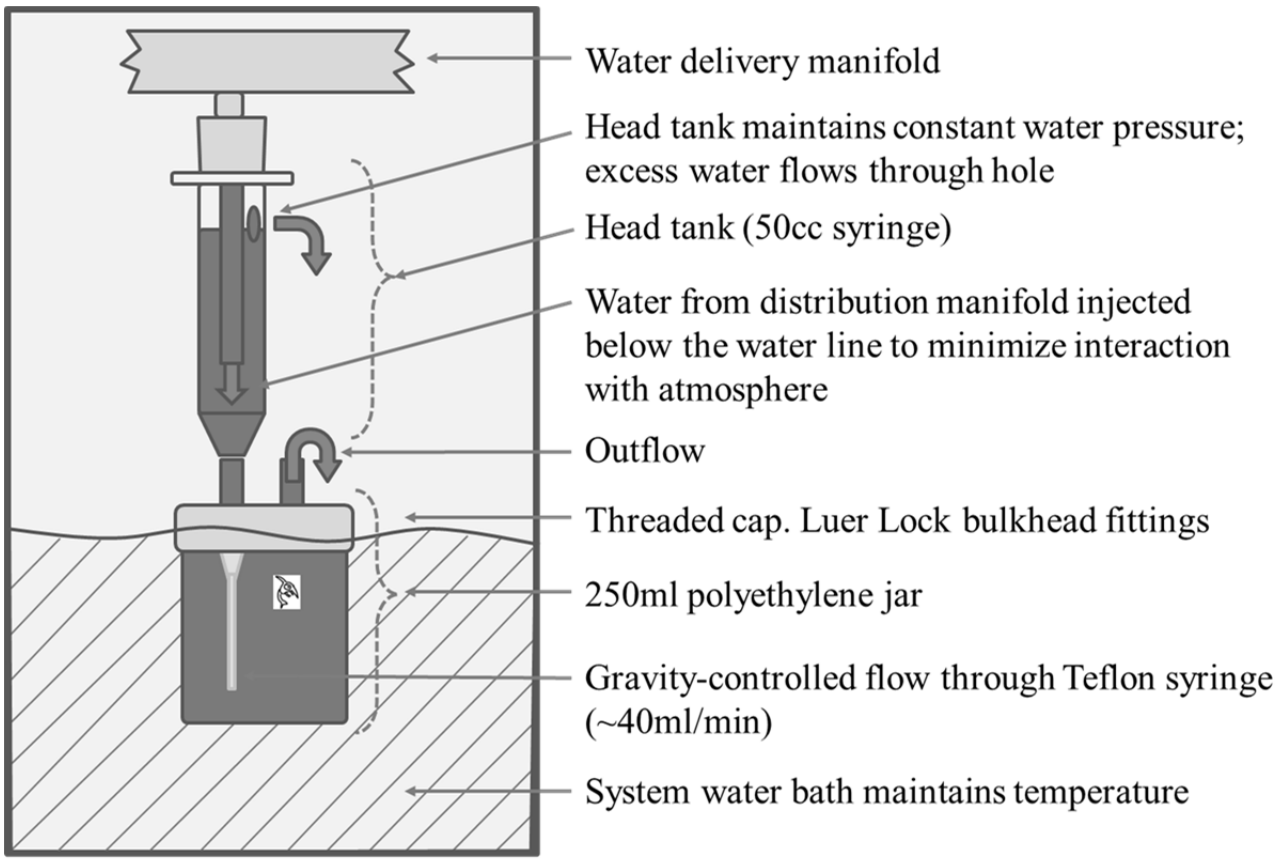

Fig. 2 Diagram of 250ml rearing jar. The small header tank was critical to maintain equal flow rates between jars. Design credit to Dr. Paul McElhany

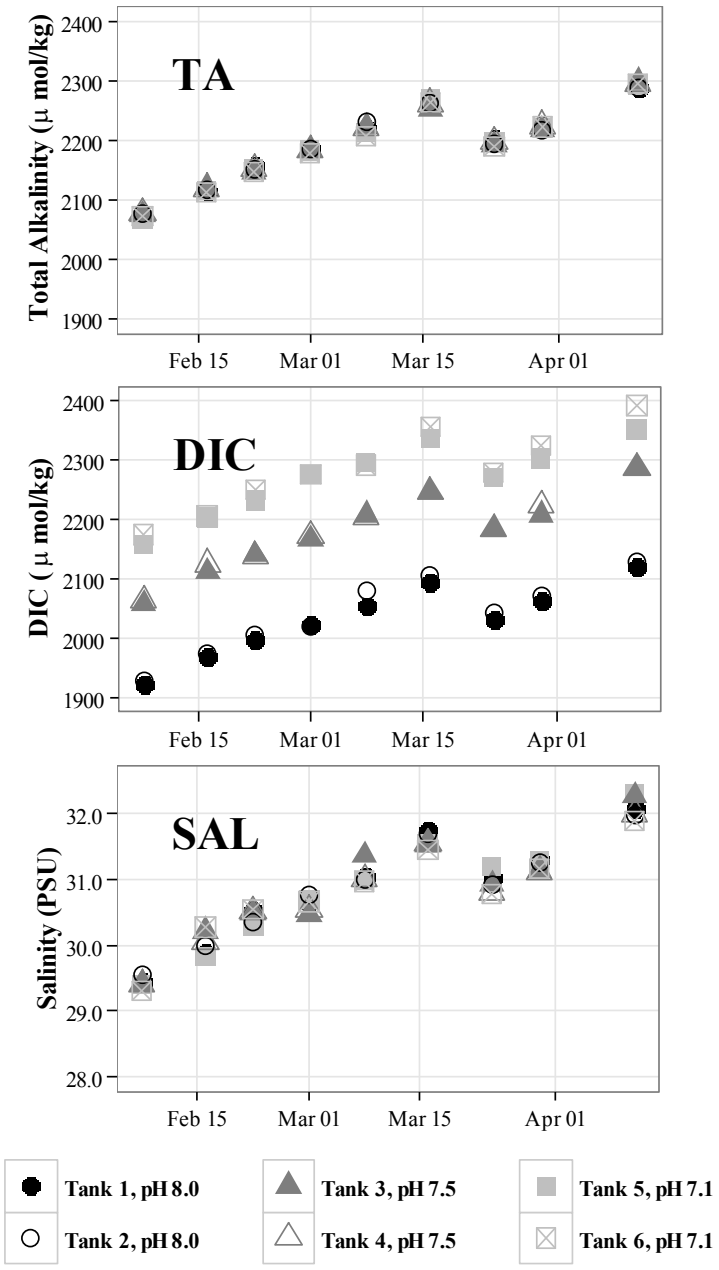

Fig. 3 **TA** Total Alkalinity, **DIC** dissolved inorganic carbon, and **SAL** salinity measured from discrete system-water samples. Changes in the measurements over time can be explained by evaporation outpacing the addition of deionized water. Three treatments, pH 8.0, pH 7.5, and pH 7.1, are each represented by two tanks in each plot. On March 19, ~8000 liters of seawater were added to the system resulting in a drop in TA, SAL and consequently DIC. Spec-pH measurements confirmed that treatment pH levels were maintained.

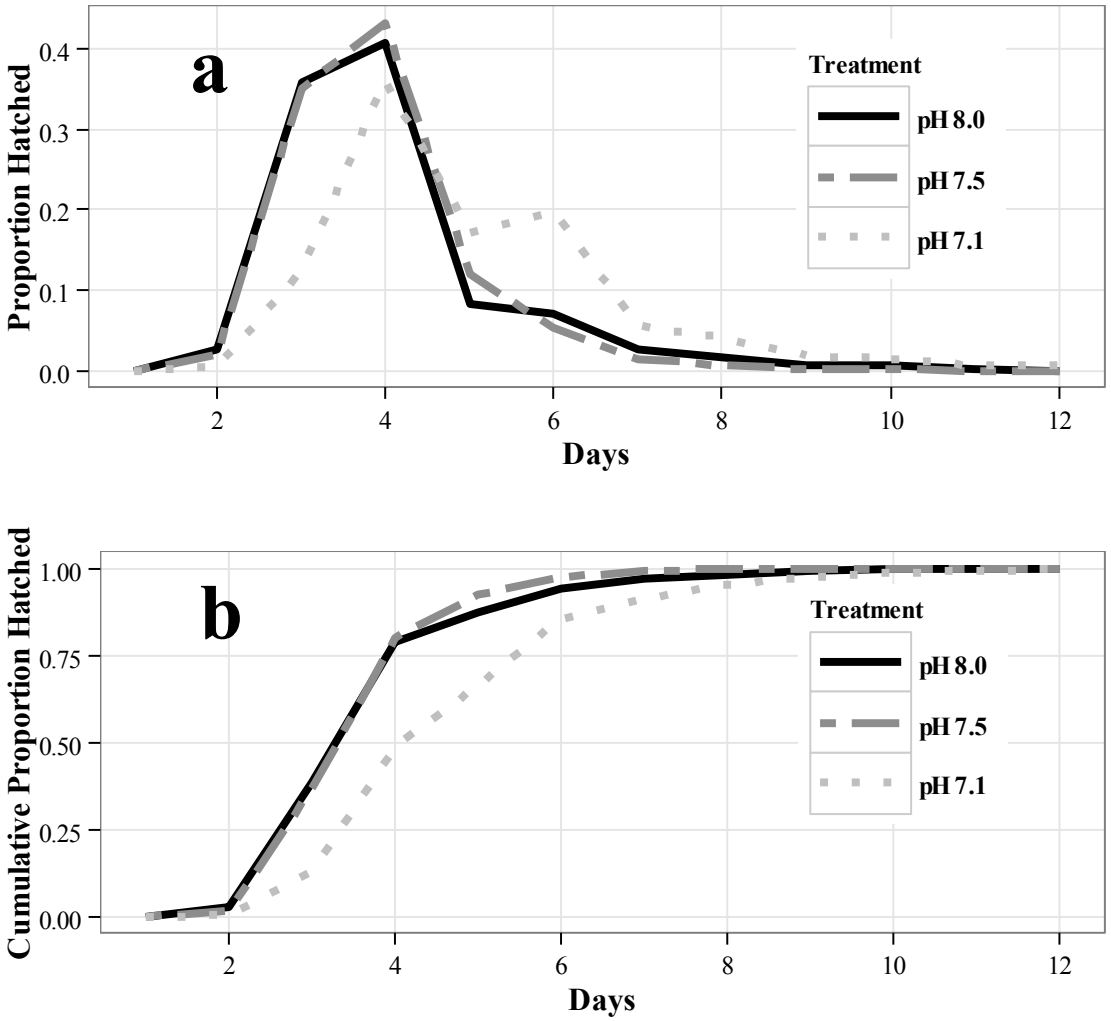

Fig. 4 a Proportion of eggs hatched each day. Peak hatching occurred three days following the first hatched egg. **b** Cumulative proportion of eggs hatched each day. The eggs hatching in the pH 7.1 treatment took longer to hatch than those in pH 8.0 by a factor of 1.24, 95%CI (1.22-1.26), while those in pH 7.5 hatched faster than those in pH 8.0 by a factor of 0.93, 95%CI (0.91-0.94)

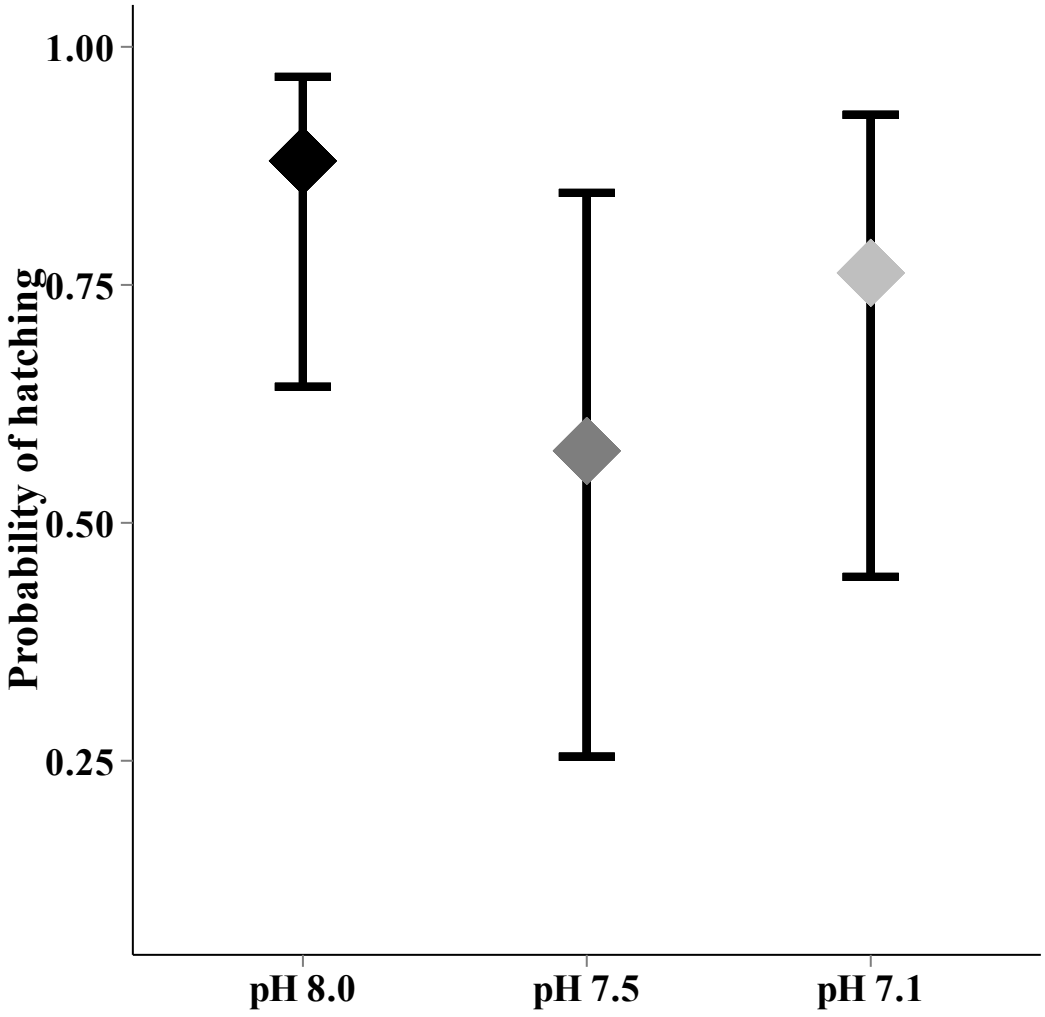

Fig. 5 Probability of hatching in each treatment. Diamonds indicate the probability of hatching for an individual egg in each treatment. Error bars show 95% confidence intervals. There were no significant differences among treatments (pH 8.0: pH 7.5, p = 0.09; pH 8.0: pH 7.1, p = 0.41; pH 7.5: pH 7.1, p = 0.39)

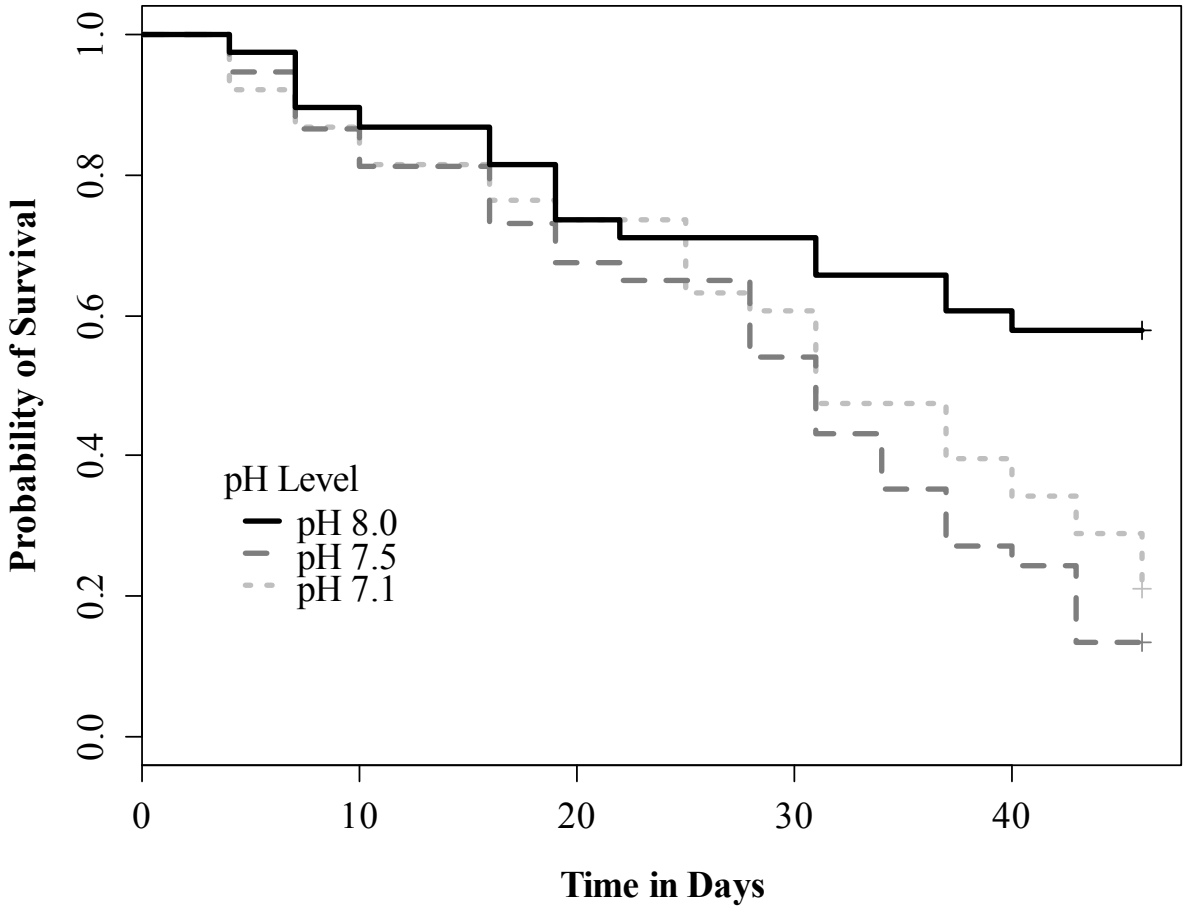

Fig. 6 The KM survival curves for the three treatment levels showing the probability of survival on each day. The initial numbers of zoeae for pH 8.0, pH 7.5, and pH 7.1 were 38, 37, and 38 respectively (three broods and two replicates per treatment were pooled). The logrank statistic and resultant pairwise comparisons indicated that larvae in pH 8.0 survived significantly higher than in each of the two low-pH treatments with no difference between the two low-pH treatments ($\chi 2 = 13.8$, 2 df, $p = 0.001$)

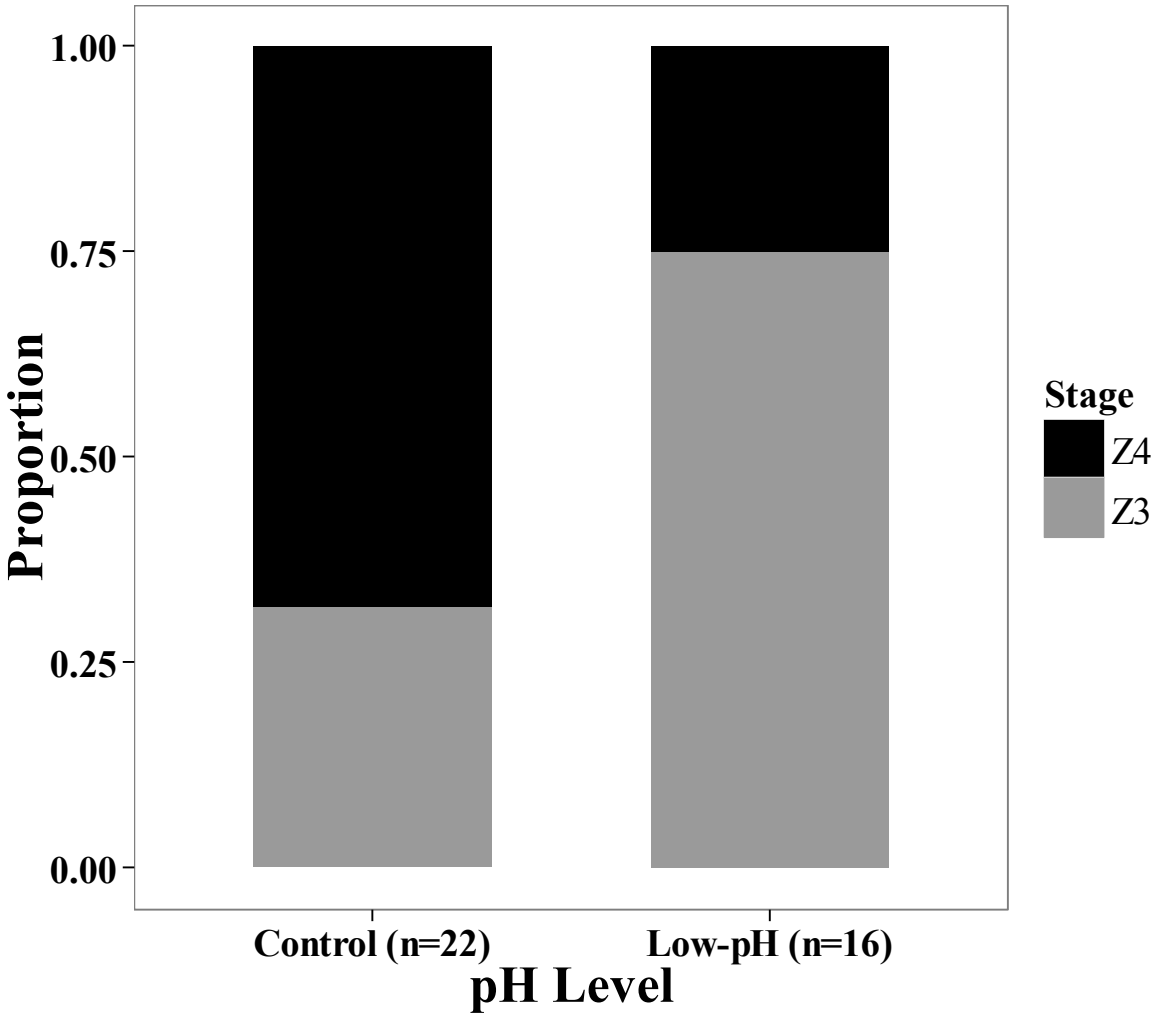

Fig. 7 Proportion of zoeae at each stage by the final day of the experiment. The control is at pH 8.0 and the low-pH category is inclusive of the pH 7.5 and pH 7.1 treatments. There was a significant difference in the ratio of stage 4 zoeae between the control and the low-pH treatments (χ^2=7.00, df=2, p=0.03)

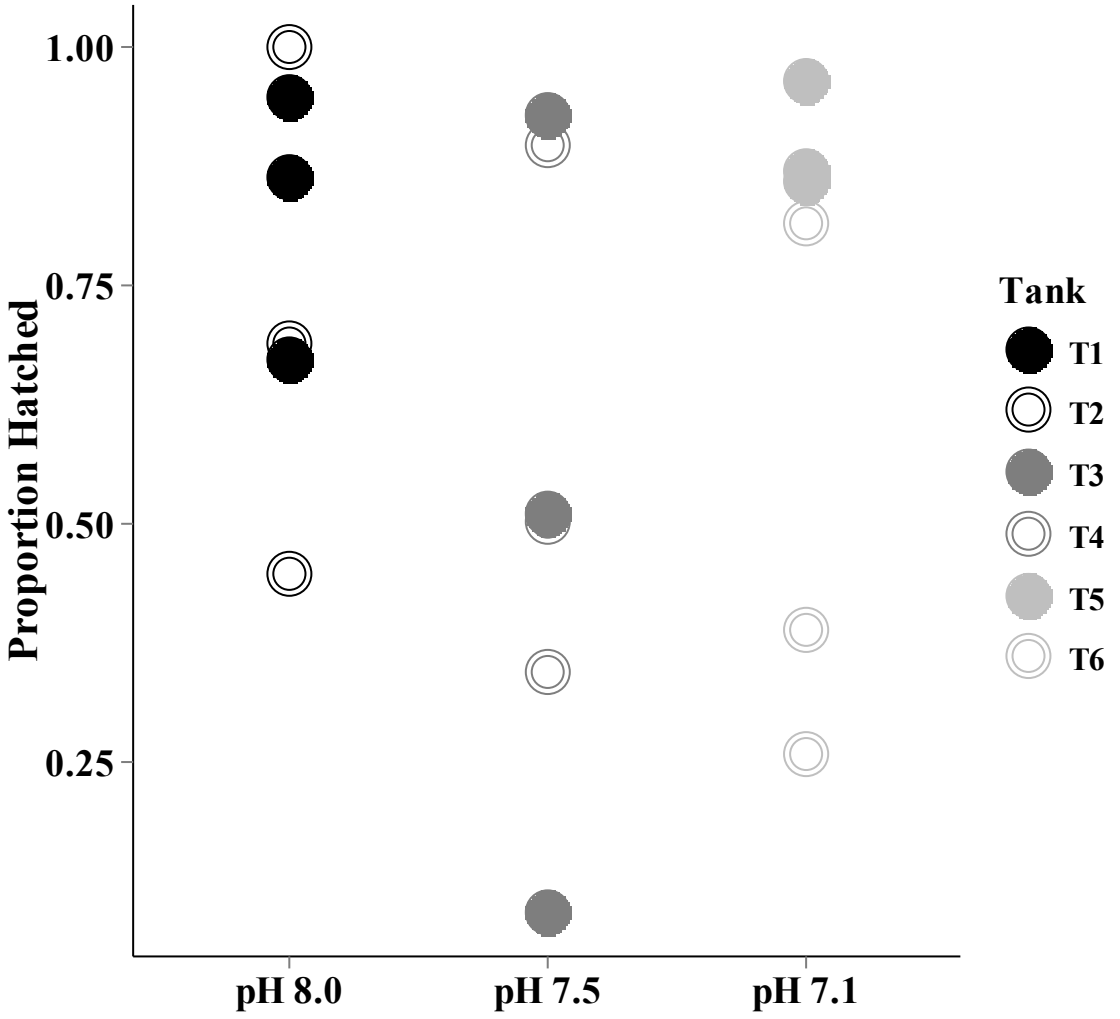

Fig. 8 Proportion of successfully hatched eggs in each jar within each tank and treatment. There was wide variation within this single brood with no significant differences between means among the six tanks ($F_{5,12} = 1.191$, $p = 0.37$)

Table 1 Summary of pH values (mean ± standard deviation) measured in the system tanks and rearing jars for each treatment level

Treatment	Tank	Mean system pH		Mean jar pH
		Durafet probe	spec-pH	spec-pH
pH 8.0	1	8.02 (0.01)	8.00 (0.01)	7.99 (0.02)
	2	8.02 (0.01)	7.97 (0.02)	7.96 (0.04)
pH 7.5	3	7.47 (0.01)	7.44 (0.03)	7.44 (0.02)
	4	7.47 (0.01)	7.44 (0.02)	7.43 (0.04)
pH 7.1	5	7.18 (0.01)	7.12 (0.04)	7.13 (0.04)
	6	7.18 (0.01)	7.08 (0.04)	7.09 (0.04)

Standard deviation in parenthesis

Table 2 Numbers of zoeae entering survival analysis and percent survival on the final day for each tank within each treatment

Treatment	Tank	$N_{t=0}$ days	$N_{t=45}$ days	% Survival	z	p value
pH 8.0	1	20	10	50.0	1.033	0.301
	2	18	12	66.7		
pH 7.5	3	19	2	10.5	0.542	0.588
	4	18	3	16.7		
pH 7.1	5	19	4	21.1	0.000	1.000
	6	19	4	21.1		

Table 3 Comparison of individual KM survival analysis results for 'each tank within a treatment' and 'each brood within each treatment' showing no 'tank' or 'brood' effect

Comparison	Logrank[a]	df	p value
pH 8.0 (tanks 1 and 2)	1.0	1	0.319
pH 7.5 (tanks 3 and 4)	$4.7\ e^{-3}$	1	0.945
pH 7.1 (tanks 5 and 6)	$2.2\ e^{-2}$	1	0.880
pH 8.0 (all three broods)	0.9	2	0.646
pH 7.5 (all three broods)	0.1	2	0.961
pH 7.1 (all three broods)	0.4	2	0.816

[a]Logrank test statistic is a chi square statistic

Table 4 KM survival analysis pairwise comparisons[a] among treatments

Comparison	χ^2	**p value**	**significant**
pH 8.0 vs pH 7.5	12.13	$5.0\ e^{-4}$	Yes
pH 8.0 vs pH 7.1	$5.8\ e^{-3}$	$5.8\ e^{-3}$	Yes
pH 7.5 vs. pH 7.1	$2.2\ e^{-2}$	0.973	No

[a]Holm-Sidak method for pairwise comparisons

Appendix A: Estimate of annual production of larvae

An approximate 'back-of-the-envelope' estimation of the number of zoeae hatching into the northeast Pacific Ocean along their entire geographic range is between 26.33×10^{12} and 78.06×10^{12}.

The estimate was made given the following observations, assumptions and calculations:

The mean ± stdev annual harvest of *C. magister* over years 2003-2012 (US and Canada) inclusive is $35.66 \times 10^6 \pm 6184$ kg year^{-1} with the weight per male typically 0.9 - 1.3kg (2-3 lbs) (FAO.org). Calculating the lower and upper range according to the weight range gives

35.66×10^6 kg / 1.3 kg = ~27,430,769 male crabs

35.66×10^6 kg / 0.9 kg = ~39,622,222 male crabs

These estimates are conservative in that annual exploitation rates for harvest-size males fluctuates around 90% (Methot and Botsford 1982, Hankin 1985, Smith and Jamieson 1989, females are not harvested). The fishery is size-limited, meaning harvestable males must be over a certain size, to ensure males get at least one chance to mate before they recruit to the fishery (Hankin et al. 1997). Thus sexually mature, under-harvest-size males also remain in the male population.

To find the number of female crabs in the population the assumption is made that the numbers of harvested males reasonably estimates the number of like-aged females (Shanks and Roegner 2007).

Setting the estimates for harvested males above on a 1:1 ratio with mature female crabs, the conservative estimate of sexually mature females is

~27,430,769 – 39,622,222 female crabs

Mating studies indicate 80-98.5% success rate for presence of sperm plugs in estuarine and open-ocean habitats (Dunn and Shanks 2012, Hankin et al 1997, Oh and Hankin 2004). The next step is to calculate the lower and upper bounds for the estimate of fertilized females

27,430,769 -> 27,430,769 females * .80 (mating success) = ~21,944,615 fertilized females

39,622,222 -> 39,622,222 females * .985 (mating success) = ~39,027,889 fertilized females

Females deposit between 1.5 and 2.5 million eggs (Rasmusson 2013). Calculating the lower and upper bounds for numbers of eggs gives

21,944,615 females * 1,500,000 eggs = ~32,916,922,800,000 eggs

39,027,889 females * 2,500,000 eggs = ~97,569,721,675,000 eggs

Egg hatching success is estimated at 80% (Ebert et al 1975). Finally, calculation for the number of larvae results in

32,915,907,000,000 * 0.80 = 26,333,538,240,000 larvae

97,569,721,675,000 * 0.80 = 78,055,777,340,000 larvae

The resultant estimate for the number of larvae is between 26.33×10^{12} and 78.06×10^{12}.

Appendix B: Chemistry summary tables

Table 1. egg exposure study

Table 2. zoeae exposure study

Table 5. System chemistry for the egg exposure experiment. Two replicate tanks were used for each treatment level. Values expressed as a mean ± std dev. N of samples are in square brackets. Individual Durafet probes (Honeywell) measured system pH and temperature (Temp). Salinity was measured using a conductivity probe (ThermoScientific Orion Star A322). Dissolved inorganic carbon (DIC) and Total Alkalinity (TA) were measured at the NWFSC OA chemistry lab.

Treatment pH level	Tank	Eggs per jar	System pH* (probe)	System pH** (spec)	Jar pH** (spec)	Temp.* (°C)	Salinity† (PSU)	DIC† (µmol/kg)	TA† (µmol/kg)	TA†† (µmol/kg)	pCO_2†† (µatm)	Ωa ††
Open ocean control	**1**	**610** ±46	**8.02** ±0.01	**7.99** ±0.01 [21]	**7.99** ±0.01 [6]	**12.07** ±0.06	**30.55** ±0.82 [6]	**2009.12** ±60.62 [6]	**2168.92** ±70.50 [21]	**2135.48** ±56.10 [21]	**439.29** ±13.69 [21]	**1.68** ±0.08 [21]
	2	**691** ±69	**8.02** ±0.01	**7.96** ±0.01 [24]	**7.94** ±0.02 [8]	**12.10** ±0.11	**30.56** ±0.77 [6]	**2018.02** ±66.26 [6]	**2171.00** ±69.40 [21]	**2145.61** ±58.63 [24]	**487.85** ±50.08 [24]	**1.57** ±0.08 [24]
Intermed-iate low	**3**	**545** ±153	**7.47** ±0.01	**7.43** ±0.03 [26]	**7.42** ±0.02 [7]	**12.05** ±0.04	**30.59** ±0.78 [6]	**2155.31** ±68.11 [6]	**2166.80** ±65.66 [21]	**2116.15** ±58.12 [26]	**1751.28** ±126.20 [26]	**0.50** ±0.04 [26]
	4	**529** ±78	**7.47** ±0.01	**7.44** ±0.03 [23]	**7.43** ±0.01 [9]	**12.13** ±0.06	**30.52** ±0.74 [6]	**2158.46** ±64.54 [6]	**2169.30** ±67.40 [21]	**2128.91** ±46.86 [23]	**1741.68** ±121.87 [23]	**0.51** ±0.03 [23]
Extreme low	**5**	**614** ±132	**7.18** ±0.01	**7.11** ±0.03 [23]	**7.10** ±0.04 [7]	**12.12** ±0.09	**30.47** ±0.78 [6]	**2250.27** ±65.30 [6]	**2166.08** ±71.82 [21]	**2113.64** ±51.30 [23]	**3734.06** ±204.02 [23]	**0.25** ±0.02 [23]
	6	**574** ±47	**7.19** ±0.01	**7.08** ±0.04 [25]	**7.05** ±0.04 [7]	**12.13** ±0.06	**30.54** ±0.72 [6]	**2259.25** ±64.82 [6]	**2164.07** ±68.34 [21]	**2101.12** ±41.14 [25]	**4027.51** ±390.54 [25]	**0.23** ±0.02 [25]

* Durafet pH and Temperature recorded every 10 minutes over the duration of the experiments

**System pH calculated at 12°C with DIC and spectrophotometric pH measurements entered into Seacarb package

† Salinity, DIC and TA directly measured from discrete water samples

†† TA, pCO2 and Ωa calculated from DIC and spectrophotometric pH measurements entered into Seacarb package

Table 6. System chemistry for the zoeal exposure experiment. Two replicate tanks were used for each treatment level. Values expressed as a mean ± std dev. N of samples are in square brackets. Individual Durafet probes (Honeywell) measured system pH and temperature (Temp). Salinity was measured using a conductivity probe (ThermoScientific Orion Star A322). Dissolved inorganic carbon (DIC) and Total Alkalinity (TA) were measured at the NWFSC OA chemistry lab.

Treatment pH level	**Tank**	**Jars per Tank**	**System pH * (probe)**	**System pH 2** (spec)**	**Jar pH** (spec)**	**Temp.***	**Salinity†**	**DIC†**	**TA†**	**TA††**	**pCO_2††**	**Ωa ††**
						(°C)	**(PSU)**	**(µmol/kg)**	**(µmol/kg)**	**(µmol/kg)**	**(µatm)**	
Open ocean control	**1**	**20**	**8.02** ±0.01	**8.00** ±0.01 [40]	**7.99** ±0.01 [28]	**12.08** ±0.07	**31.20** ±0.57 [7]	**2054.06** ±42.79 [7]	**2220.73** ±44.70 [7]	**2214.14** ±37.70 [40]	**449.65** ±11.99 [40]	**1.78** ±0.07 [40]
	2	**18**	**8.02** ±0.01	**7.97** ±0.03 [44]	**7.96** ±0.03 [26]	**12.12** ±0.11	**31.14** ±0.56 [7]	**2064.41** ±44.56 [7]	**2218.59** ±47.20 [7]	**2212.92** ±35.72 [44]	**486.24** ±36.06 [44]	**1.68** ±0.11 [44]
Intermed-iate low	**3**	**19**	**7.47** ±0.01	**7.44** ±0.02 [46]	**7.44** ±0.02 [26]	**12.06** ±0.05	**31.17** ±0.63 [7]	**2205.91** ±49.05 [7]	**2216.45** ±49.34 [7]	**2185.45** ±37.95 [46]	**1785.79** ±100.44 [46]	**0.53** ±0.03 [46]
	4	**18**	**7.47** ±0.01	**7.43** ±0.02 [43]	**7.43** ±0.04 [25]	**12.14** ±0.07	**31.08** ±0.54 [7]	**2208.01** ±49.49 [7]	**2219.60** ±48.15 [7]	**2185.97** ±34.64 [43]	**1806.92** ±102.27 [43]	**0.53** ±0.03 [43]
Extreme low	**5**	**19**	**7.18** ±0.01	**7.13** ±0.02 [46]	**7.14** ±0.02 [25]	**12.15** ±0.08	**31.19** ±0.64 [7]	**2295.29** ±41.02 [7]	**2218.62** ±48.98 [7]	**2169.30** ±32.72 [46]	**3691.85** ±182.11 [46]	**0.26** ±0.02 [46]
	6	**19**	**7.18** ±0.01	**7.08** ±0.03 [47]	**7.09** ±0.02 [26]	**12.13** ±0.07	**31.07** ±0.48 [7]	**2310.53** ±50.02 [7]	**2215.17** ±50.96 [7]	**2165.31** ±37.38 [47]	**4097.92** ±244.56 [47]	**0.24** ±0.02 [47]

* Durafet pH and Temperature recorded every 10 minutes over the duration of the experiments

**System pH calculated at 12°C with DIC and spectrophotometric pH measurements entered into Seacarb package

† Salinity, DIC and TA directly measured from discrete water samples

†† TA, pCO2 and Ωa calculated from DIC and spectrophotometric pH measurements entered into Seacarb package

Appendix C: R code for analyses

Chemistry

#Chemistry calculations were carried out in Seacarb package (Lavigne and Gattuso 2013). I used spectrophotometric pH and dissolved inorganic carbon measurements.

#Import your data file. Mine was named, "crabpHALK_DIC.csv"

#This was my path. Yours will likely be different. I named mine such that I would remember the two parameters that I used.

>crabpHDIC <- read.csv("D:/Ocean Acidification Studies/Dungeness_Crab/D Crab CHEMISTRY/crabpHALK_DIC.csv")

#make note of the list of different constants you can use. A couple of mine were defaults so did not need to be listed.

>crabpHDIC_out<-carb(9,crabpHDIC$pH, crabpHDIC$DIC_II,S=crabpHDIC$ALK_AVGSAMPSAL,T=25)

>crabpHDIC_out<-cbind(crabpHDIC$SAMPLE_ID,crabpHDIC_out)

#Again, this path is unique to my computer set-up. It is handy to use this bit of code if you are looking to place your results directly into excel. Keep in mind that Seacarb has an MS Excel version as well that some find easier to use.

write.table(crabpHDIC_out,"D:/Ocean Acidification Studies/Dungeness_Crab/D Crab CHEMISTRY/crabpHDIC_out.csv", sep=",",row.names=FALSE)

Eggs, probability of hatching analysis

#Use Lme4 package. Expanding egg data out for 0-1 scoring (binary) for no-hatch and hatched, make sure to save data file as "eggs". TotalHatch is the number of hatched inclusive of alive and dead (post hatch). Egg_count is the number of eggs counted from the final sample vials. This analysis gives a value of 1 for hatching and a zero for not hatching. In this analysis my datafiles were organized by target CO2 levels, so you will see treatment levels at 400, 1600, and 3200 µatm (the actual CO2 levels were different from this). "400", "1600" and "3200" are actually pH 8.0, pH 7.5, and pH 7.1, respectively, in the thesis.

>hatch_data = eggs[0,]

```
>for (i in 1:length(eggs$TotalHatch))
>{if(eggs$TotalHatch[i]>0)
{dataset.add.succ = eggs[rep(i, eggs$TotalHatch[i]), ]
dataset.add.succ$TotalHatch=1
dataset.add.succ$Egg_Count=0
hatch_data=rbind(hatch_data, dataset.add.succ)}
if(eggs$Egg_Count[i]>0)
{dataset.add.fail = eggs[rep(i, eggs$Egg_Count[i]), ]
dataset.add.fail$TotalHatch=0
dataset.add.fail$Egg_Count=1
hatch_data=rbind(hatch_data, dataset.add.fail)}}
```

#Use glmer here to create a mixed model with treatment as a fixed variable. Tank and Jar are random variables. Tank, JarID2 are the random variable column headings

```
> cm1 <- glmer(TotalHatch ~ 1 + TX + (1|Tank) + (1|JarID2), family=binomial, data=hatch_data)
> summary(cm1)
```

#this is output

```
Generalized linear mixed model fit by the Laplace approximation
Formula: TotalHatch ~ 1 + TX + (1 | Tank) + (1 | JarID2)
   Data: hatch_data
  AIC  BIC logLik deviance
 9393 9430  -4692     9383
Random effects:
 Groups Name        Variance   Std.Dev.
 JarID2 (Intercept) 3.0012e+00 1.7324e+00
 Tank   (Intercept) 7.5493e-12 2.7476e-06
```

Number of obs: 10684, groups: JarID2, 18; Tank, 6

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)	
(Intercept)	1.9915	0.7164	2.780	0.00544	**
TX[T.1600]	-1.6853	1.0080	-1.672	0.09453	.
TX[T.3200]	-0.8314	1.0080	-0.825	0.40947	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	TX[T.1
TX[T.1600]	-0.711	
TX[T.3200]	-0.711	0.505

#I then removed Tank as a random variable and noticed the AIC went down a little. I renamed the model, cm2_400

> cm2_400 <- glmer(TotalHatch ~ 1 + TX + (1|JarID2), family=binomial, data=hatch_data)

> summary(cm2_400)

#output

Generalized linear mixed model fit by the Laplace approximation

Formula: TotalHatch ~ 1 + TX + (1 | JarID2)

Data: hatch_data

AIC	BIC	logLik	deviance
9391	9420	-4692	9383

Random effects:

Groups Name Variance Std.Dev.

JarID2 (Intercept) 3.0012 1.7324

Number of obs: 10684, groups: JarID2, 18

Fixed effects:

Estimate Std. Error z value Pr(>|z|)

(Intercept) 1.9914 0.7164 2.780 0.00544 **

TX[T.1600] -1.6852 1.0079 -1.672 0.09455 .

TX[T.3200] -0.8311 1.0080 -0.825 0.40962

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

(Intr) TX[T.1

TX[T.1600] -0.711

TX[T.3200] -0.711 0.505

#The next step is to look at the pairwise comparisons between the different treatments and that calls for releveling the fixed variable, treatment (TX). First off I relevel for 1600.

>hatch_data$TX <- relevel(hatch_data$TX, ref = “1600”)

#make sure that quote marks are entered in R. Copy/paste does not do the trick if the font in Word is different.

>cm2_1600 <- glmer(TotalHatch ~ 1 + TX + (1 | Tank) + (1|JarID2), family=binomial, data= hatch_data)

>summary (cm2_1600)

#Output

Generalized linear mixed model fit by the Laplace approximation

Formula: TotalHatch ~ 1 + TX + (1 | Tank) + (1 | JarID2)

Data: hatch_data

AIC BIC logLik deviance

9393 9430 -4692 9383

Random effects:

Groups Name Variance Std.Dev.

JarID2 (Intercept) 3.0012 1.7324

Tank (Intercept) 0.0000 0.0000

Number of obs: 10684, groups: JarID2, 18; Tank, 6

Fixed effects:

Estimate Std. Error z value Pr(>|z|)

(Intercept) 0.3062 0.7090 0.432 0.6658

TX[T.0400] 1.6852 1.0079 1.672 0.0945 .

TX[T.3200] 0.8541 1.0027 0.852 0.3943

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

(Intr) TX[T.0

TX[T.0400] -0.703

TX[T.3200] -0.707 0.497

#Releveled for 3200

> hatch_data$TX <- relevel(hatch_data$TX, ref = "3200")

```
> cm2_3200<-glmer(TotalHatch ~ 1 + TX + (1|Tank) + (1|JarID2), family=binomial,
data=hatch_data)

> summary(cm2_3200)
```

#output

```
Generalized linear mixed model fit by the Laplace approximation

Formula: TotalHatch ~ 1 + TX + (1 | Tank) + (1 | JarID2)

   Data: hatch_data

  AIC  BIC logLik deviance

 9393 9430  -4692     9383

Random effects:

 Groups Name        Variance   Std.Dev.

 JarID2 (Intercept) 3.0012e+00 1.73240750

 Tank   (Intercept) 1.4296e-09 0.00003781

Number of obs: 10684, groups: JarID2, 18; Tank, 6


Fixed effects:

            Estimate Std. Error z value Pr(>|z|)

(Intercept)   1.1609     0.7090   1.637    0.102

TX[T.1600]   -0.8546     1.0027  -0.852    0.394

TX[T.0400]    0.8298     1.0080   0.823    0.410


Correlation of Fixed Effects:

          (Intr) TX[T.1

TX[T.1600] -0.707

TX[T.0400] -0.703  0.497
```

#Results support the null hypothesis that the numbers of "hatched" eggs (inclusive of alive and dead post-hatch) in each treatment (400, 1600, 3200) are not different from each other suggesting that treatment had no effect on numbers of hatched eggs.

#The next step was to calculate the confidence intervals around the central probability of hatching in each treatment. These estimates were pulled from each of the models above (cm2_400, cm2_1600, and cm2_3200) to create this table.

	estimate	std.e	z-value	Pr
(400TX)	**1.9914**	**0.7164**	**2.780**	**0.00544**
(1600TX)	**0.3062**	**0.7090**	**0.432**	**0.6658**
(3200TX)	**1.1609**	**0.7090**	**1.637**	**0.102**

#Confidence interval summaries are in bold below

mean_400<-plogis(1.9914)

#0.880

lowerconf_400<-plogis(1.9914 – (1.96*0.7164))

#0.643

upperconf_400<-plogis(1.9914 + (1.96*0.7164))

#0.968

#TX400->0.880, 95%CI (0.643,0.968)

mean_1600<-plogis(0.3062)

#0.576

lowerconf_1600<-plogis(0.3062 – (1.96*0.7090))

#0.253

upperconf_1600<-plogis(0.3062 + (1.96*0.7090))

#0.845

#TX1600->0.576 95%CI (0.253,0.845)

mean_3200<-plogis(1.1609)

#0.761

lowerconf_3200<-plogis(1.1609– (1.96*0.7090))

#0.443

upperconf_3200<-plogis(1.1609 + (1.96*0.7090))

#0.928

#TX3200->0.761, 95%CI (0.443,0.928)

Accelerated Failure Time model for 'time to hatch' among egg treatments, Weibull distribution

#Expand dataset, "CumHatchbyTX" such that only the individual eggs that hatched are represented in the data set.

>hatch_data = CumHatchbyTX[0,]

>for (i in 1:length(CumHatchbyTX$TotalHatch))

>{if(CumHatchbyTX$TotalHatch[i]>0)

{dataset.add.succ = CumHatchbyTX[rep(i, CumHatchbyTX$TotalHatch[i]),]

dataset.add.succ$TotalHatch=1

hatch_data=rbind(hatch_data, dataset.add.succ)}}

#Will need to install 'Survival' Package from Therneau 2013, 'frailtypack' Rondeau et al. 2013, and 'survC1' Uno 2013. I did not end up using these in this particular analysis, but they can come in handy with mixed effect models.

>hatch2_wei<-survreg(Surv(DAYs,TotalHatch)~factor(TX), data=hatch_data, dist="weibull")

>summary(hatch2_wei)

#Output

Call:

survreg(formula = Surv(DAYs, TotalHatch) ~ TX, data = hatch_data,

dist = "weibull")

Value Std. Error z p

(Intercept) 1.5175 0.00622 243.80 0.00e+00

TX1600 -0.0756 0.00953 -7.93 2.18e-15

TX3200 0.2117 0.00859 24.63 5.58e-134

Log(scale) -1.1390 0.00790 -144.09 0.00e+00

Scale= 0.32

Weibull distribution

Loglik(model)= -13074.9 Loglik(intercept only)= -13575.8

Chisq= 1001.79 on 2 degrees of freedom, p= 0

Number of Newton-Raphson Iterations: 5

n= 7448

#Here are the 95% CIs and AFT ratios
TX1600:TX400 = exp(-0.0756) = 0.93
TX3200:TX400 = exp(0.2117) = 1.24

95%CI 1600:400-> exp(-0.0756-/+1.96(0.00953) = exp(-0.0756-0.0186788), exp(-0.0756+0.0186788) = 0.91, 0.94

95%CI 3200:400-> exp(0.2117-/+1.96(0.00859) = exp(0.2117 – 0.0168364), exp (0.2117+0.0168364) = 1.22, 1.26

#plot of the curve

>curve(pweibull(x, scale=exp(coef(hatch2_wei)[1]), shape=1/hatch2_wei$scale, lower.tail=FALSE), from=0, to=max(hatch_data$DAYs), ylim=c(0,1), col=1, ylab =expression(hat(S)(t)), xlab='t')

>curve(pweibull(x, scale=exp(coef(hatch2_wei)[1]+ coef(hatch2_wei)[2]), shape=1/hatch2_wei$scale, lower.tail=FALSE), from=0, to=max(hatch_data$DAYs), ylim=c(0,1), add=T, col=2)

>curve(pweibull(x, scale=exp(coef(hatch2_wei)[1]+ coef(hatch2_wei)[3]), shape=1/hatch2_wei$scale, lower.tail=FALSE), from=0, to=max(hatch_data$DAYs), ylim=c(0,1), add=T, col=3)

>legend('topright',paste('TX',c('400','1600','3200')), col=1:3, lty=1, cex=.9, bty='n')

Zoeal survival: Kaplan Meier analysis

#Use the Survival package from Therneau 2013 and splines

#Used IDCZS_2013 comma delimited data file. The first analysis I am running combines all tanks and moms. There were six tanks. Two tanks for each of three treatments: 400, 1600, 3200.

> IDCZS_2013 <- read.csv("C:/Ocean Acidification Studies/Dungeness_Crab/D Crab 2013 Zoeae Individual Cup Study 2013/IDCZS_2013.csv")

#Change TX column to read as a factor

> IDCZS_2013$TX <- factor(IDCZS_2013$TX, labels=c('0400', '1600', '3200'))

#Next step is to run the survfit formula. The conf.type = log is a default. I'm not sure what this does nor am I sure what greenwood error does.

> surTX <- survfit(Surv(DAY, MORT) ~ TX, conf.type="log", conf.int=0.95, type="kaplan-meier", error="greenwood", data=IDCZS_2013)

#events records the number of mortalities from the binary column of "MORT" in the IDCZS_2013 data file.

> surTX

Call: survfit(formula = Surv(DAY, MORT) ~ TX, data = IDCZS_2013, conf.type = "log",

conf.int = 0.95, type = "kaplan-meier", error = "greenwood")

	records	n.max	n.start	events	median	0.95LCL	0.95UCL
TX=0400	38	38	38	16	NA	37	NA
TX=1600	37	37	37	32	31	28	37
TX=3200	38	38	38	30	31	25	43

#I decided to paste it directly from the Rstudio window and keep the formatting to make it easier to see the layout. Here is the summary of surTX. Note that the 95% confidence intervals below for TX 400 don't overlap with the other two treatments' confidence intervals while TX 1600 and TX 3200 do overlap.

> summary(surTX)
Call: survfit(formula = Surv(DAY, MORT) ~ TX, data = IDCZS_2013, conf.type = "log",

```
  conf.int = 0.95, type = "kaplan-meier", error = "greenwood")

          TX=0400
time n.risk n.event survival std.err lower 95% CI upper 95% CI
  4    38       1    0.974  0.0260        0.924        1.000
  7    37       3    0.895  0.0498        0.802        0.998
 10    34       1    0.868  0.0548        0.767        0.983
 16    33       2    0.816  0.0629        0.701        0.949
 19    31       3    0.737  0.0714        0.609        0.891
 22    28       1    0.711  0.0736        0.580        0.870
 31    27       2    0.658  0.0770        0.523        0.827
 37    25       2    0.605  0.0793        0.468        0.782
 40    23       1    0.579  0.0801        0.441        0.759

          TX=1600
time n.risk n.event survival std.err lower 95% CI upper 95% CI
  4    37       2    0.946  0.0372       0.8758        1.000
  7    35       3    0.865  0.0562       0.7614        0.982
 10    32       2    0.811  0.0644        0.6939        0.947
 16    30       3    0.730  0.0730        0.5998        0.888
 19    27       2    0.676  0.0770        0.5405        0.845
 22    25       1    0.649  0.0785        0.5117        0.822
 28    24       4    0.541  0.0819        0.4016        0.728
 31    20       4    0.432  0.0814        0.2990        0.626
 34    16       3    0.351  0.0785        0.2268        0.544
 37    13       3    0.270  0.0730        0.1592        0.459
 40    10       1    0.243  0.0705        0.1378        0.429
 43     9       4    0.135  0.0562        0.0598        0.305

          TX=3200
time n.risk n.event survival std.err lower 95% CI upper 95% CI
  4    38       3    0.921  0.0437        0.839        1.000
  7    35       2    0.868  0.0548        0.767        0.983
 10    33       2    0.816  0.0629        0.701        0.949
 16    31       2    0.763  0.0690        0.639        0.911
 19    29       1    0.737  0.0714        0.609        0.891
 25    28       4    0.632  0.0783        0.495        0.805
 28    24       1    0.605  0.0793        0.468        0.782
 31    23       5    0.474  0.0810        0.339        0.662
 37    18       3    0.395  0.0793        0.266        0.585
 40    15       2    0.342  0.0770        0.220        0.532
 43    13       2    0.289  0.0736        0.176        0.476
 46    11       3    0.211  0.0661        0.114        0.390
```

#Figure creation. Kaplan-Meier plot

```
> plot(surTX, col=1:3, lty=1:3, mark.time=TRUE, ylab="Probability of Survival", xlab="Time in Days")
> legend("bottomleft", legend=c("0400", "1600", "3200"), title="Dissolved CO2 Treatment", col=1:3, lty=1:3, bty="n")
```

Zoeal Accelerated Failure Model, loglogistic distribution

#Using survival package in R, Therneau and a frailty term for the random effect of TANK, a good reference is:

#Kleinbaum DG, Klein M (2005) Survival Analysis. A self-learning text. 2nd ed. Springer Science, New York, NY pp 257-327

#Import IDCZS_2013 and make sure that the TX column is treated as a factor.

```
> IDCZS_2013$TX<-as.factor(IDCZS_2013$TX)
```

#Here is the model

```
> crab_loglog<-survreg(Surv(DAY,MORT)~TX + frailty(TANK), dist="loglogistic", data=IDCZS_2013)
```

#the print command brings this up. It shows a Chisq test and interestingly shows the same test for the frailty(TANK) term. It comes up with its own degrees of freedom for the random effect and shows the variance

```
> print(crab_loglog)
Call:
survreg(formula = Surv(DAY, MORT) ~ TX + frailty(TANK), data = IDCZS_2013,
    dist = "loglogistic")

                coef   se(coef) se2   Chisq  DF   p
(Intercept)    3.875 0.180    0.173 462.42 1.00 0.000
TX1600        -0.591 0.236    0.225   6.25 1.00 0.012
TX3200        -0.466 0.237    0.226   3.86 1.00 0.049
frailty(TANK)                 0.11  0.29 0.470
```

```
Scale= 0.525

Iterations: 10 outer, 33 Newton-Raphson
    Variance of random effect= 0.00504   I-likelihood = -132.3
Degrees of freedom for terms= 0.9 1.8 0.3 1.0
Likelihood ratio test=7.8  on 2 df, p=0.0207  n= 113
```

#The summary shows the WALD test z statistic. Log(scale) is exactly that, it is the ln(0.525). The inverse of the scale is the "shape factor".

```
> summary(crab_loglog)

Call:
survreg(formula = Surv(DAY, MORT) ~ TX + frailty(TANK), data = IDCZS_2013,
   dist = "loglogistic")
             Value Std. Error     z        p
(Intercept)  3.875      0.180 21.50 1.43e-102
TX1600      -0.591      0.236 -2.50  1.24e-02
TX3200      -0.466      0.237 -1.96  4.94e-02
Log(scale)  -0.645      0.098 -6.58  4.63e-11

Scale= 0.525

Log logistic distribution
Loglik(model)= -366.3   Loglik(intercept only)= -370.2
             Chisq= 7.8 on 2 degrees of freedom, p= 0.021
Number of Newton-Raphson Iterations: 10 33
n= 113
```

#Acceleration factor calculation:

ϒ (400:1600) = exp (-0.591) = 0.544, 95%CI = exp(-0.591 +/- 1.96(0.236) = (0.349,0.879)

ϒ (400:3200) = exp(-0.466) = 0.628, 95%CI = exp(-0.466 +/- 1.96(0.237) = (0.394,0.999)

#Odds ratio:

take the inverse of the "scale" to get the shape parameter *p*.

scale= 0.525

p = 1/0.525 = 1.90476

#To obtain the odds ratio, (I used the 95% CI's from the acceleration factor calculation and carried those numbers through the same odds ratio calcuations)

B (1600:400) = exp(-(-0.591)(1.90476)) = exp(1.126) = 3.08 (95%CI=1.28,7.44)

B (3200:400) = exp(-(-0.466)(1.90476)) = exp(0.888) = 2.43 (95%CI=1.003,5.89)

Zoeal stage analysis for calculating odds of being stage 4 zoea in control vs. low-pH treatments

 # summary of original model, using Lme4 package

```
df<-Stages_Final_dayII

m1 <- glmer(BinStage~TXII + (1|TANK), family = binomial, link=logit, data=df)
```

```
Generalized linear mixed model fit by maximum likelihood ['glmerMod']
 Family: binomial ( logit )
Formula: BinStage ~ TXII + (1 | TANK)
   Data: df

    AIC      BIC   logLik deviance
 52.6828  59.2331 -22.3414  44.6828

Random effects:
 Groups Name        Variance  Std.Dev.
 TANK   (Intercept) 2.596e-13 5.095e-07
Number of obs: 38, groups: TANK, 6

Fixed effects:
            Estimate Std. Error z value Pr(>|z|)
(Intercept)   0.7621     0.4577   1.665   0.0959 .
TXIIT1600    -1.1676     1.0212  -1.143   0.2529
TXIIT3200    -2.2662     0.9059  -2.502   0.0124 *
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:
```

```
        (Intr) TXIIT1
TXIIT1600 -0.448
TXIIT3200 -0.505  0.226
```

 # set a priori contrasts, there are k-1 orthogonal contrasts where k is the number of factor levels

```
> contrasts(df$TXII) <-cbind( c(2, -1, -1), c(0, 1, -1))
```

 # double check what they look like

```
> contrasts(df$TXII)
     [,1] [,2]
T0400    2    0
T1600   -1    1
T3200   -1   -1
     [,1] [,2]
T0400    2    0
T1600   -1    1
T3200   -1   -1
```

 # run again with updated contrasts, new model called, m2

#summary of updated model (m2)

```
Generalized linear mixed model fit by maximum likelihood ['glmerMod']
 Family: binomial ( logit )
Formula: BinStage ~ TXII + (1 | TANK)
   Data: df

    AIC      BIC   logLik deviance
 52.6828  59.2331 -22.3414  44.6828

Random effects:
 Groups Name        Variance Std.Dev.
 TANK   (Intercept) 0        0
Number of obs: 38, groups: TANK, 6

Fixed effects:
            Estimate Std. Error z value Pr(>|z|)
(Intercept)  -0.3825     0.4287  -0.892    0.372
TXII1         0.5723     0.2518   2.273    0.023 *
TXII2         0.5493     0.6009   0.914    0.361
```

```
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
     (Intr) TXII1
TXII1 -0.528
TXII2  0.144 -0.122
```

 # shows overall p-value of updated model verses the null

```
> drop1(m2, test="Chisq")

Single term deletions

Model:
BinStage ~ TXII + (1 | TANK)
      Df    AIC    LRT Pr(Chi)
<none>    52.683
TXII    2 55.687 7.0047 0.03013 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

 # Calculate the Odds Ratio control:treatments

```
pi = exp(s1$coefficients[2,1])/(1+exp(s1$coefficients[2,1]))

[1] 0.6392946

se = pi*(1-pi) # just for info

[1] 0.230597

odds = pi/(1-pi)

[1] 1.772345


print(paste("odds = ", round(odds,2), ":1", sep=""))


[1] "odds = 1.77:1"
```