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THE COLLEGE OF NEW JERSEY
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P.O. BOX 7718

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14. ABSTRACT

The effect of environment on adhesion and biomineralization was assessed in the barnacle Amphibalanus amphitrite. Adhesive properties were robust to pH, but reduced pH resulted in an increase in shell growth and a decrease in shell toughness. Increased temperature moderated the effect of reduced pH. The response to salinity depended on larval-batch. The project sheds light on fundamental mechanisms of adhesion and biomineralization in barnacles and provides information that may be useful as the Navy assesses conditions that reduce the fouling load.

15. SUBJECT TERMS

Barancle, biofouling, adhesion, biomineralization, ocean acidification, shell mechanical properties

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Final Technical Report

GRANT #: N00014-14-1-0491
PRINCIPAL INVESTIGATOR: Dr. Gary H. Dickinson
INSTITUTION: The College of New Jersey, Department of Biology
GRANT TITLE: Assessing the effect of environment on barnacle settlement, adhesion and biomineralization
AWARD PERIOD: 15 April 2014 - 28 February 2018

OBJECTIVES: For barnacles grown under varying environmental conditions:

1. Assess adhesion strength and shell micromechanical properties
2. Characterize glue composition
3. Analyze the chemical composition and structure of shells
4. Quantify cyprid settlement intensity

APPROACH: We created conditions that emulated what the barnacle *Amphibalanus amphitrite* might experience in a typical estuary. Barnacle larvae (provided by Dr. Dan Rittschof at the Duke Marine Lab) were settled on T2 silicone coated panels either at Duke or in house at TCNJ. At TCNJ, barnacles were held in small aquaria with seawater equilibrated to the conditions of interest. Each batch of barnacles was exposed to experimental conditions for several months. Throughout experimental exposures, mortality and growth were monitored. At the conclusion of the exposure, adhesion strength of each barnacle was quantified using a digital force gauge, following ASTM standards. Base plate area, shell dimensions, shell mass and tissue mass were quantified. We then used a subset of animals to assess: 1) glue protein abundance and composition; 2) shell mechanical properties; and 3) shell structure and composition.

ACCOMPLISHMENTS: Over the award period, we conducted four long-term exposure assays assessing the effects of: 1) salinity alone; 2) pH alone; 3) the combination of pH and temperature; and 4) the combination of pH and salinity over two generations. Each exposure ran for several months and included hundreds of barnacles. We were also able to conduct a number of projects complementary to the award, which advanced our basic understanding of barnacle adhesion and mineralization.

1) Salinity: Juvenile barnacles (11 days post metamorphosis) were gradually acclimated to eight target levels of salinity, ranging from 10 – 45 in steps of 5, while keeping pH and temperature as consistent as possible. Barnacles were exposed for 16 weeks to these conditions. Over the course of the experiment, mortality was low and was not significantly affected by salinity, although tissue mass was reduced at salinities above and below 35 (the salinity at the site of breeding stock collection). Sexual maturity (determined by the presence of eggs) was not affected by salinity. At the conclusion of the exposure period, barnacle adhesion strength was assessed in shear following ATSM standards. The effect of salinity on adhesion strength was significant, but non-linear; adhesion strength was significantly lower in barnacles held at 35 & 45 as compared to those at 15. Trends in adhesion strength did not correlate with the presence of soft, thick ("gummy") glue, and no effect of salinity was found on gummy glue production. SDS-PAGE analysis of glue proteins revealed a 130 kDa protein that was consistently more abundant at 35 ppt, as compared to lower salinity levels. In terms of biomimeralization, salinity did not exert a significant overall effect on shell mass, thickness, micromechical properties, or base plate area.

2) pH: We assessed if a reduction in seawater pH, at levels predicted within the next 200 years would alter physiology, adhesion, and shell formation in *A. amphitrite*. Juvenile barnacles, settled on silicone substrates, were exposed to one of three levels of pH_T, 8.0, 7.8 or 7.5, for 13 weeks. We found that barnacles were robust to reduced pH, with no effect of pH on physiological metrics (mortality, tissue mass, and the presence of eggs). Likewise, adhesive properties (adhesion strength, morphology of the adhesive plaque, and the composition of glue proteins expressed) were not affected by reduced pH. Shell formation, however, was affected by seawater pH. Shell mass and base plate area were found to be higher in barnacles exposed to even moderately reduced pH; barnacles grown at pH_T 8.0 exhibited approximately 30% lower shell mass and 20% smaller base plate area as compared to barnacles grown at pH_T 7.5 or 7.8. Enhanced growth at reduced pH appears to be driven by the increased size of the calcite crystals that comprise the shell, with calcite crystals within the base plate approximately 95% smaller in barnacles grown at pH_T 8.01 as compared to those grown at pH 7.5, and calcite crystals within the parietal plate approximately 35% smaller in barnacles grown at pH_T 8.0 as compared to those grown at pH 7.5. Despite enhanced growth, mechanical properties of the base plate (but not the parietal plates) were compromised at the lowest pH level. Barnacle base plates at pH_T 7.5 broke more easily and crack propagation, measured through microhardness testing, was significantly affected by seawater pH, suggesting that the toughness of base plates grown at the lowest pH level was reduced. Other shell metrics (plate thickness, relative crystallinity, and atomic disorder) were not affected by seawater pH, but magnesium content of the shells was reduced at pH 7.5. Hence, a reduction in pH resulted in larger barnacles but with base plates that cracked more readily.

3) Combination of pH and temperature: We conducted an 18-week multi-stressor experiment to assess the interaction of pH and temperature on barnacle physiology, shell formation and adhesion. Following conservative climate models, we exposed barnacles to ambient (26°) or elevated (30°) temperature at ambient (8.0) or reduced (7.8) pH, levels that are predicted for the year 2100. Mortality was not affected by pH or temperature, but tissue mass was reduced when both stressors (increased temperature and decreased pH) were combined. We found that neither pH nor temperature significantly affected barnacle adhesion strength or glue morphology, and the interaction of pH and temperature was not significant. Proteomic assessments of glue proteins from these barnacles are currently underway at NRL. Base plate area also was not significantly affected by pH or temperature independently, but in this case, the interaction of pH X temperature was significant. This suggest that the barnacle's response to pH in terms of shell growth depends on the exposure temperature. At ambient temperature (26°), base plate area was larger in barnacles grown at reduced pH, consistent with our previous observations. At elevated temperature (26°), however, this trend was absent with no difference in area between the two pH levels. Hence the response of barnacles to pH is temperature dependent. In terms of shell mechanical properties, barnacles were found to be robust to the moderate reduction in pH and increase in temperature assessed. Neither pH nor temperature exerted a significant impact on shell microhardness or crack propagation. Shells from all exposure conditions were structurally similar and in all cases shell plates were comprised of calcite crystallites. The shells were comprised of ~40% calcium by weight, and calcium content did not differ among treatments in either the base or parietal plates. Magnesium comprised ~0.4% of the shells by weight. There was a small, but significant, effect of temperature on both base and parietal plate magnesium content, with higher temperature resulting in slightly higher magnesium content. This minor

difference in magnesium content did not appear to manifest itself in altered mechanical properties.

4) Combination of pH and salinity: A multigenerational exposure was conducted to assess the interactive effects of pH and salinity over two successive generations. Larvae were collected from wild-type barnacles, juveniles were exposed to conditions of interest for 14 weeks, and then larvae were reared from these animals to produce a second generation. Two pH levels (8.0 and 7.5) and two salinities (35 and 15) were assessed. In the first generation, neither salinity nor pH significantly affected barnacle mortality. Tissue mass, however, was significantly lower in barnacles held at low salinity (at both pH levels) as compared to those at 35 ppt. Reduced tissue mass could indicate altered energy budgets due to salt and water balance. Base plate area was significantly affected by both salinity and pH. The base plate of barnacles at low salinity were significantly smaller, than those at full salinity; barnacles at low pH were significantly larger than those at ambient pH. Increased shell growth at low pH is consistent with previous exposures, suggesting that, at least for *A. amphitrite*, low pH (predicted in coming years) will result in larger barnacles. The strong effect of salinity observed, however, is somewhat surprising, given that in a previous exposure assessing varied salinity alone, salinity did not influence shell growth or properties. Unlike pH, the response to salinity appears to be strongly larval-batch dependent. For this exposure, neither salinity nor pH significantly affected barnacle adhesive properties. Both adhesion strength and the proportion of barnacles expressing atypical "gummy" adhesive varied among panels and was not driven by exposure conditions. The suite of proteins produced by these barnacles also did not differ among treatments.

We successfully obtained nauplii from all seawater conditions, and for all conditions at least some nauplii metamorphosed into cyprids. The viability of the cyprids, however, differed among seawater conditions. Few cyprids successfully settled for barnacles raised at low pH from either salinity and of the cyprids that settled, none of them survived the first week. Although pH did not affect mortality of juvenile barnacles in the first generation, it appears to have a strong impact on the viability of their offspring. Hence only the effect of salinity could be assessed in the second generation. Consistent with generation 1, reduced salinity did not affect adhesive strength or properties, but significantly reduced shell area and mass.

5) Other complementary work:

We completed analyses of phosphoproteins in barnacle glue. Phosphoproteins were identified using phosphoprotein-specific gel staining, Western blotting, and immunohistochemistry. The presence and abundance of phosphoproteins were variable among barnacles, suggesting cyclical changes in the glue secretion overtime. Localization studies showed that phosphoproteins were associated with the capillary glue ducts. Mineralization assays on separated proteins showed that phosphoproteins can induce mineralization. Phosphoproteins form strong ionic bonds with mineral surfaces and could be involved in both the adhesion and biomineralization processes.

A new area of investigation was initiated to assess the effects of hydrophobic coatings on adult barnacle adhesion. Barnacles were reattached to hydrophilic glass, and to glass slides modified with a monolayer of PFOTS or MTS, making them hydrophobic. Surprisingly, barnacles adhered more strongly to the hydrophobic surfaces than to unmodified, hydrophilic glass slides. We attributed this trend to exclusion of water on hydrophobic coatings, which creates a protected environment for glue release thereby enhancing molecular interactions between the glue and the surface. For this project, we also developed a novel assay for assessing the amount of glue

remaining on a surface after barnacle removal. Residual glue after barnacle removal was stained with a general protein stain, the substrate was scanned with an optical scanner, and pixel density was quantified using ImageJ. We found a strong, positive correlation between staining density and adhesion strength. This assay could be useful for experiments where a force gauge might not be available, where the samples do not meet the ASTM standard for measurement of adhesion strength (e.g., if the barnacles are less that 5 mm basal diameter or if the base plate breaks upon removal) or when simply analyzing glue remnants in terms of adhesive characteristics.

Lastly, we leveraged techniques developed through the funded work to assess the effect of environment on shell formation in other crustaceans (specifically juvenile red and blue king crabs). Similar to what was found in *A. amphitrite*, shell mechanical properties were reduced with reduced pH in crabs. Importantly, we found that mechanical properties do not scale directly with mineral abundance, suggesting that the composition and organization of the organic matrix plays a strong role in determining shell mechanical responses.

CONCLUSIONS: Variations in environmental parameters (salinity, pH, and temperature) can significantly affect growth and development of the cosmopolitan barnacle *A. amphitrite*. The effect of salinity was strongly larval batch dependent and may vary based on genetics of the breeding population. Adhesive properties were consistently robust to pH, suggesting that the barnacle controls the pH of the adhesive interface, or that the suite of proteins present in the glue is insensitive to pH. Exposure to reduced pH, though, consistently resulted in an increase in shell growth. At least at the lowest pH assessed (7.5) this increase in shell growth was associated with a decreased in shell toughness. Increased temperature moderated the effect of reduced pH, suggesting an interactive effect of these two stressors. It is yet to be determined if such changes would alter the survival of *A. amphitrite* in the field, but changes in the abundance of this ecologically dominant species would undoubtedly affect the composition of biofouling communities.

SIGNIFICANCE: Overall, the project sheds light on fundamental mechanisms of adhesion and biomineralization in barnacles. It provides specific information how environmental factors affect barnacle growth and development, which may be useful as the Navy assesses conditions that may be favorable in terms of reducing the fouling load. Importantly, the project provided substantial training to students and young researchers (17 in total), prompting many of them to pursue STEM careers.

PATENT INFORMATION: No patents were filed for this project.

AWARD INFORMATION:

- Project PI, Gary H. Dickinson, tenured and promoted to Associate Professor.
- Project PI, Gary Dickinson, served as session co-chair, for a Biofouling Session at Aquaculture 2016 (Triennial Meeting of the National Shellfisheries Association), and currently serves as session chair, for the Macro and Integrative Biology Session at ICMCF 2018, June 24-29, 2018.
- Project PI, Gary H. Dickinson, awarded competitive teaching release time for research ("Support of Scholarly Activities" – SOSA) in 2015 and 2017, and competitive internal funding to support summer research students (TCNJ's MUSE program) in 2017 & 2018 (total funding for these internal award ~$41,000).

- Undergraduate researcher, Shrey Patel, awarded an NREIP internship to work with Chris Spillman's group at the Naval Research Laboratory; Undergraduate researchers Kyle Siegel and Ahmed Mahmoud accepted to summer research programs at Thomas Jefferson University and Stanford University.
- Undergraduate researcher, Kyle Siegel, accepted to a PhD Program at Northwestern University in Interdisciplinary Biological Sciences; Undergraduate researchers Julian Sison and Aparna Yarram accepted to Medical School.
- Undergraduate student Kyle Siegel received a Phi Kappa Phi Honors Society Faculty-Student Research Award, for his ONR related research (June 2016).
- Technician, Conall McNicholl, accepted to a PhD Program at Florida Atlantic University in Marine Science; Technician, Jessica Nardone, accepted to a Master's Program at Tufts University in Ecology, Evolution, and Behavior

PUBLICATIONS and ABSTRACTS (for total period of grant):

A. Refereed Journal Articles:

1. Nardone JA, Patel S, Siegel KR, Tedesco D, McNicjoll CG, O'Malley J, Herrisk J, Metzler RA, Orihuela B, Rittschof D, and Dickinson GH. Assessing the impacts of ocean acidification on adhesion and shell formation in the barnacle *Amphibalanus amphitrite*. *Frontiers in Marine Science. In review*.
2. Figueroa MA, Schablik JD, Mastroberte M, Singh LJ, and Dickinson GH. 2017. The effect of hydrophobic alkyl silane self-assembled monolayers on adult barnacle adhesion. *Marine Technology Society (MTS) Journal*. 51: 39-48.
3. Coffey WD, Nardone JA, Yarram A, Long WC, Swiney KM, Foy RJ, and Dickinson GH. 2017. Ocean acidification leads to altered micromechanical properties of the mineralized cuticle in juvenile red and blue king crabs. *J. Exper. Marine Biology and Ecology.* 495: 1-12.
4. Dickinson GH, Yang X, Wu F, Orihuela B, Rittschof D and Beniash E. 2016. Localization of phosphoproteins within the barnacle adhesive interface. *Biological Bulletin.* 230: 233-242.
5. Stafslien SJ, Sommer S, Webster DC, Bodkhe R, Pieper R, Daniels J, Wal LV, Callow MC, Callow JA, Ralston E, Swain G, Brewer L, Wendt D, Dickinson GH, Lim CS and Teo SLM. 2016. Comparison of laboratory and field testing performance evaluations of siloxane-polyurethane fouling-release marine coatings. *Biofouling.* 8: 949-968.
6. Lim CS, Dickinson GH**,** Sommer S, Teo SLM, Bodkhe R, Webster DC and Loo YY. 2015. A small scale waterjet test method for screening novel foul-release coatings *J. Coatings Technology & Research.* 12: 533-542.

B. Books or Chapters

1. Sokolova IM, Matoo OB, Dickinson GH, and Beniash E. Physiological effects of ocean acidification on animal calcifiers. 2016. In: Stressors in the marine environment: physiological and ecological responses and societal implications, Solan M and Whiteley N, Eds. Oxford University Press, Oxford, UK. Pp. 36-55.

C. Workshop and Conference Participation/Proceedings

1. Dickinson GH, Nardone JA, Patel S, Siegel KR, Tedesco D, McNicholl CG, O'Malley J, Herrick J, Metzler RA, Orihuela B, Rittschof D. 2018. Assessing the impacts of ocean acidification on adhesion and shell formation in the barnacle *Amphibalanus amphitrite*. 47th Benthic Ecology Meeting, Corpus Christi, TX.

2. Patel S, Spillman CM, Wang C, Schultzhaus J, Dickinson GH, Orihuela B, Rittschof D. 2018. Building a barnacle cement protein profile using immunohistochemistry. 47th Benthic Ecology Meeting, Corpus Christi, TX.
3. Figueroa M and Dickinson GH. 2017. Characterization of adult barnacle adhesion upon reattachment to hydrophobic surfaces. American Vacuum Society 64th International Symposium, Tampa, FL.
4. Siegel KR, Nardone JA, Tedesco D, Patel S, Karra L, Orihuela B, Rittschof D and Dickinson GH. 2017. Examining the interactive effects of salinity and ocean acidification on the physiology of the barnacle *Amphibalanus amphitrite*. 46th Benthic Ecology Meeting, Myrtle Beach, SC.
5. Tedesco D, Nardone JA, Siegel KR, Patel S, Orihuela B, Rittschof D, Dickinson GH. 2017. Effects of ocean acidification and temperature on biomineralization and adhesion in the barnacle, *Amphibalanus amphitrite*. 46th Benthic Ecology Meeting, Myrtle Beach, SC.
6. Patel S, Nardone JA, Tedesco D, Siegel KR, Orihuela B, Rittschof D and Dickinson GH. 2017. Determining the impacts of ocean acidification on shell mechanics and structure in the barnacle *Amphibalanus amphitrite*. 46th Benthic Ecology Meeting, Myrtle Beach, SC.
7. Dickinson GH, Nardone JA, McNicholl CG, Siegel KR, Tedesco D, Patel S, Orihuela B, and Rittschof D. 2016. Barnacle adhesion and biomineralization in a changing ocean: assessing the effects of seawater salinity and pH. 18th International Congress on Marine Corrosion and Fouling, Toulon, France.
8. Nardone JA, McNicholl CG, Siegel KR, Tedesco D, Orihuela B, Rittschof D and Dickinson GH. 2016. Evaluating the effects of environment on barnacle adhesion and biomineralization. 45th Benthic Ecology Meeting, Portland, ME.
9. Dickinson GH, McNicholl CG, Nardone JA, Siegel KR, Tedesco D, Khan T, Orihuela B, and Rittschof D. 2016. Assessing the effects of salinity and acidification on barnacle adhesion, growth and biomineralization. Aquaculture 2016, Triennial Meeting of the National Shellfisheries Association, 108th Annual Meeting, Las Vegas, NV.
10. Figueroa MA, Shablik J, and Dickinson GH. 2016. Structural effects of hydrophobic coatings on barnacle adhesion. Aquaculture 2016, Triennial Meeting of the National Shellfisheries Association, 108th Annual Meeting, Las Vegas, NV.
11. Siegel KR, McNicholl CG, Tedesco D, Nardone JA, Orihuela B, Rittschof D and Dickinson GH. 2016. Effects of salinity on adhesive and shell properties in the barnacle *Amphibalanus amphitrite*. 45th Benthic Ecology Meeting, Portland, ME.
12. Tedesco D, Nardone JA, Siegel KR, McNicholl CG, Orihuela B, Rittschof D and Dickinson GH. 2016. Ocean acidification affects shell formation but not adhesion in the barnacle Amphibalanus amphitrite. 45th Benthic Ecology Meeting, Portland, ME.
13. Dickinson GH, McNicholl CG, Orihuela B, and Rittschof D. 2015. Assessing the effect of salinity on barnacle adhesion and biomineralization. 107th National Shellfisheries Association Annual Meeting, Monterey, CA.
14. McNicholl CG, Orihuela B, Rittschof D, and Dickinson GH. 2015. Assessing the effect of environment on barnacle adhesion and biomineralization. 3rd U.S. Ocean Acidification PI Meeting, Woods Hole, MA.
15. Sison J, Orihuela B, Rittschof D, and Dickinson GH. 2015. Characterizing Key Proteins that Contribute to Barnacle Adhesion. 107th National Shellfisheries Association Annual Meeting, Monterey, CA.

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Assessing the Impacts of Ocean Acidification on Adhesion and Shell Formation in the Barnacle Amphibalanus amphitrite

Jessica A. Nardone[1], Shrey Patel[1], Kyle R. Siegel[1], Dana Tedesco[1], Conall G. McNicholl[1], Jessica O'Malley[2], Jack Herrick[2], Rebecca A. Metzler[2], Beatriz Orihuela[3], Daniel Rittschof[3], Gary H. Dickinson[1*]

[1]Department of Biology, The College of New Jersey, United States, [2]Department of Physics and Astronomy, Colgate University, United States, [3]Marine Science and Conservation, Duke University Marine Lab, Nicholas School of the Environment, United States

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

JN developed the seawater exposure system, collected and compiled data on seawater chemistry, physiology and adhesive metrics, and oversaw daily operations of the exposure. SP assessed shell thickness, mechanical properties, and crystallinity. KS and DT collected data on mortality, egg presence, adhesive properties, and shell mass and area. CM developed a prototype seawater exposure system and initiated a pilot study. JM, JH, and RM prepared shells and collected data on atomic disorder and RM analyzed atomic disorder data. BO and DR provided juvenile barnacles and algae and provided expertise on barnacle growth and assessment throughout the exposure. GD conceived of the experiments, analyzed final datasets, oversaw the experiment, and wrote the manuscript. All authors contributed to editing of the manuscript.

Keywords

Barnacle, Adhesion, Biomineralization, ocean acidification, Shell formation, Mechanical Properties, Amphibalanus amphitrite, Balanus amphitrite

Abstract

Word count: 350

Barnacles are dominant members of marine intertidal communities. Their success depends on firm attachment provided by their proteinaceous adhesive and protection imparted by their calcified shell plates. Little is known about how variations in the environment affect adhesion and shell formation processes in barnacles. Increased levels of atmospheric CO2 have led to a reduction in the pH of ocean waters (i.e. ocean acidification), a trend that is expected to continue into the future. Here, we assessed if a reduction in seawater pH, at levels predicted within the next 200 years, would alter physiology, adhesion, and shell formation in the cosmopolitan barnacle Amphibalanus amphitrite. Juvenile barnacles, settled on silicone substrates, were exposed to one of three levels of pHT, 8.01, 7.78 or 7.50, for 13 weeks. We found that barnacles were robust to reduced pH, with no effect of pH on physiological metrics (mortality, tissue mass, and presence of eggs). Likewise, adhesive properties (adhesion strength and gross morphology of the adhesive plaque) were not affected by reduced pH. Shell formation, however, was affected by seawater pH. Shell mass and base plate area were higher in barnacles exposed to reduced pH; barnacles grown at pHT 8.01 exhibited approximately 30% lower shell mass and 20% smaller base plate area as compared to those at pHT 7.50 or 7.78. Enhanced growth at reduced pH appears to be driven by the increased size of the calcite crystals that comprise the shell. Despite enhanced growth, mechanical properties of the base plate (but not the parietal plates) were compromised at the lowest pH level. Barnacle base plates at pHT 7.5 broke more easily and crack propagation, measured through microhardness testing, was significantly affected by seawater pH. Other shell metrics (plate thickness, relative crystallinity, and atomic disorder) were not affected by seawater pH. Hence, a reduction in pH resulted in larger barnacles but with base plates that would crack more readily. It is yet to be determined if such changes would alter the survival of A. amphitrite in the field, but changes in the abundance of this ecologically dominant species would undoubtedly affect the composition of biofouling communities.

Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

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This study was carried out in accordance with standard procedures for invertebrates.

Assessing the Impacts of Ocean Acidification on Adhesion and Shell Formation in the Barnacle *Amphibalanus amphitrite*

Jessica A. Nardone[1], Shrey Patel[1], Kyle R. Siegel[1], Dana Tedesco[1], Conall G. McNicholl[1], Jessica O'Malley[2], Jack Herrick[2], Rebecca A. Metzler[2], Beatriz Orihuela[3], Daniel Rittschof[3], and Gary H. Dickinson[1*]

[1]Department of Biology, The College of New Jersey, 2000 Pennington Rd., Ewing, NJ 08628, USA

[2]Department of Physics and Astronomy, Colgate University, 13 Oak Dr., Hamilton, NY 13346, USA

[3]Duke University Marine Laboratory, Marine Science and Conservation, 135 Duke Marine Lab Rd., Beaufort, NC, 28516

*Corresponding author: Gary H. Dickinson, dickinga@tcnj.edu

Keywords: barnacle, adhesion, biomineralization, ocean acidification, shell formation, mechanical properties, *Amphibalanus amphitrite*, *Balanus amphitrite*

ABSTRACT

Barnacles are dominant members of marine intertidal communities. Their success depends on firm attachment provided by their proteinaceous adhesive and protection imparted by their calcified shell plates. Little is known about how variations in the environment affect adhesion and shell formation processes in barnacles. Increased levels of atmospheric CO_2 have led to a reduction in the pH of ocean waters (i.e. ocean acidification), a trend that is expected to continue into the future. Here, we assessed if a reduction in seawater pH, at levels predicted within the next 200 years, would alter physiology, adhesion, and shell formation in the cosmopolitan barnacle *Amphibalanus amphitrite*. Juvenile barnacles, settled on silicone substrates, were exposed to one of three levels of pH_T, 8.01, 7.78 or 7.50, for 13 weeks. We found that barnacles were robust to reduced pH, with no effect of pH on physiological metrics (mortality, tissue mass, and presence of eggs). Likewise, adhesive properties (adhesion strength and gross morphology of the adhesive plaque) were not affected by reduced pH. Shell formation, however, was affected by seawater pH. Shell mass and base plate area were higher in barnacles exposed to reduced pH; barnacles grown at pH_T 8.01 exhibited approximately 30% lower shell mass and 20% smaller base plate area as compared to those at pH_T 7.50 or 7.78. Enhanced growth at reduced pH appears to be driven by the increased size of the calcite crystals that comprise the shell. Despite enhanced growth, mechanical properties of the base plate (but not the parietal plates) were compromised at the lowest pH level. Barnacle base plates at pH_T 7.5 broke more easily and crack propagation, measured through microhardness testing, was significantly affected by seawater pH. Other shell metrics (plate thickness, relative crystallinity, and atomic disorder) were not affected by seawater pH. Hence, a reduction in pH resulted in larger barnacles but with base plates that would crack more readily. It is yet to be determined if such changes would alter the survival of

A. amphitrite in the field, but changes in the abundance of this ecologically dominant species would undoubtedly affect the composition of biofouling communities.

INTRODUCTION

Barnacles are dominant members of marine biofouling communities throughout much of the world's oceans. They settle and tenaciously adhere to nearly any inert surface in the marine environment, including ship hulls and maritime facilities, and once established can serve as a substrate for less tenacious species. This results in a tremendous cost burden for Naval and maritime industries in the form of coating application, cleaning and maintenance, as well as lost operational time (Callow and Callow, 2011; Schultz et al., 2011).

The success of Balanomorph barnacles depends on the firm attachment provided by their secreted adhesive and the protection imparted by their heavily calcified outer shell plates. Adult barnacles adhere using a largely proteinaceous glue which forms adhesive bonds with surfaces and cures (Walker, 1972; Naldrett, 1993; Kamino et al., 2000; Kamino, 2008). The glue is comprised of at least ten major proteins, which are thought to play differing but specific roles in the adhesion process, including displacement of water from the substratum, integrating the cement with the base plate, adsorption to the substratum, assembly, and curing (Kamino, 2016; So et al., 2016). Shell calcification in juvenile barnacles occurs soon after metamorphosis (LeFurgey et al., 1995; Metzler et al. *in prep*). In the genus *Amphibalanus*, barnacles are protected by six parietal (i.e. lateral) shell plates that sit atop a calcified basal plate (Pitombo, 2004). Shell plates are composed of calcite, held within a matrix of chitin, acidic proteins, and sulfate-rich polymers (Fernandez et al., 2002; Rodriguez-Navarro et al., 2006; Khalifa et al., 2011). The base plate, and a narrow uncalcified growth region, are glued to the substrate. The calcified base plate is composed of layered structures, with grain size of calcite crystallites increasing with distance from the glue layer (Lewis et al., 2014; De Gregorio et al., 2015).

Although we are beginning to understand the biochemical mechanisms involved in barnacle adhesion and shell formation, relatively little is known about how variations in the environment, for example in seawater pH, affect barnacle adhesion and shell formation. This topic is particularly relevant given current and predicted changes in the pH of ocean waters, a process known as ocean acidification (OA). Resulting from the absorption of CO_2 by the world's oceans, the pH of global surface waters has decreased by 0.1 pH units since the industrial revolution and is projected to drop a further 0.3 – 0.5 pH units by the year 2100 (Caldeira and Wickett, 2003; Doney et al., 2009). Such changes will be more extreme in coastal regions, due to a decreased buffering capacity of coastal waters and biological CO_2 production (Waldbusser et al., 2011; Baumann et al., 2015). Effects of reduced seawater pH or associated changes in carbonate chemistry (i.e. reduced calcium carbonate saturation states) have previously been found to affect adhesion and shell formation in other marine invertebrates. For example, in marine mussels reduced pH led to diminished attachment strength and changes in the expression of proteins that comprise the byssus and adhesive plaques (O'Donnell et al., 2013; Zhao et al., 2017). Alterations in calcification, growth, and shell properties resulting from decreased pH or calcium carbonate saturation states have been documented in a broad range of calcifying organisms (Kroeker et al., 2010; Kroeker et al., 2013).

Given the general sensitivity of protein conformation to pH, and evidence of a reduction in marine mussel attachment with reduced pH, we hypothesized that the barnacle adhesive system is sensitive to seawater pH. Specifically, we predicted that barnacles grown under different levels of pH would vary in: 1) adhesive strength, and 2) gross morphology of the adhesive plaque (i.e.

whether the adhesive plaque was thin and transparent, or thick and opaque when grown on silicone: Berglin and Gatenholm, 2003; Wiegemann and Watermann, 2003; Holm et al., 2005). The effect of reduced pH (to pH_{NBS} 7.4) on barnacle adhesion strength was tested previously in the barnacle *Amphibalanus amphitrite* by McDonald et al. (2009), but in this experiment, barnacles were grown on glass and all barnacles broke upon removal. Therefore, force recordings reflected mechanical properties of the shell rather than adhesive properties per se. Here, we assessed if pH affects barnacle adhesion strength when barnacles were grown on silicone coatings and measured following the ASTM "Standard test method for measurement of barnacle adhesion strength in shear" (ASTM, 2005). In this test, barnacles whose shells break during removal are excluded from analysis, and therefore force measurements are solely dependent on properties of the adhesive bond with the substrate and cohesive properties of the glue itself.

Interestingly, McDonald et al. (2009) observed an overall increase in exoskeleton calcification at pH_{NBS} 7.4 as compared to the ambient level of 8.2, despite weakened mechanical strength of the parietal plates in barnacles exposed to low pH. We predicted that alterations in the shell formation and maintenance processes in barnacles would occur even under more moderately reduced seawater pH. Specifically, we tested the shell size, mass, and plate thickness of barnacles exposed to seawater at moderately reduced pH_T levels of 7.78 and 7.50, values that are predicted global averages for oceanic surface waters in the years ~2100 and ~2200, respectively, and compared this to a current average oceanic level of pH_T 8.01 (Caldeira and Wickett, 2003; Doney et al., 2009). Shell mechanical properties were assessed in both the base and parietal plates using microhardness testing. Further, using a combination of scanning electron

120 microscopy and FTIR spectroscopy, we tested if alterations in calcification or mechanical
121 properties are driven by changes in ultrastructure, composition, crystallinity, or atomic disorder
122 of the shell plates.
123
124 To address these predictions, we exposed juvenile barnacles, *Amphibalanus (=Balanus)*
125 *amphitrite*, to one of three pH_T levels (8.01, 7.78, or 7.5) for a total of 13 weeks. *A. amphitrite* is
126 a cosmopolitan intertidal species, inhabiting tropical and semi-tropical waters, and both larvae
127 and adults of this species have been found to tolerate reduced pH (McDonald et al., 2009).
128 Therefore, in addition to the adhesion and shell formation metrics described, we also assessed if
129 pH affects the general physiology of *A. amphitrite.* This was done by monitoring mortality
130 throughout the exposure period and assessing tissue mass and the presence of eggs at the
131 conclusion of the experiment. Overall, the goal of this work is to provide a comprehensive
132 assessment of the effect of seawater pH on *A. amphitrite*; alterations in the presence or
133 abundance of this common species could alter the composition of intertidal and biofouling
134 communities.
135

136 **MATERIALS AND METHODS**

137 **Animal collection, larval culture, and experimental exposure**
138 Barnacle larvae were reared from field-collected adult barnacles following the methods of
139 Rittschof et al. (1984). Adult *Amphibalanus amphitrite* were collected from dock pilings at the
140 Duke University Marine Laboratory, Beaufort, NC, USA (34.7° N, 76.7° W). Larval culture was
141 conducted using aged, natural seawater. Barnacle cyprid larvae were settled on T2 silicone-
142 coated glass panels (15.2 x 7.6 cm) on July 22, 2015. Panels with settled barnacles were placed

individually in 1 L plastic bins (12.5 x 10.5 x 10.5 cm) filled with aged seawater. Barnacles were fed daily with a mix of *Skeletonema costatum* and *Dunaliella tertiolecta.* At 11 days post-settlement, barnacles were shipped overnight mail to The College of New Jersey (TCNJ; Ewing, NJ, USA). Immediately upon arrival, panels with juvenile barnacles were held individually in 1 L plastic bins filled with Artificial Seawater (Instant Ocean, mixed to a salinity of 35). At TCNJ barnacles were fed daily with *Dunaliella tertiolecta,* and brine shrimp (*Artemia sp.*) were added to their diet at approximately 15 days post settlement. At approximately 30 days post-settlement, barnacles were large enough to feed exclusively on brine shrimp and addition of *Dunaliella tertiolecta* was discontinued. Throughout the experiment, a small number of barnacles were randomly culled to prevent overgrowth.

Panels with juvenile barnacles were randomly assigned to one of three target pH_T treatments, 8.01, 7.78 or 7.50, with a total of 8 panels per pH treatment. Replicate panels were split evenly between two, replicate 5 gallon glass aquaria per pH treatment. Panels were placed into their assigned pH treatment on August 19, 2015 (26 days post-settlement). For each aquarium, a modified polypropylene test tube rack was used to hold the panels in place in an upright position. To prevent accumulation of organic matter in tanks and to enhance feeding, panels were removed from the aquaria once a day, 6 days a week, for feeding. Panels were placed individually in 1 L plastic bins (12.5 x 10.5 x 10.5 cm) that had been filled with seawater from the specific aquarium from which that panel had been taken. Barnacles were allowed to feed for approximately an hour per day and were always fed in excess. The presence of dead barnacles and those that had fallen off of panels was recorded during each feeding period and immediately removed from the panels. For the first week of the exposure, when barnacles were fed with both

algae and brine shrimp, water contained in feeding bins was discarded after feeding and this volume (~ 4 L) was replaced with freshly mixed seawater. Once barnacles were feeding exclusively on brine shrimp, seawater from two of the four bins per aquarium was run through a 180 μm mesh filter to remove brine shrimp and particulate matter and added back to the respective tank. Seawater from the other two bins per aquarium was discarded and this volume (~ 2 L) was replaced with freshly mixed seawater. In all cases, new artificial seawater was added at the beginning of the feeding interval, to enable equilibration of seawater to the pH treatment.

Throughout the experimental exposure aquarium water was tested weekly for ammonia, nitrite and nitrate using a saltwater aquarium test kit (API Saltwater Master Kit, Mars Fishcare North America). Levels of all nitrogen cycle components remained at negligible levels (detected at 0 ppm) through the first 12 weeks of the experimental exposure. Within the last week of the experiment, nitrate levels rose slightly in most aquaria. At this point, half of the water from each aquarium was replaced with freshly mixed seawater, and after every remaining feeding, water from all four bins per aquaria was discarded and replaced with fresh seawater. The experimental exposure lasted for a total 13 weeks (91 days).

Adjustment and monitoring of seawater conditions

Experimental exposures at TCNJ were run in artificial seawater (Instant Ocean), mixed to a salinity of 35. Seawater was prepared in 32 gallon LLDPE (linear low density polyethylene) garbage cans, and continuous mixing was accomplished using a 1300 GPH aquarium pump (Marineland Maxi-Jet Pro water pump). Since Instant Ocean is formulated with total alkalinity (TA) above what is found in natural seawater, TA was reduced to ~2200 μmol kg^{-1} SW by

addition of 12 M HCl (Lunden et al., 2014). The value of ~2200 μmol kg^{-1} SW was chosen to approximate typical TA values in Beaufort, NC where the barnacle broodstock was collected. The TA of artificial seawater was measured following SOP 3b (Dickson et al., 2007) on an automated titrator (Hanna Instruments, HI902) with 0.1 M HCl (Fluke #35335, certified volumetric) as a titrant, and values were checked against certified reference material from the Dickson Laboratory (Scripps Institution of Oceanography, La Jolla, CA). All TA samples were run at least in duplicate.

Target pH and temperature levels were achieved using an automated aquarium control system (Apex AquaController, Neptune Systems), which functioned as both a pHstat and thermostat. Temperature was held at 25°C for all aquaria. Each aquarium was equipped with a temperature probe (Neptune Systems, Extended Life Temperature Probe) and a 50-watt submersible aquarium heater (Aqueon 06105 Pro). Heaters were activated through the aquarium control system when aquarium temperature dropped below the 25°C set point. Room temperature was always kept well below 25°C, preventing water temperature from rising substantially above this level. Each tank was also equipped with a pH probe (Neptune Systems, Lab Grade pH Probe). Seawater pH was brought to the set point for each aquarium (8.01, 7.78, or 7.50) by addition of pure CO_2 gas (AirGas, food grade) or CO_2-free air. A CO_2 regulator with an electronic solenoid (Azoo CO_2 Pressure Regulator) enabled the aquarium control system to release CO_2 when pH exceeded the set point. CO_2-free air was generated using an in-line soda lime column attached to a 1 L/min aquarium air pump (JW Pet Company, Fusion Air Pump 200) and was added to tanks when pH was below the set point. Both CO_2 and CO_2-free air lines were split into two lines, which entered the water at opposite ends of the aquarium and each terminated with an airstone.

Water within each aquarium was continuously circulated using an 80 GPH submersible aquarium pump (Patuoxun 80 GPH Submersible Pump).

Seawater conditions (salinity, pH_T, and temperature) were measured 6 days per week using a handheld multiparameter meter (YSI, Professional Plus). Measurements were taken each day immediately before removing barnacles for feeding. The multiparameter meter was calibrated monthly for salinity using conductivity standards (#3168 & 3169, YSI). pH was calibrated weekly against TRIS and AMP buffers, enabling determination of pH_T following SOP 6a (Dickson et al., 2007). A summary of seawater conditions is provided in Table 1 and reflects multiparameter meter readings. TA was measured weekly as described above. TA measurements were taken immediately after water collection from aquaria. Other components of the carbonate system were calculated using CO2Calc (Robbins et al., 2010). Calculations incorporated the CO_2 constant of Lueker et al. (2000), KSO_4^- constant of Dickson (1990), total boron of Lee et al. (2010), and air-sea flux of Wanninkhof (1992).

To ensure consistency of temperature and pH monitoring among replicate aquaria, the aquarium control system was calibrated against the multiparameter meter readings. Temperature values were consistent to within 0.05°C among replicate tanks. If necessary, an offset was programmed for individual aquaria using the aquarium control system's software to ensure that control system readings matched that of the multiparameter meter. The aquarium control system would not allow calibration of pH probes with TRIS and AMP buffers. Instead, NBS buffers were used (#3821, 3822, & 3823, YSI) and an offset was programmed for each aquarium to ensure that aquarium control system readings matched pH_T readings of the multiparameter meter.

235

236 **Adhesion and growth assessments**

237 Adhesion strength in shear (critical shear stress) was measured in all barnacles after 13 weeks
238 exposure. Adhesion testing followed ASTM D 5618-94 (ASTM, 2005). Barnacles were removed
239 from panels using a hand-held digital force gauge (Shimpo, FGE-5X). Force values were
240 discarded if damage to the barnacle shell or to the silicone panel occurred during removal. To
241 enable determination of base plate area, base plate diameter was measured on each barnacle in
242 two dimensions (along and perpendicular to the operculum) using a digital caliper. Removal
243 force values were normalized to base plate area. The height of each barnacle was also measured
244 with a digital caliper as the perpendicular distance from the bottom of the base plate to the top of
245 the highest parietal plate. Lastly, the presence or absence of gummy, opaque glue (as described
246 in Berglin and Gatenholm, 2003; Wiegemann and Watermann, 2003; Holm et al., 2005) was
247 recorded for each barnacle.

248

249 Following assessments, barnacles from each panel were placed in shallow glass finger bowls
250 with seawater taken from the aquarium in which that panel had been held. Within 72 hours of
251 removal, barnacles were individually removed from water and dissected to remove the soft body
252 from the shell plates. Opercular plates were removed from the soft tissue and were not included
253 in subsequent analyses. Dissected barnacle bodies were placed individually on pre-weighed
254 pieces of weigh paper, allowed to dry for 48 hours at room temperature and then dried in a
255 vacuum oven at 45°C, 25 in. Hg. Dried barnacle bodies were weighed individually on an
256 analytical balance with 0.02 mg precision (Metler-Toledo, XSE105DU) to determine tissue
257 mass. During dissections, the presence or absence of eggs within the mantle cavity was recorded.

Eggs, if present, were removed from the mantle cavity but were not included in tissue mass measurements. Remaining barnacle shells (base and all parietal plates) were then cleaned thoroughly with water to ensure all tissue had been removed. Shells were dried overnight at room temperature, then dried in a vacuum oven at 45°C, 25 in Hg, for 24 hours, and then weighed individually on an analytical balance with 0.02 mg precision. Visibly damaged shells were not included in shell mass measurements.

Structural and mechanical assessments

All barnacle shells were inspected after shell mass measurements using a stereomicroscope (Leica, S8Apo) and any damage to the base plate that had not been observed by eye (e.g. micro-scale cracks or holes) was recorded. Two undamaged barnacle shells per panel (16 per pH treatment) were then randomly selected for structural and micromechanical assessments. These assessments required embedding and polishing of the shell as shown in Fig. 1. Individual shells were secured to the bottom of a 1.25 in. mounting cup (#198-10000, Allied High Tech Products) using superglue (Loctite, Control Gel). Shells were oriented with the opercular axis parallel to, and the base plate perpendicular to the bottom of the mounting cup. This orientation enabled a cross-section of both the base and parietal plates to be produced as successive layers of epoxy were removed during grinding and polishing. Mounting cups were filled with a two-part epoxy (#145-20000, Allied High Tech Products), which cured overnight. Embedded samples were ground to the mid-line of the barnacle on a manual grinding/polishing machine (Allied High Tech Products, M-Prep 5). Grinding and polishing followed the procedure described in Coffey et al. (Coffey et al., 2017).

Thickness of barnacle shell plates was measured on polished shell cross-sections using an upright reflected light microscope (Zeiss AxioScope.A1) equipped with a digital camera (Zeiss Axiocam 105 color). Images were taken of the entire shell cross-section at 2.5 X magnification. Typically, this required 2-4 images per sample, depending on the size of the barnacle. Using the camera's analysis software (Zeiss Zen 2), a 100 x 100 μm grid was placed on each image. Thickness of the base or parietal plate was measured at each point the grid crossed the plate, resulting in about 40 measurements per shell plate. For the base plate, replicate measurements were averaged to determine the mean base plate thickness for each sample. At least two separate parietal plates were visible in each sample, and measurements were taken within all plates. Replicate measurements were combined among parietal plates, and values were averaged to determine the mean parietal plate thickness for each sample.

Micromechanical properties were quantified using a microindentation hardness tester (Mitutoyo HM-200). Indents were made at 20 g load, 5 seconds dwell time. Testing was conducted in the base and one of the parietal plates, with 10 replicate indents made in each plate. For the base plate, 5 indents were made on each side of the plate, spaced about 200 μm apart, starting at approximately 500 μm from the distal edge of the plate on each of the two sides (Fig. 1A). Replicate indents were typically made throughout the length of a single parietal plate. Individual indents were measured directly on the hardness tester at 50 X magnification in two dimensions, and Vickers microhardness values were automatically calculated. An image of each indent was taken on the hardness tester using a digital microscope camera (Moticam 2.0MP), which enabled quantification of crack propagation (Fig. 1B). Crack length was determined as the radius of a circle emanating from the center of the indent and encompassing all visible cracks. Replicate

measurements within a shell plate were averaged to determine the mean microhardness and crack propagation for each shell plate.

SEM imaging, elemental analysis, and calcite crystal area assessment

After micromechanical testing, scanning electron microscopy (SEM) imaging was conducted on all polished shell cross-sections. Imaging was conducted on uncoated samples, at low vacuum (50 Pa), in back-scattered electron mode on a field emission SEM (Hitachi America, SU5000). An accelerating voltage of 15 kV was used at a working distance of approximately 8 μm. For each sample, images were taken within two separate regions of the base plate, and two regions of one of the parietal plates. In all cases, images were taken in close proximity (typically within 100 μm) to the indents made during micromechanical testing. Images were taken at 1,000 and 5,000 X magnification.

Elemental analysis was conducted on all samples at 1,000X magnification using an EDAX EDS detector (AMTEK Materials Analysis Division, Octane Plus). Imaging conditions resulted in a count rate of 5,000-10,000 counts per second. For each region, a total of five point spectra were taken across the region. Replicate spectra were averaged within and between the two regions per plate, to determine the mean elemental composition for each shell plate.

Individual calcite crystals were readily resolved at 5000 X magnification (Fig. 1C). The area of individual calcite crystals was determined on SEM images using image analysis software (ImageJ, Ver. 1.49). For each image, a total of 15 different calcite crystals were randomly selected for area determination. For selection, a 2 μm^2 grid was first placed on the image. Pairs

of random numbers were generated using a random number generator, and each pair was used as coordinates to identify a specific calcite crystal on the image. The perimeter of each identified crystal was traced by hand using the polygon tool in ImageJ and area within the traced region was automatically determined. One SEM image per shell plate was assessed, and replicate area measurements within the image were averaged to provide a mean crystal area for each shell plate.

FTIR analysis

FTIR spectroscopy was used to assess: 1) the polymorph of calcium carbonate present in barnacle shells; 2) relative crystallinity of shells and; 3) atomic disorder of shells. Spectra were collected for polymorph and relative crystallinity determination using a PerkinElmer Spectrum Two spectrometer and for atomic disorder using a PerkinElmer Spectrum 100 spectrometer. In all cases, individual barnacle shells that had been cleaned of soft tissue were powdered using a mortar and pestle. For these assessments, the base and all parietal plates were included for each sample, but the opercular plates had been removed. Powdered sample was placed directly on the instrument's ATR crystal and compressed to a uniform force with a built-in anvil. Spectra were taken at 4 wavenumbers resolution, with 32 scans per sample. Spectra were normalized and baseline corrected within the 600–2000 cm^{-1} region. The ratio of ν_2 to ν_4 peak absorbance was used as a measure of crystallinity of the shells (Beniash et al., 1997). For atomic disorder assessments, each sample was ground into a coarse powder for the first spectrum. Following the first spectrum the sample was then ground with the mortar and pestle to make a slightly finer powder, and a second spectrum was acquired. This process was carried out 5-8 times, depending on how much the spectrum changed after each subsequent grind. Each spectrum was ATR

corrected and background removed before peak height measurements were acquired following Regev et al. (2010).

Statistical analysis

Statistical analyses were conducted using SPSS (V. 23, IBM Analytics). Categorical data (mortality, presence of eggs, expression of gummy glue) were assessed using chi-square tests. All other data were assessed using one-way ANOVA followed by Tukey HSD post-hoc testing. Prior to analyses, outliers were calculated for all metrics as values greater than three times the interquartile range above or below the third or first quartile, respectively, and were removed from the dataset. Following removal of outliers, assumptions of normality and equal variance were assessed using Shapiro-Wilk and Levene tests, respectively, and data were log transformed if necessary to meet these assumptions. If assumptions of normality or equal variance could not be met after log transforming data, a non-parametric Kruskal-Wallis test was used in place of the parametric ANOVA. In all cases, individual barnacles within a pH treatment were pooled among panels and tanks and treated as individual replicates. Testing for both panel and tank effects was conducted for metrics yielding a significant response to pH. No significant panel or tank effects were found.

RESULTS

Seawater chemistry

A summary of seawater chemistry over the 13 week exposure is provided in Table 1. pH targets were met in all treatments throughout the duration of the exposure. As expected, pCO_2 increased

with decreasing pH. Seawater remained supersaturated with respect to calcite for all treatments. Total alkalinity tended to be higher in pH 7.50 aquaria as compared to those at pH 7.78 and 8.01.

Physiology

Mortality of barnacles was low and variable throughout the experimental exposure and was not significantly influenced by pH treatment (chi-square: $p > 0.05$). Cumulative mortality was 21.0, 25.3 and 14.7% for the 7.50, 7.78, and 8.01 pH treatments, respectively. The value for the 8.01 treatment excludes a single panel in which 85% of barnacles died during the third week of the exposure. The reason for this die-off is unknown, but given that this level of mortality was not observed in any of the other panels, it is unlikely that this was driven by pH treatment.

Barnacles were dissected at the conclusion of the experiment, enabling quantification of soft body tissue mass and identification of eggs within the mantle cavity. The effect of pH treatment on tissue dry mass was not significant (Table 2; Fig. 2). At the conclusion of the experiment, nearly all barnacles had eggs within the mantle cavity (96.4, 95.2 and 94.6% of barnacles in the 7.50, 7.78, and 8.01 pH treatments, respectively); the proportion of ovigerous barnacles was not affected by pH treatment (chi-square: $p > 0.05$).

Adhesion

Adhesion strength, measured as critical shear force, was assessed after the 13 week exposure. Adhesion strength was not affected by pH treatment (Table 2; Fig. 3). Likewise, the proportion of barnacles expressing opaque, gummy glue was not affected by pH treatment (chi-square: $p >$

0.05; Fig. 3). Gummy glue was found on the base of most barnacles, with 72.9, 80.8 and 62.9% of barnacles exhibiting gummy glue in the 7.50, 7.78, and 8.01 pH treatments, respectively.

Shell formation

Assessments of shell growth and materials properties were conducted following adhesion assays. Exposure pH was found to significantly affect barnacle shell mass and the area of the base plate (Table 2; Figs. 2 & 4). Barnacles grown at pH 8.01 exhibited approximately 30% lower shell mass and 20% smaller base plate area as compared to barnacles grown at pH 7.50 or 7.78. Height of the barnacles, measured from the base plate to the highest parietal plate, was not affected by exposure pH (Table 2; Figs. 4). Shell thickness was measured in both the base and parietal plates on polished shell cross-sections (see Fig. 1C). Exposure pH did not significantly influence thickness in either the base or parietal plates. SEM imaging of polished cross-sections revealed a cobblestone like composite of calcite crystals (Fig. 1). Exposure pH was found to significantly alter calcite crystal area in both the base and parietal plates (Table 2; Fig. 4). In the base plate, calcite crystals were approximately 95% smaller in barnacles grown at pH 8.01 as compared to barnacles grown at pH 7.50 or 7.78. The difference in calcite crystal area was less pronounced in the parietal plates, but on average calcite crystals were 35 and 23% smaller in barnacles grown at pH 8.01 as compared to barnacles grown at pH 7.50 or 7.78, respectively.

Qualitative assessments of barnacle base plates following adhesion testing and dissection suggested that damage to the base plate (identified by the presence of cracks or holes) occurred more often in barnacles exposed to pH 7.5 as compared to those exposed to pH 7.78 or 8.01. Base plate damage was identified in 38.6% of barnacles grown at pH 7.50 as compared to 21.3%

417 and 18.8% at pH 7.78 and 8.01, respectively. Rigorous assessments of micromechanical
418 properties were conducted on polished shell cross-sections. Mechanical testing revealed that
419 microhardness was not affected by exposure pH in the base or parietal plates (Table 2; Fig. 5).
420 Crack propagation resulting from microindentation, however, was significantly affected by pH
421 when tested in the base plate (Table 2; Fig. 5). Within the base plate, cracks radiating from
422 indents were, on average, 28% longer in barnacles exposed pH 7.50 as compared to those at pH
423 8.01 (Fig. 5; Tukey HSD: $p = 0.058$). Crack propagation was not affected by exposure pH when
424 tested in the parietal plates.
425
426 FTIR spectroscopy was used to assess the polymorph of calcium carbonate present in barnacle
427 shells, as well as their relative crystallinity and atomic disorder. Spectroscopy was conducted on
428 powdered shell samples and both base and all parietal plates were included in each sample. At all
429 pH levels, identified peaks were characteristic of calcite with no other polymorphs of calcium
430 carbonate present (Fig. 6). Relative crystallinity of the shells (assessed as the ratio of the ν_2 to ν_4
431 absorbance) was not affected by pH (Table 2; Fig. 6). Atomic disorder, assessed through FTIR
432 grinding curves, varied among samples and was not significantly affected by pH (Table 2; Fig.
433 6).
434
435 Elemental composition was assessed on polished shell cross-sections using EDS. Eight elements
436 were identified in all samples: in order of abundance, Ca, O, C, S, Sr, Mg, Na, and Cl. The vast
437 majority of the shell was comprised of Ca, O and C, with all other elements occurring at less than
438 1 wt %. Exposure pH did not affect calcium content when assessed in the base or parietal plates
439 (Table 2; Fig. 7). While no elements showed a significant effect of pH, magnesium content

tended to be lower (on average by 19%) in barnacles exposed pH 7.50 as compared to those at pH 8.01 (Fig. 7; Tukey HSD: $p = 0.052$).

DISCUSSION

Barnacles are dominant members of marine intertidal communities and their success depends on both the firm attachment provided by their proteinaceous adhesive and the protection imparted by their calcified shell plates. Here we assessed if a reduction in seawater pH, at levels predicted within the next 200 years (i.e. to pH_T 7.78 and 7.50, based on oceanic projections), would alter physiology, adhesion, and shell formation in the cosmopolitan barnacle *A. amphitrite*. Changes in the abundance of *A. amphitrite* could affect the composition of biofouling communities. We found that barnacles were generally robust to reduced pH, with no effect of pH on physiological metrics, and, contrary to our prediction, adhesive properties were not affected by reduced pH. Shell mass and base plate area were found to be higher in barnacles exposed to even moderately reduced pH, a trend that appears to be driven by increased size of the calcite crystals that comprise the shell. Although microhardness of the shell plates, a measure of resistance to permanent or plastic deformation, was not affected by pH, the length of cracks propagating from indents in the base plate was, suggesting that the toughness of base plates grown at the lowest pH level was reduced. Hence, a reduction in pH resulted in larger barnacles but with base plates that would crack more readily.

Physiology

Over the course of 13 weeks, we found no effect of seawater pH on cumulative mortality, tissue mass, or egg production in *A. amphitrite*. Assessments of ocean acidification responses in

juvenile or adult *A. amphitrite* are limited, but consistent with our observations, McDonald et al. (2009) found no effect of pH on *A. amphitrite* egg production after 11 weeks exposure to pH_{NBS} 7.4. Campanati et al. (2016) exposed *A. amphitrite* larvae to reduced pH (pH_{NBS} 7.6) and tracked survival of juveniles settled from these larvae for 11 days. Mortality was actually reduced at low pH as compared to the pH_{NBS} 8.2 control when assessed at 3 and 9 days post-settlement. At their final time point (11 days post settlement), there was no effect of seawater pH on juvenile survival.

Amphibalanus improvisus (Pansch et al., 2014; Eriander et al., 2016) and *Elminius modestus* (Findlay et al., 2010a) have also been found to be robust to ocean acidification, as least in terms of survival and reproduction. Eriander et al. (2016) found no effect of reduced seawater pH (to pH_{NBS} 7.7) on mortality in *A. improvisus* over 12 weeks, either when the pH reduction was kept at a stable level or when pH was allowed to fluctuate over the course of the day to mimic diurnal pH cycles. Pansch et al. (2014) similarly found no effect of reduced pH on *A. improvisus* mortality or reproduction (larval release rate) after 20 weeks in juveniles collected from a field site that shows natural variability in seawater pH (Kiel Fjord, Germany). In contrast though, juveniles collected from a field site with limited variability in seawater pH (Tjärnö Archipelago, Sweden) showed increased mortality at the lowest pH tested (pH_{NBS} 7.2). Exposure of *Elminius modestus* to reduced pH (pH_{NBS} 7.7) for 30 days did not affect mortality when tested at 14 or 19°C (Findlay et al., 2010a).

Semibalanus balanoides, a boreoarcitic species, appears considerably more sensitive to ocean acidification. Increased mortality has been shown in both short (30 days) and longer term (80 or

104 days) exposures to reduced pH (pH_{NBS} 7.7-7.8)(Findlay et al., 2009; 2010a; Harvey and Moore, 2017). Barnacles in these studies were collected near the southern limit of the range for *S. balanoides*. Similar to what was shown for *A. improvisus*, environmental conditions of the collection site impact responses to ocean acidification (Findlay et al., 2010b). When Findlay et al. (2010) exposed *S. balanoides* collected near the northern limit of their range to reduced pH (to pH_{NBS} 7.7 or 7.3) for 20 days, survival of juveniles did not differ from the pH_{NBS} 8.1 control. Hence, barnacles show a mixed response to seawater pH. Individual responses vary by species as well as within a species based on local conditions of the breeding population.

In general, crustaceans tend to be more tolerant of the effects of ocean acidification as compared to other taxa that build a mineralized shell (e.g. mollusks, corals: Kroeker et al., 2013) and such tolerance has been hypothesized to be due to their capacity for ion and acid-base regulation (Melzner et al., 2009; Whiteley, 2011). Populations that are routinely exposed to varying environmental conditions are likely to exhibit a considerable scope for physiological adjustment to a changing environment, making them well-adapted physiologically to changes in seawater pH (Wong et al., 2011; Pansch et al., 2012; Pansch et al., 2013). In *A. amphitrite* larvae for example, expression of energy metabolism related proteins was altered at reduced pH, illustrating the potential for proteomic plasticity in this robust species, and a possible mechanism for mediating the stress of ocean acidification (Wong et al., 2011). Variations among species and populations may stem from differences in ion and acid-base regulation ability, as well as differences in the ability to adjust energy metabolism (Whiteley, 2011).

Adhesion

We hypothesized that the barnacle adhesive system is sensitive to seawater pH, and based on this hypothesis, tested the prediction that barnacles grown under different levels of pH would vary in adhesive strength and gross morphology of the adhesive plaque. Previous work with marine mussels (*Mytilus trossulus*) found a reduction in both strength and extensibility of byssal threads when animals were exposed to reduced pH (tested at a range of pH_T values from 8.1 to 7.5: O'Donnell et al., 2013). Such changes in mechanical properties of the byssal threads were calculated to lead to a 35-41% reduction in attachment tenacity, which could substantially affect a mussel's ability to anchor itself in high energy environments. O'Donnell et al. (2013) suggest that this response is due to sensitivity of DOPA to pH. DOPA is a major component of byssal threads that is involved in cross-linking and adhesion. A similar response was found in the mussel *Mytilus coruscus* (Zhao et al., 2017). Decreased pH (tested at a range of pH_{NBS} values from 8.1 to 7.4) led to a decrease in byssal thread breaking force and toughness, as well as a reduction in the number of threads produced. Incorporating mechanical data with thread counts per mussel, Zhao et al. (2017) predicted a 60-65% reduction in attachment tenacity. Reduced seawater pH also led to significant alteration in expression of byssal thread related genes, thereby providing a mechanism that, in combination with direct effects of pH on DOPA chemistry, could explain the response of byssal threads to reduced pH.

Data collected here did not support the hypothesis that the barnacle adhesive system is sensitive to seawater pH; neither adhesive strength (measured in shear following the ASTM standard for measurement of barnacle adhesive strength: ASTM, 2005) nor the gross morphology of the adhesive plaque (i.e. if the glue layer was thin and hard or thick and gummy) were affected by seawater pH. A number of factors may contribute to the observed difference in sensitivity to pH

between marine mussels and the barnacle (*A. amphitrite*) tested here. First, the Balanomorph barnacle adhesive system is fundamentally different from that of marine mussels in that the adhesive interface is relatively protected from the external environment. Glue is delivered directly to the substrate at the periphery of the base and parietal plates (Saroyan et al., 1970; Burden et al., 2014). Although the cured glue layer is partially hydrated (Barlow et al., 2009), there is no component of the adhesive system that is constantly exposed to seawater, as is the case for a mussel's byssal threads. Second, the chemistry of barnacle glue differs from that of marine mussels. Barnacle glue is composed of at least ten major proteins, which play differing but specific roles in the adhesion process (Kamino, 2016; So et al., 2016). DOPA, which is pH sensitive (O'Donnell et al., 2013), has not been identified in barnacle glue (Naldrett, 1993; Kamino et al., 1996; Naldrett and Kaplan, 1997), although evidence of oxidative activity and cross-linking has been found (Dickinson, 2008; Golden et al., 2016; Essock-Burns et al., 2017; So et al., 2017). The sensitivity of isolated barnacle glue proteins to altered pH has yet to be assessed. Last, reduced seawater pH may lead to changes in the suite of glue proteins expressed, either in terms of the specific proteins expressed or the relative abundance of these proteins. Such a mechanism has not been tested in barnacles, but hypothetically could compensate for altered structure and activity of individual glue proteins. Recent advances in sequencing of the barnacle glue proteome (Wang et al., 2015; So et al., 2016) will enable direct assessment of proteomic responses to varied seawater conditions.

In our study, barnacles were settled on silicone substrates, which enabled them to be removed intact. Individuals with broken shells were excluded from analysis, and therefore the response to pH described here reflects only adhesive and cohesive properties of the adhesive plaque. When

555 barnacles were settled on hydrophilic glass beakers, the force required to shear the barnacles
556 from the glass was actually enhanced in barnacles exposed to reduced pH (McDonald et al.,
557 2009). In this case, all barnacles broke upon removal. On hydrophilic substrates, mechanical
558 properties of the shell plates are weaker than the adhesive bond between the glue layer and the
559 substrate, and therefore shear removal measurements reflect integrity of the lower shell plates
560 where the force is applied. McDonald et al. (2009) suggest that thickening of the growing edge
561 of the barnacle was responsible for the observed difference in shear removal force. Clearly
562 surface chemistry, and hence whether failure will occur within the adhesive layer or within the
563 shell plates, will mediate the effect of seawater pH on attachment tenacity. Assessments of
564 barnacle responses to varied pH when grown on substrates that naturally occur in the marine
565 environment may shed light on if ocean acidification will affect attachment ability in the field.
566

567 **Shell formation**

568 Consistent with what was found previously for *A. amphitrite* (McDonald et al., 2009), reduced
569 seawater pH resulted in elevated shell formation. We found that even a moderate reduction in
570 seawater pH (to pH_T 7.78) resulted in greater shell mass as compared to the pH_T 8.01 control.
571 After 13 weeks growth, barnacle base plates were larger at reduced pH. Thickness of shell plates,
572 measured directly on cross-sectioned shells, was not affected by seawater pH, although thickness
573 of the parietal plates did tend to increase with decreasing pH. Enhanced shell formation at
574 reduced seawater pH has been documented in several other crustaceans (i.e. *Callinectes sapidus*,
575 *Penaeus plebejus*, *Homarus americanus*: Ries et al., 2009). Given that bicarbonate ion
576 concentration increases under ocean acidification (increased pCO_2), enhanced growth in
577 crustaceans may stem from their ability to utilize bicarbonate in the mineralization process

578 (Cameron and Wood, 1985; Whiteley, 2011; Roleda et al., 2012). Enhanced growth though, is
579 not universal in crustaceans (e.g. mixed growth responses have been observed in *A. improvisus*
580 and *S. balanoides*: Findlay et al., 2010b; Pansch et al., 2014) and likely depends on an
581 individual's capacity to mitigate the stress of reduced pH (see physiology section above).
582
583 Scanning electron microscopy imaging of cross-sectioned barnacle shells revealed that the shell
584 plates were composed of crystals, with dimensions on the order of 1 μm. FTIR spectroscopy
585 confirmed that these crystallites were composed of calcite with no other polymorphs of calcium
586 carbonate present, and EDS spectroscopy identified both magnesium and strontium within the
587 calcitic shell plates. Consistent with previous assessments of *A. amphitrite* shells, individual
588 crystallites were irregular in shape, did not take on a uniform orientation, and the boundaries of
589 larger crystals appear rough, suggestive of smaller crystallites on the surface of the larger
590 crystals (Khalifa et al., 2011; Lewis et al., 2014). The organic matrix surrounding calcite crystals
591 is composed of chitin, acidic proteins, and sulfate-rich polymers (Fernandez et al., 2002;
592 Rodriguez-Navarro et al., 2006; Khalifa et al., 2011), and in *A. amphitrite* comprises
593 approximately 2 wt% of the shell (Khalifa et al., 2011). We did not assess the organic matrix
594 specifically in this study, but observations of a double peak at 1145 cm^{-1} in the FTIR spectrum
595 (taken on whole, crushed shells) are suggestive of sulfate-rich polymers within the organic
596 matrix (Khalifa et al., 2011).
597
598 Although seawater pH did not alter the overall shape or orientation of calcite crystals, the size of
599 individual crystals increased dramatically in barnacles grown at reduced seawater pH. Calcite
600 crystals comprising the base plate were nearly twice as large in barnacles at reduced pH (pH_T

601 7.50 or 7.78) as compared to the pH_T 8.01 control. Seawater pH resulted in a graded response in
602 parietal plates, with the largest calcite crystals at the lowest seawater pH, a trend that closely
603 followed parietal plate thickness. Shell formation in barnacles is directed by cells of the mantle
604 epithelium, which participate in deposition of organic matrix and calcium transport (Nousek,
605 1984; Fernandez et al., 2002; Rodriguez-Navarro et al., 2006; Gohad et al., 2009). Crystal
606 nucleation, structure, and orientation of calcite crystals is proposed to be controlled by the
607 organic matrix (Fernandez et al., 2002; Khalifa et al., 2011).
608
609 At present, the mechanisms driving such differences in calcite crystal size are unclear. Increased
610 size of calcite crystals under reduced seawater pH may reflect differences in the process of
611 organic matrix deposition by mantle cells or the rate by which matrix deposition occurs. Similar
612 to what was observed here, an increase in the size of shell microstructures (folia) was observed
613 in eastern oysters (*Crassostrea virginica*) when exposed to pH_{NBS} 7.5 for 20 weeks (Beniash et
614 al., 2010). In this case it was proposed that energy limitations could impede organic matrix
615 deposition or cell division. Here though, we did not observe an effect of seawater pH on *A.*
616 *amphitrite* soft tissue mass, and therefore it does not appear as though animals at reduced pH
617 were functioning under an energy deficit. An alternative hypothesis is that reduced seawater pH
618 led to differences in intracellular pH, which could alter the ability of cells to participate in the
619 mineralization process. In another crustacean (the Tanner crab, *Chionoecetes bairdi*), a reduction
620 in intracellular pH was observed in animals held at reduced pH, a response that was proposed to
621 have implications on the shell formation process (Meseck et al., 2016). Direct assessments of
622 intracellular pH in barnacles grown at reduced pH as well as further investigation into the role of

623 cells in the shell formation process in barnacles would be helpful in evaluating these
624 mechanisms.
625
626 Changes in the shell formation process under reduced pH may have implications in terms of
627 functionality of the shell. The base plates of barnacles grown at the lowest pH tested (pH_T 7.50)
628 tended to break more easily than those of barnacles grown at pH_T 7.78 or 8.01. Rigorous
629 mechanical testing supported this observation. When tested in the base plate, hardness, a
630 material's ability to resist plastic or permanent deformation, was not affected by pH, but the
631 cracks that propagated from mechanical tests were longer at low pH, indicating that reduced
632 seawater pH led to a reduction in toughness. This response may partially be driven by the
633 dramatic increase in calcite crystal size at low pH. Larger crystals would imply a lower ratio of
634 organic to inorganic material within the shell. Organic matrix plays an important toughening role
635 in biological materials, serving to trap and deflect cracks (Fratzl et al., 2007; Beniash et al.,
636 2010; Meyers and Chen, 2014). Therefore, as the ratio of organic matrix to inorganic mineral
637 decreases, crack deflection ability is also diminished. This mechanism, however, cannot fully
638 explain the trends observed, given that animals at pH_T 7.78 showed larger calcite crystal size but
639 did not exhibit a reduction in base plate toughness.
640
641 Several additional factors could contribute to the observed reduction in base plate toughness.
642 Assessment of the shell organic matrix was beyond the scope of this study, but given the ability
643 of the organic matrix to trap and deflect cracks (Fratzl et al., 2007; Meyers and Chen, 2014)
644 alterations in the composition or density of the organic matrix could also lead to changes in
645 toughness. Considering the calcite crystals themselves, two factors could have influenced

mechanical properties. First, organic constituents (e.g. amino acids) can be occluded within biogenic calcite crystals and can dramatically impact mechanical properties via alterations in the crystal lattice (Cho et al., 2016; Kim et al., 2016). If the identity or quantity of these organic inclusions was altered at low pH, this could influence shell mechanical properties. Second, inclusion of magnesium into the calcite crystal lattice can have a major impact on mechanical properties of shells, with the addition of even small amounts of magnesium leading to enhanced mechanical properties (Kunitake et al., 2012; Long et al., 2014). Correspondingly, in the base plate of pH_T 7.50 barnacles, we observed a trend ($p = 0.056$) toward decreased magnesium content as compared to animals at pH_T 7.78 or 8.01. Such a reduction in magnesium content could contribute to the observed reduction in toughness. At this point it is unclear if the reduced magnesium content at low pH is due to lower uptake and incorporation of magnesium, or increased dissolution of weekly bound magnesium (Findlay et al., 2009).

A reduction in the force needed to break parietal plates was observed previously in *A. amphitrite* (McDonald et al., 2009) and *A. improvisus* (Pansch et al., 2013) after exposure to reduced pH. Force needed to break parietal plates was not assessed in this study, but at the micro-scale, we did not observe an effect of seawater pH on parietal plate hardness or crack propagation. Hence differences in force needed to break parietal plates may stem from structural changes in the plates (e.g. local dissolution of mineral: McDonald et al., 2009), rather than differences in their material properties. We did not observe damage or erosion of parietal plates for any of the treatments in this study, although alkalinity was consistently elevated at pH_T 7.50, suggesting that some dissolution of shells at this pH may have occurred.

669 Neither shell crystallinity nor atomic disorder, both measures of the level of structural order
670 within a crystal at the atomic scale, were affected by seawater pH. Changes in formation
671 conditions or the composition of molecules at the time of crystal formation could affect these
672 metrics (Khalifa et al., 2011). Interestingly, although the mean values of these metrics were not
673 significantly affected by seawater pH, variance around the mean was dramatically greater at
674 reduced pH as compared to pH_T 8.01 (e.g. for atomic disorder, standard error at reduced pH was
675 4-5 times greater than at pH_T 8.01). This suggests that reduced pH may increase variability
676 among individuals in their ability to control the environment in which mineral forms. A similar
677 response in terms of increased variability in crystal properties among individuals at decreased pH
678 was observed previously in the marine mussel, *Mytilus californianus* (McCoy et al., 2018).
679

680 **CONCLUSIONS**

681 A reduction in seawater pH at levels predicted within the next 200 years (i.e. to pH_T 7.78 and
682 7.50, based on oceanic projections: Caldeira and Wickett, 2003; Doney et al., 2009) had little
683 impact on physiological and adhesive metrics in the barnacle *A. amphitrite*. Shell growth,
684 though, was significantly enhanced at reduced pH, while toughness of the base plate was
685 diminished at pH_T 7.50. If these changes impact the survival of *A. amphitrite* in the field is yet to
686 be determined, but alterations in the abundance of this ecologically dominant species would
687 undoubtedly affect the composition of biofouling communities. Given the economic impact of
688 marine biofouling (Callow and Callow, 2011; Schultz et al., 2011), additional assessments of *A.*
689 *amphitrite* under changing environmental conditions are warranted. Multi-stressor and
690 transgenerational assessments, as well as experiments that test natural sources of mortality in

691 barnacles (e.g. predators or hydrodynamic stresses) would be especially helpful in predicting
692 population level responses in *A. amphitrite.*
693
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698
699 **AUTHOR CONTRIBUTIONS**
700 JN developed the seawater exposure system, collected and compiled data on seawater chemistry,
701 physiology and adhesive metrics, and oversaw daily operations of the exposure. SP assessed
702 shell thickness, mechanical properties, and crystallinity. KS and DT collected data on mortality,
703 egg presence, adhesive properties, and shell mass and area. CM developed a prototype seawater
704 exposure system and initiated a pilot study. JM, JH, and RM prepared shells and collected data
705 on atomic disorder and RM analyzed atomic disorder data. BO and DR provided juvenile
706 barnacles and algae and provided expertise on barnacle growth and assessment throughout the
707 exposure. GD conceived of the experiments, analyzed final datasets, oversaw the experiment,
708 and wrote the manuscript. All authors contributed to editing of the manuscript.
709
710 **Conflicts of interest statement**
711 The authors declare that the research was conducted in the absence of any commercial or
712 financial relationships that could be construed as a potential conflict of interest.
713

Data availability statement

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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TABLE AND FIGURE LEGENDS

Table 1. Seawater chemistry parameters (means ± SD). pH_T, temperature, and salinity were measured six days per week (n = 76), total alkalinity was measured weekly (n = 12), all other parameters were calculated using CO2Calc.

Table 2. Assessment of the effects of seawater pH on physiology, adhesion, and shell formation in the barnacle *Amphibalanus amphitrite*. ANOVA results are shown for all metrics except shell dry mass, crystallinity, parietal plate thickness, and parietal plate crack propagation, where Kruskal-Wallis tests were applied. Significant p-values are shown in bold.

Fig. 1. A. Assessments of barnacle shell properties were conducted on polished cross-sections of individual barnacle shells, which exposed both the base and parietal plates. **B.** Mechanical testing was conducted using microindentation, which results in a diamond shaped indent and induces crack formation (arrows). Image was taken under polarized light, revealing organization of

calcite crystals. **C.** Shell ultrastructure was assessed using SEM. Shells are comprised of a cobblestone-like network of calcite crystallites, the area of which can be measured using image analysis software.

Fig. 2. Tissue and shell mass of barnacles, *A. amphitrite*, exposed to one of three levels of pH_T for 13 weeks (mean ± s.e.m.). Groups marked with different letters are significantly different as shown by Tukey HSD post-hoc analysis. N is indicated on each bar and represents individual barnacles.

Fig. 3. Adhesion strength (critical shear stress) and gummy glue expression of barnacles, *A. amphitrite*, exposed to one of three levels of pH_T for 13 weeks (mean ± s.e.m.). N is indicated on each bar. For adhesion strength, this represents individual barnacles. Gummy glue expression was calculated as the percent of barnacles per panel with any visible gummy glue.

Fig. 4. Shell structural assessments of barnacles, *A. amphitrite*, exposed to one of three levels of pH_T for 13 weeks (mean ± s.e.m.). Groups marked with different letters are significantly different as shown by Tukey HSD post-hoc analysis. N is indicated on each bar and represents individual barnacles.

Fig. 5. Micromechanical assessments of barnacles, *A. amphitrite*, exposed to one of three levels of pH_T for 13 weeks. Mean ± s.e.m. is shown for microhardness and crack propagation. N is indicated on each bar and represents individual barnacles. pH significantly affected crack

propagation in the base plate (ANOVA: $p < 0.05$). Bottom images show indents and cracks (arrows) for the base plate of a pH 8.01 barnacle (left) and 7.50 barnacle (right).

Fig. 6. Representative FTIR spectra of a powdered barnacle shell with peaks characteristic of calcite (left). The ratio of ν_2 to ν_4 peak absorbance was used as a proxy of crystallinity (center), and FTIR grinding curves were used to assess atomic disorder (right). Mean ± s.e.m. is shown for crystallinity and atomic disorder, and pH did not significantly affect either metric (ANOVA: $p < 0.05$). N is indicated on each bar and represents individual barnacles.

Fig. 7. Calcium and magnesium content of the shells of barnacles, *A. amphitrite*, that were exposed to one of three levels of pH_T for 13 weeks (mean ± s.e.m.). N is indicated on each bar and represents individual barnacles.

975 **TABLES**

976 **Table 1.** Seawater chemistry parameters (means ± SD). pH_T, temperature, and salinity were
977 measured six days per week (n = 76), total alkalinity was measured weekly (n = 12), all other
978 parameters were calculated using CO2Calc.

	Treatment		
	8.01	**7.78**	**7.50**
pH_T	8.01 ± 0.03	7.78 ± 0.04	7.50 ± 0.04
Temperature (°C)	25.0 ± 0.1	25.0 ± 0.1	25.0 ± 0.1
Salinity	35.8 ± 0.5	35.7 ± 0.5	35.8 ± 0.5
pCO_2 (µatm)	412.8 ± 35.5	810.4 ± 119.5	1747.7 ± 263.4
DIC (µmol kg^{-1} SW)	1889.6 ± 41.3	2015.5 ± 49.7	2330.6 ± 92.5
HCO_3^- (µmol kg^{-1} SW)	1687.5 ± 40.5	1871.7 ± 53.5	2203.9 ± 89.8
CO_3^{2-} (µmol kg^{-1} SW)	190.5 ± 14.3	120.9 ± 17.1	77.4 ± 10.4
Total alkalinity (µmol kg^{-1} SW)	2166.6 ± 47.7	2174.7 ± 49.6	2394.1 ± 86.3
$\Omega_{Calcite}$	4.55 ± 0.33	2.89 ± 0.41	1.85 ± 0.25

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983 **Table 2.** Assessment of the effects of seawater pH on physiology, adhesion, and shell formation
984 in the barnacle *Amphibalanus amphitrite*. ANOVA results are shown for all metrics except shell
985 dry mass, crystallinity, parietal plate thickness, and parietal plate crack propagation, where
986 Kruskal-Wallis tests were applied. Significant p-values are shown in bold.

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Parameter	df	Test Statistic	p
Physiology			
Tissue dry mass	134	1.860	0.160
Adhesion			
Adhesive strength	168	1.363	0.259
Shell formation			
Whole shell			
Dry mass	**2**	**8.366**	**0.015**
Height	172	0.938	0.393
Crystallinity	2	1.088	0.581
Atomic disorder	9	1.021	0.408
Base plate			
Area	**171**	**6.016**	**0.003**
Thickness	46	2.019	0.145
Microhardness	47	0.367	0.695
Crack propagation	**46**	**3.244**	**0.049**
Calcite crystal area	**47**	**16.736**	**0.000**
Ca content	47	0.094	0.911
Mg content	47	3.083	0.056
Parietal plate			
Thickness	2	2.642	0.267
Microhardness	47	0.354	0.704
Crack propagation	2	1.108	0.575
Calcite crystal area	**47**	**7.080**	**0.002**
Ca content	47	0.076	0.927
Mg content	47	0.620	0.542

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Figure 1.JPEG

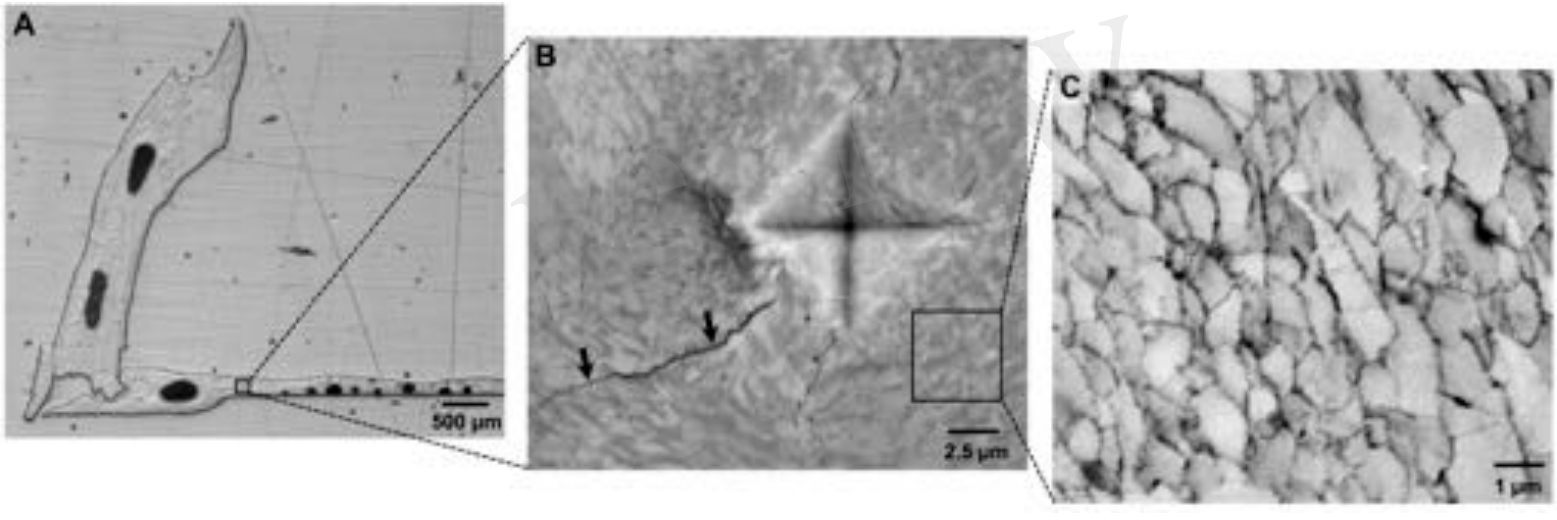
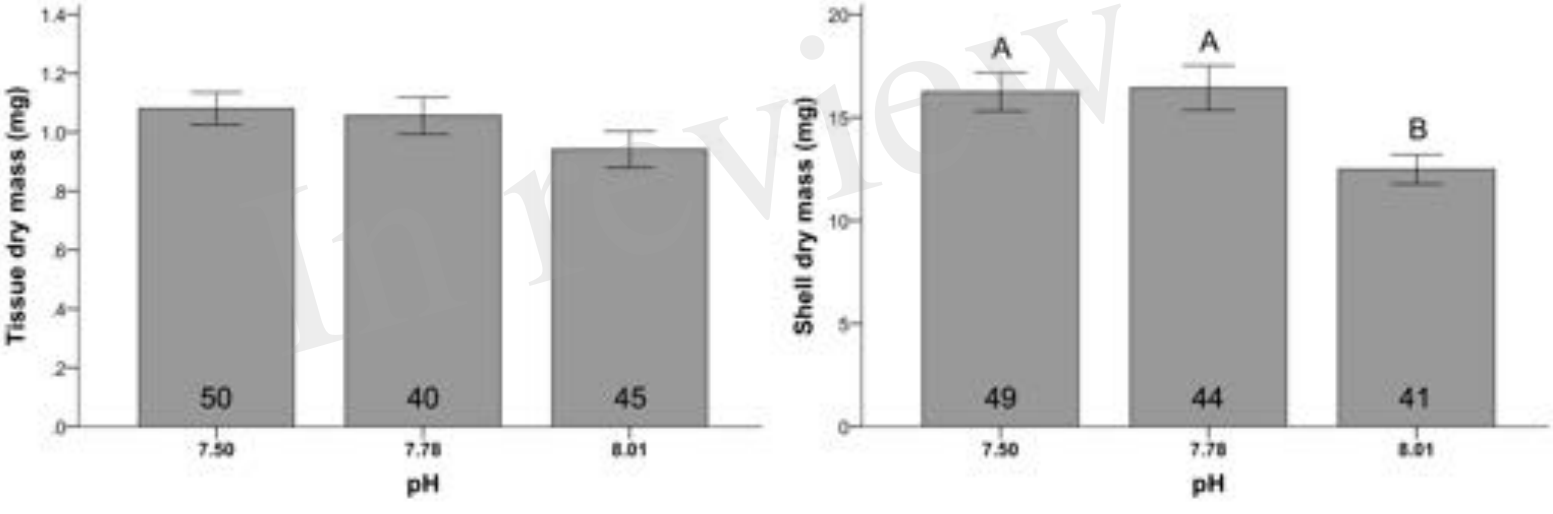


Figure 2.JPEG



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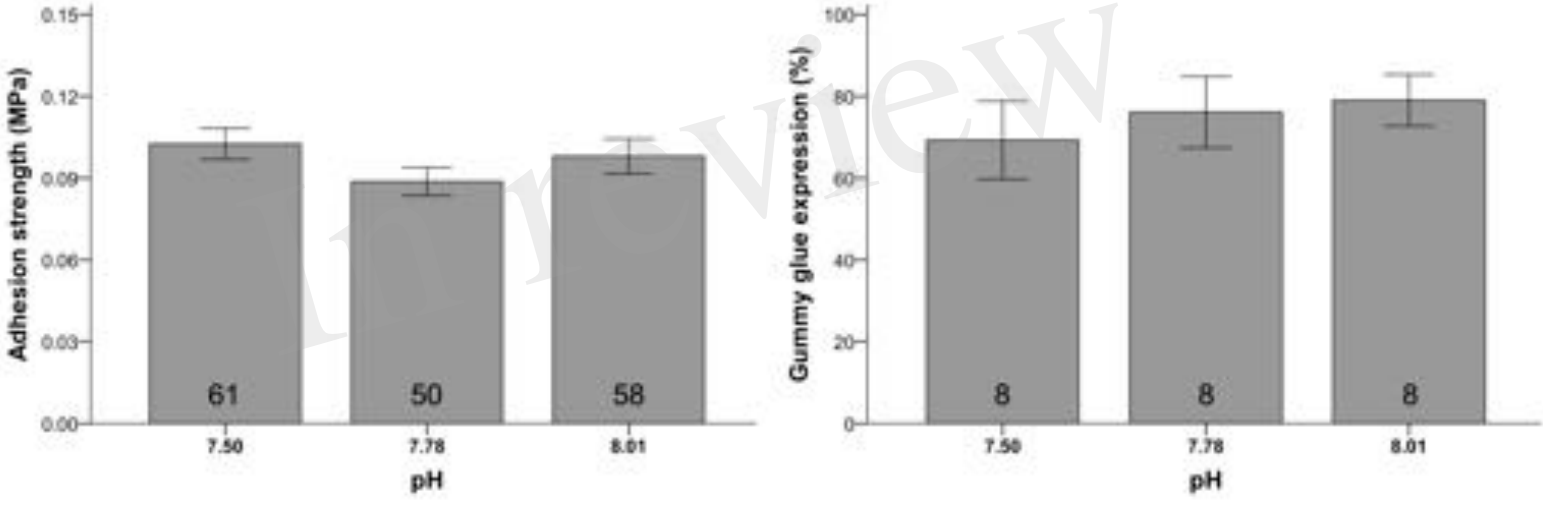

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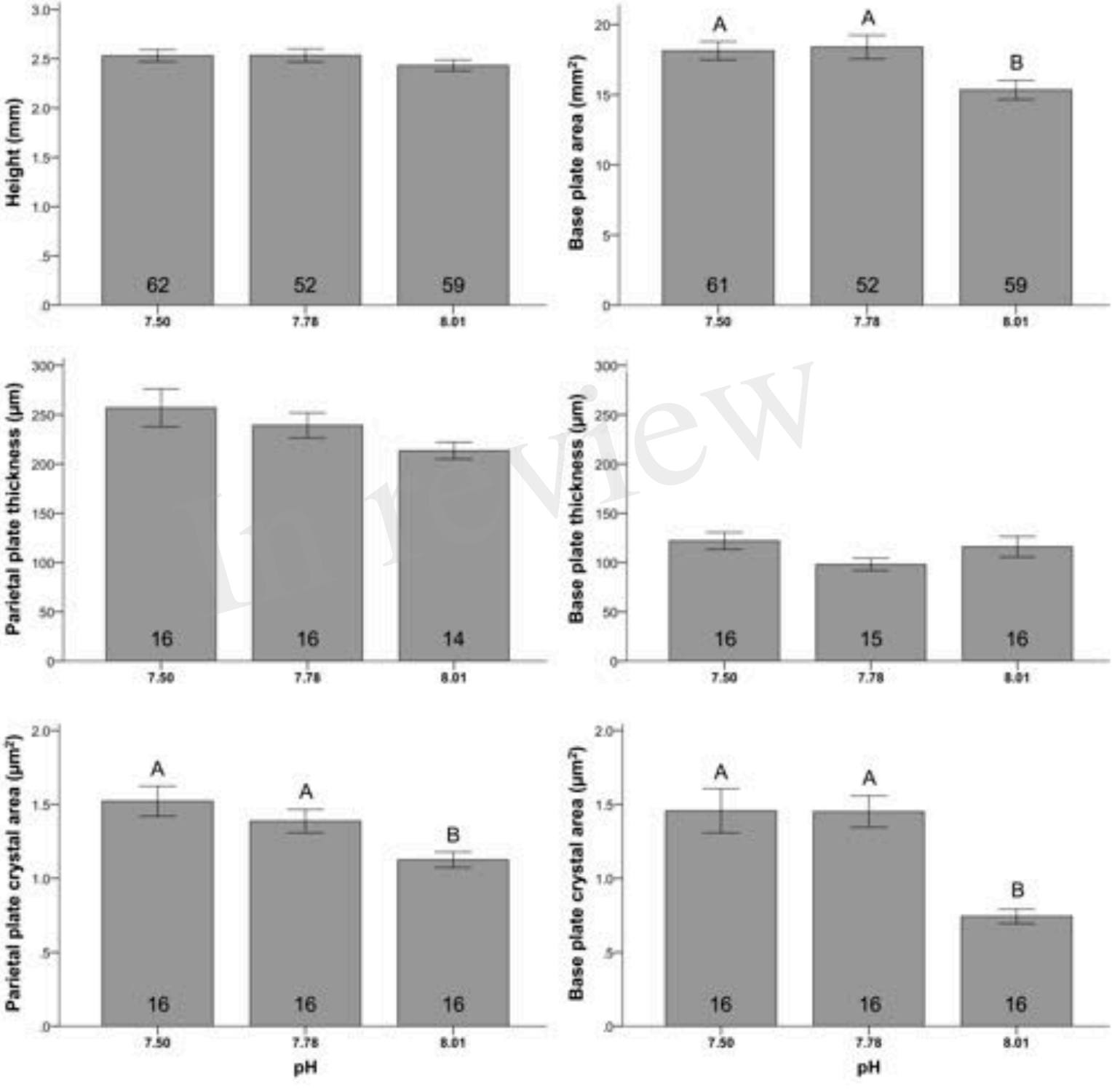

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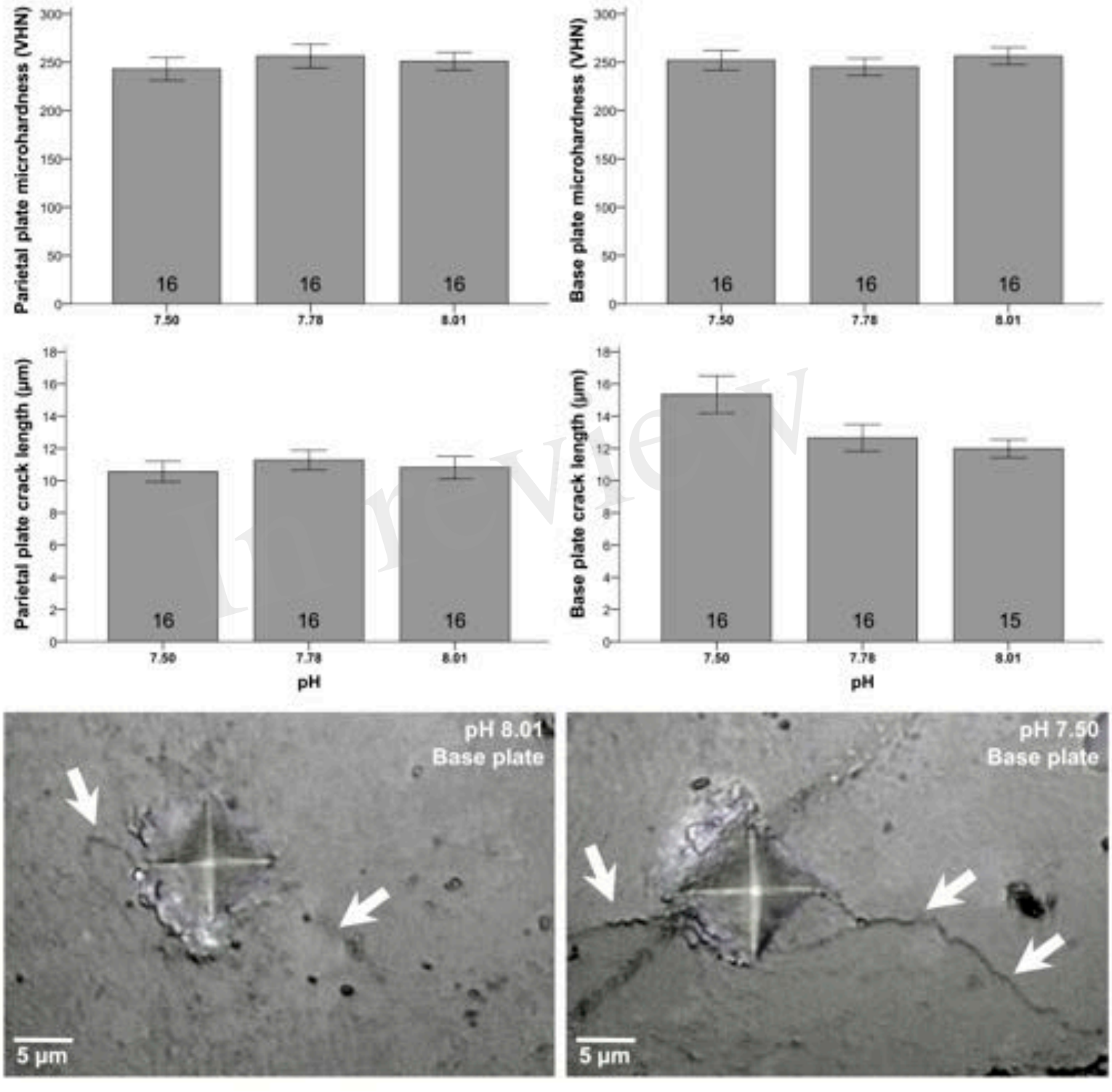

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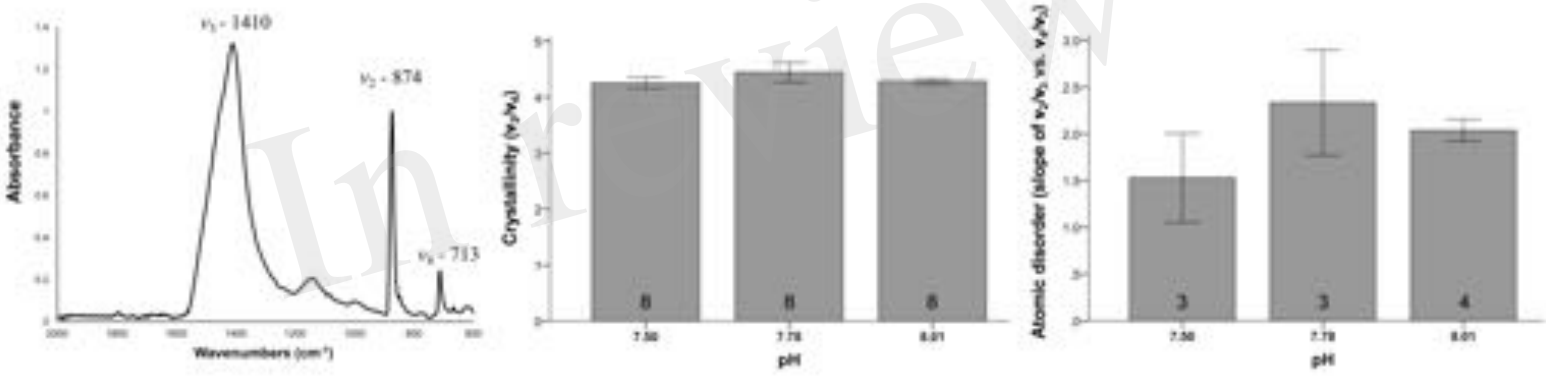
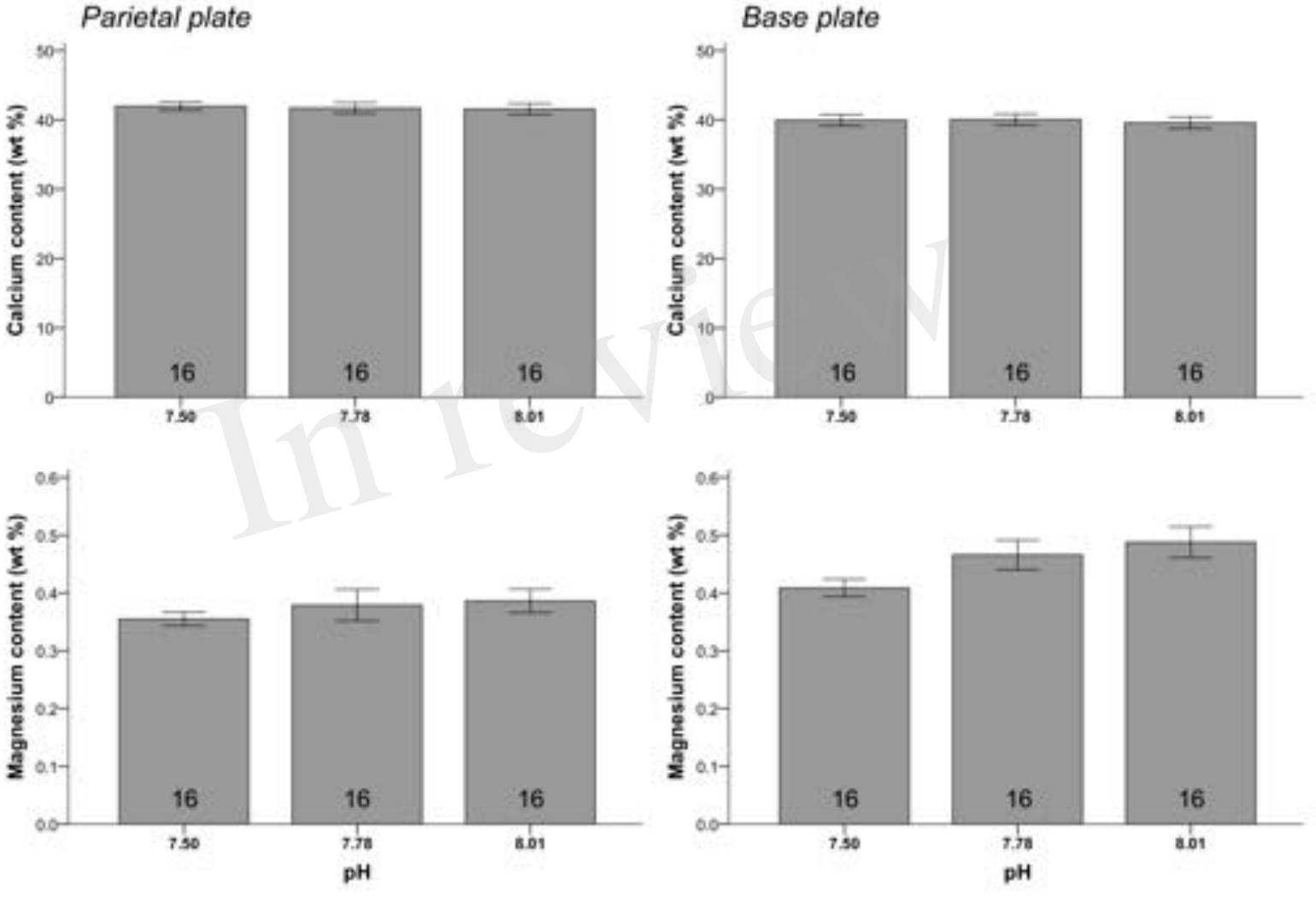


Figure 7.JPEG



source by Oregon Physics that replaced, for the first time, Duoplasmatron on ADEPT1010 Dynamic SIMS System by Physical Electronics.

The FIB sample preparation procedure is discussed in detail and the first back side SIMS results compared to the front side depth profiles.

11:40am **AS+BI+MI-MoM11 Phase Quantification of Mixed TiO_2 Powders by X-ray Photoemission Valence Band Analysis and Raman Spectroscopy**, ***Paul Mack***, *T.S. Nunney,* Thermo Fisher Scientific, UK*; R.G. Palgrave,* University College London, United Kingdom of Great Britain and Northern Ireland

Titanium dioxide is one of the most studied materials in surface science. It has applications in heterogeneous catalysis, dye-sensitised solar cells, bone implants and self-cleaning windows. Many polymorphs of TiO_2 are known to exist but only two occur naturally in abundance: rutile and anatase. Rutile is the more thermodynamically stable form but anatase is more energetically favourable when forming nanoparticles at atmospheric temperature and pressure. The anatase polymorph has been recognised as more photoactive than rutile, although recent research indicates that the greatest photovoltaic efficiencies are achieved in devices that contain a mixture of anatase and rutile. The degree of mixing between two polymorphs influences other material properties, such as catalytic activity. This raises the question: how can one determine the polymorph ratio in a sample that contains a mixture of anatase and rutile?

Quantitative phase analysis of anatase-rutile mixtures by two experimental methods is presented in this work. Spectra of pure reference anatase and rutile were acquired X-ray Photoelectron Spectroscopy (XPS) and Raman spectroscopy. These spectral shapes were then used to fit similar data from mixed phase samples. XPS and Raman spectroscopy give information from different depth regions in a sample. The surface sensitive character of XPS yields a surface phase fraction of anatase and rutile. Mixed phase samples were prepared from high and low surface area anatase and rutile powders. In this work, the surface phase fraction of anatase was found to be linearly correlated with photocatalytic activity of the mixed phase samples, even for samples with very different anatase and rutile surface areas.

Biomaterial Interfaces Division
Room 12 - Session BI-MoM

Engineering a Paradigm Shift in Control of Microbes and Fouling

Moderators: Joe Baio, Oregon State University, Daniel Barlow, US Naval Research Laboratory

8:20am **BI-MoM1 Characterization of Adult Barnacle Adhesion Upon Reattachment to Hydrophobic Surfaces**, ***Manuel Figueroa***, *G. Dickinson,* The College of New Jersey

Although a wide range of environmentally friendly surface coatings can reduce biofouling on marine structures, there is still not a fundamental understanding of barnacle adhesion upon reattachment. This study assessed the effect of hydrophobicity on adhesion in the barnacle *Amphibalanus amphitrite*, an abundant and widespread biofouler. Self-assembled monolayers were made on glass slides from alkyl silanes with methylated and fluorinated terminal groups to produce hydrophobic surfaces. Coated and uncoated glass slides underwent a 2-week barnacle reattachment assay. Barnacles were removed using a force gauge and critical shear stress was calculated for each substrate. Following reattachment assays, a Coomassie Blue G250 protein stain was used to quantify the amount of glue remaining on substrates by measuring pixel density with ImageJ software on glue scans.

Critical shear stress was found to be significantly higher for both hydrophobic surfaces as compared to the hydrophilic uncoated glass, and correspondingly the density of residual glue was higher on hydrophobic surfaces. Given that hydrophobic substrates can exclude water from the surface, they may provide a protected environment for glue release that is favorable for adhesive bond formation with the substrate as well as inter and intramolecular bonding within the glue layer. Critical shear stress showed a strong positive correlation with residual glue density, suggesting barnacle release occurs primarily via cohesive failure. Scanning electron microscope micrographs depicted a diverse mixture of features in the glue remnants depending on the coating and its location under the base plate. These features, which included a sponge-like matrix, globular structures, viscous fingering and nanoscale fibers contribute to adhesion strength. The design of marine coatings must continue to consider the nanoscale topography as an essential attribute to reducing biofouling as well as the ability of a coating to exclude water from the surface.

8:40am **BI-MoM2 Constructing and Deconstructing the Barnacle Adhesive Interface**, *C.R. So, K.P. Fears,* US Naval Research Laboratory*; H. Ryou,* ASEE Research Fellow at US Naval Research Laboratory*; D.E. Barlow, D.H. Leary, J.A. Wollmershauser, C.M. Spillmann,* ***Kathryn Wahl***, US Naval Research Laboratory

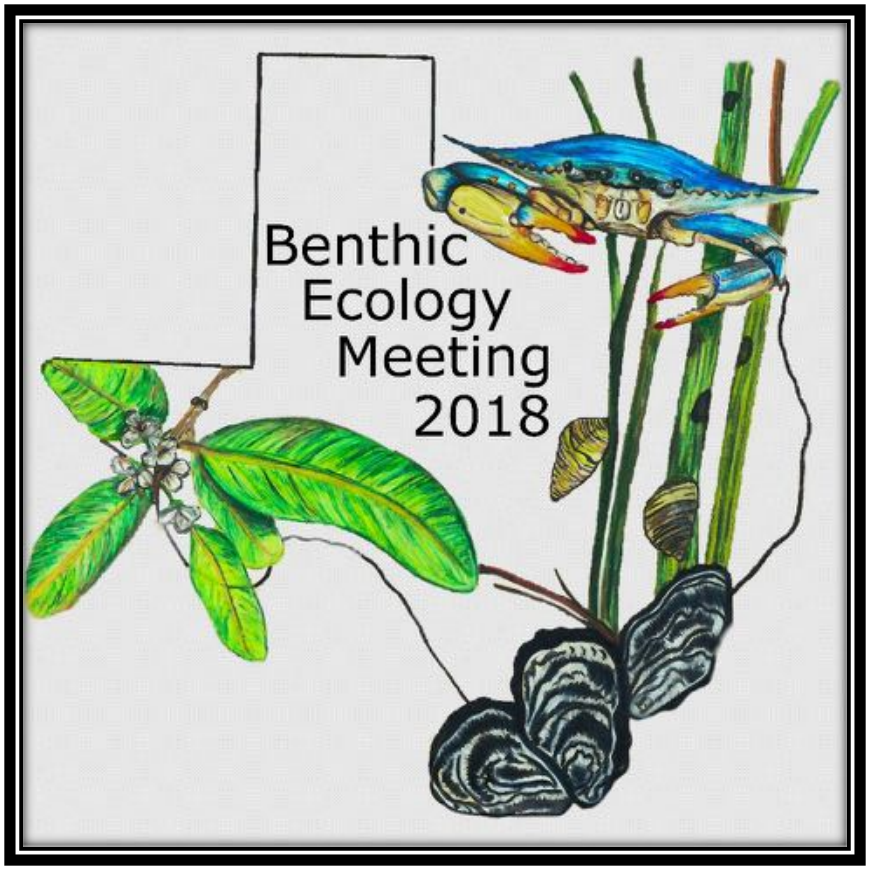
Barnacles are sessile marine arthropods that live and reproduce on nearly any available surface in the ocean. They adhere via a thin adhesive layer developed through a multistep secretory process synchronized with growth via molting. Unlike other arthropods, the combination of expansion, molting and protein secretion within the narrow adhesion interface leads to a nanofibrillar protein layer manipulated by shear stresses, protected by calcite, and containing a cocktail of chemically active molecules and proteins. Here we use *in vivo* imaging, mechanics, and spectroscopy of barnacle growth and development, coupled with mass spectrometry and proteomics to reveal much about the biophysics and biochemistry of barnacle adhesion. We will discuss the role of interfacial processes, self-assembly, amino acid composition, and chemical manipulation in the construction and function of the adhesive.

9:00am **BI-MoM3 Live Confocal Microscopy of *Balanus Amphitrite* Reveals Anti-Fouling Strategy of a Marine Fouler**, ***Kenan Fears***, US Naval Research Laboratory*; B. Orihuela, D. Rittschof,* Duke University Marine Laboratory*; K.J. Wahl,* US Naval Research Laboratory

The adhesion of hard foulers (e.g., barnacles and tubeworms) has plagued the maritime community for as long as mankind has been setting sail. Since the biological processes responsible for adhesion occur at buried interfaces, elucidating the mechanisms by which foulers adhere is challenging. Through the use of multiple fluorescent probes, peptides, and antibodies, we have been able to discern an unprecedented level of detail about biological processes that occur at the interface between acorn barnacles (Balanus Amphitrite) and the underlying substratum during the barnacle growth cycle. Barnacles secrete a lipidaceous substance around the outside of their shell, prior to expansion that dislodges microorganisms and biofilms to present a cleaned surface. During molting, epithelia cells build a new interfacial cuticular layer, which becomes autofluorescent as it is sclerotized, above the existing cuticle whose degradation coincides with the exuviation of the main body's cuticle. Rather than being directly secreted onto the substrate, nanostructured barnacle cement accumulates in the space in between the new and old cuticle. As the barnacle expands, the cuticular layers are stretched and pulled around the outside of the side plate. The strain causes the old cuticle to randomly tear, allowing the new cuticle to deposit cement into the interface as it is dragged across the substrate. Furthermore, antibody staining allowed us to spatially and temporally identify where different cement proteins are presenet. These results illustrate that the methodologies we have developed to break down and analyze barnacle cement collection are yielding a more accurate representation of the proteins at the buried interface, and providing insight on their roles which will lead to improved strategies to both combat and mimic barnacle adhesives.

9:20am **BI-MoM4 Considering the Consequences of a Paradigm Shift in Biofouling Management**, ***Daniel Rittschof***, *B. Orihuela,* Duke University*; K. Efimenko, J. Genzer,* NC State University

Present Fouling Management Strategies that use long-lived, broad-spectrum biocides are not sustainable because they alter ecosystem services and threaten food security. As globalization continues, human populations increase and wild fisheries collapse there will be increasing pressure and genuine need for less environmentally damaging approaches. A question that should be asked up front for any new fouling management technology is what are the environmental, food security and human health consequences if a technology gains market share. Information on impacts of industrial grade components, acute and chronic toxicity, breakdown and non-toxic biological effects such as teratogenicity, carcinogenicity and environmental steroid activity should be evaluated. This presentation looks at a few of the details of basic silicone coatings which have had their components purified and then tested for acute toxicity, impacts on a hydrolytic enzyme and teratogenicity. Some components like catalysts and small cyclics are extremely toxic. Other components impact enzyme activity, some inhibit activity other potentiate activity. Terratogenicity assays are so sensitive that even effects of medical grade silicones can be demonstrated. This information needs to be taken as preliminary factual information that can be used by engineers in developing risk benefit analysis.



47TH Annual Benthic Ecology Meeting

ABSTRACT BOOK

March 27-30, 2018
Corpus Christi, TX

Hosted by:
Harte Research Institute for Gulf of Mexico Studies,
Texas A&M University – Corpus Christi

TEXAS A&M UNIVERSITY
CORPUS CHRISTI

Assessing the impacts of ocean acidification on adhesion and shell formation in the barnacle *Amphibalanus amphitrite*

Dickinson, G. H.*[,1]; Nardone, J. A.[1]; Patel, S.[1]; Siegel, K. R. [1]; Tedesco, D.[1]; McNicholl, C. G.[1]; O'Malley, J.[2]; Herrick, J.[2]; Metzler, R. A.[2]; Orihuela, B.[3]; Rittschof, D.[3]
[1]Department of Biology, The College of New Jersey
[2]Department of Physics and Astronomy Colgate University
[3]Duke University Marine Laboratory, Marine Science and Conservation

Barnacles are dominant members of intertidal communities. Their success depends on firm attachment provided by their adhesive and protection imparted by calcified shell plates. We assessed if reduction in seawater pH would alter physiology, adhesion, and shell formation in the barnacle *Amphibalanus amphitrite*. Juvenile barnacles were exposed to one of three levels of pH_T, 8.01, 7.78 or 7.50, for 13 weeks. We found that barnacles were robust to reduced pH, with no effect of pH on mortality, tissue mass, or egg presence. Likewise, adhesive strength and morphology were not affected by reduced pH. Shell formation, however, was affected by seawater pH. Shell mass and base plate area were higher in barnacles exposed to reduced pH. Enhanced growth at reduced pH was driven by increased size of calcite crystals that comprise the shell. Despite enhanced growth, barnacles grown at pH_T 7.5 had base plates that broke more easily, suggesting reduced shell toughness. Shell thickness, crystallinity, and atomic disorder were not affected by pH. It is yet to be determined if such changes would alter survival of *A. amphitrite* in the field, but changes in abundance of this ecologically dominant species would undoubtedly affect composition of biofouling communities. Authors acknowledge ONR funding.
Presenting author contact info: dickinga@tcnj.edu

Direct and indirect effects of disturbance and eutrophication on SW Florida seagrasses

Douglass, J.*; Sang, A.
Florida Gulf Coast University

Shallow estuaries and lagoons along the SW coast of Florida have historically supported extensive seagrass beds, but anthropogenic pressures increasingly threaten these vulnerable habitats. This presentation reviews the status of SW Florida seagrass ecosystems, highlighting alarming declines in the Caloosahatchee Estuary and Estero Bay. The causes of these declines are discussed and categorized as: 1) disturbances stemming from the interaction of extreme weather events and altered watershed hydrology, 2) chronic stresses from nutrient loading and water quality degradation, and 3) indirect effects of these stressors mediated by benthic algae, epifauna, infauna, macrograzers, and microbes. We identify gaps and uncertainties in our current understanding of the mechanisms of seagrass decline in the region, and we describe ongoing and anticipated studies investigating these mechanisms.
Presenting author contact info: jdouglass@fgcu.edu

Building a barnacle cement protein profile using immunohistochemistry

Patel, S.*[,1,2]; Spillmann, C. M.[1]; Wang, C.[1]; Schultzhaus, J.[1]; Dickinson, G. H.[2]; Orihuela, B.[3]; Rittschof, D.[3]

[1] Naval Research Laboratory

[2] Department of Biology, The College of New Jersey

[3] Duke University Marine Laboratory

Acorn barnacles are able to adhere to a range of surfaces using secreted cement. In the barnacle *Amphibalanus amphitrite*, secretion of proteinaceous cement occurs at the periphery of the basal plate through a complex process where biomineralization and cuticle formation occur. Previously, cured cement was partially solubilized and analyzed where 19, 43 and 114 kDa cement proteins were identified and polyclonal antibodies prepared against them. The purpose of this experiment was to determine where these cement proteins are localized within the barnacle. Transverse sections of barnacle tissue were prepared, beginning just above the cement-substrate interface and continuing to the operculum and exposed to antibodies. No cement proteins were found in exterior muscle bundles or penis. Positive staining against Aacp43 was found within testes and in longitudinal canals, which run through parietal plates of the shell. Staining against Aacp19 was found lining the interior side of mantle tissue, and in granular-like structures in longitudinal canals. Antibody for Aacp114 tended to aggregate, so the staining profile was inconclusive. Overall, we demonstrate positive staining against cement proteins in barnacle sections, indicating their presence in areas other than the region in contact with the underlying interface. Authors acknowledge support from NRL. S.P. acknowledges NREIP.

Presenting author contact info: patels111@tcnj.edu

Nutrient loads explains spatial checker boarding of oysters and seagrass along the US Gulf of Mexico coastline

Patrick, C. J.*[,1]; Lister, J.[1]

[1]Department of Life Sciences, Texas A&M University Corpus Christi

While areas of the eastern United States have experienced large declines in biogenic foundation habitat over the last century, the estuaries of the Gulf Coast have some of the best remaining oyster reef habitat in North America and extensive seagrass beds. Using gulf coast estuaries, federal databases and geospatial information about foundation habitats, infauna, estuary characteristics, climate, nutrient loads, and upstream watershed land use were summarized to build a structural equation model of factors driving biogenic habitat distributions ($P = 0.069$, Chi-Square = 30.666, df = 38). Oysters and seagrass covaried among estuaries due to differential responses to nitrogen loads and air temperature. Effects also cascaded to the density and richness of the infaunal assemblages. Nutrient loads predicted water column chl *a*. Oyster coverage was positively related with chl *a*, possibly because of its food value. In contrast, chl *a* reduced water clarity and was negatively related with seagrass coverage. Temperature effects may be a proxy for hyposaline conditions during summer. Oysters were lower in high salinity, while seagrass were not due to successional turnover from *Thallasia testudinum* to *Halodule wrightii* and *Syringodium filiforme.* These patterns demonstrate a macroscale interaction between nutrient loads, climate, and living resources.

Presenting author contact info: christopher.patrick@tamucc.edu