Manuscript submitted to CBP part D, Genomics and Proteomics, special aquaculture issue, September 28th, 2018

Title

*Pacific geoduck (*Panopea generosa*) resilience to natural pH variation*

Authors

Laura H Spencer[a], Micah Horwith[b], Alexander T. Lowe[c], Yaamini R. Venkataraman[a], Emma Timmins-Schiffman[d], Brook L. Nunn[d], Steven B. Roberts[a]

[a]University of Washington, School of Aquatic and Fishery Sciences, 1122 NE Boat St, Seattle, WA 98105, United States
[b]Washington State Department of Natural Resources, 1111 Washington St SE, MS 47027, Olympia, WA 98504, United States
[c]University of Washington, Biological Sciences, 24 Kincaid Hall, Seattle, WA 98105, United States
[d]University of Washington, Genome Sciences, William H. Foege Hall, 3720 15th Ave NE, Seattle, WA 98195, United States

Corresponding Author: Laura H Spencer, lhs3@uw.edu, (206) 919-0736

Declarations of interest: none

Figures: All figures are provided in color.

Suggested reviewers

- Hollie Putnam, University of Rhode Island, hputnam@uri.edu
- Morgan Kelly, Louisiana State University, morgankelly@lsu.edu
- Jerome LePeyre, Louisiana State University, JLapeyre@agcenter.lsu.edu
- Marta Gomez-Chiarri, University of Rhode Island, gomezchi@uri.edu
- Charlotte Corpereau, Ifremer, charlotte.corporeau@ifremer.fr
- Emma Thompson, University of Sydney, e.thompson@sydney.edu.au
- David Raftos, Macquarie University, david.raftos@mq.edu.au
- Vengatesen Thiyagarajan (Rajan), University of Hong Kong, rajan@hku.hk

Abstract

Pacific geoduck aquaculture is a growing industry, however little is known about how geoduck respond to varying environmental conditions, or how production might be impacted by low pH associated with ocean acidification. Ocean acidification research is increasingly incorporating multiple environmental drivers and natural pH variability into biological response studies for more complete understanding of the effects of projected ocean conditions. In this study, eelgrass habitats and environmental heterogeneity across four estuarine bays were leveraged to examine low pH effects on geoduck under different natural regimes, using proteomics to assess physiology. Juvenile geoduck were deployed in eelgrass and adjacent unvegetated habitats for 30 days while pH, temperature, dissolved oxygen, and salinity were monitored. Across the four bays pH was lower in unvegetated habitats compared to eelgrass habitats, however this did not impact geoduck growth, survival, or proteomic expression patterns. However, across all sites temperature and dissolved oxygen corresponded to growth and protein expression patterns. Specifically, three protein abundance levels (trifunctional-enzyme subunit β-subunit, puromycin-sensitive aminopeptidase, and heat shock protein 90-α) and shell growth positively correlated with dissolved oxygen variability and inversely correlated with mean temperature. These results demonstrate that geoduck are resilient to low pH in a natural setting, and other abiotic factors (i.e. temperature, dissolved oxygen variability) may have a greater influence on geoduck physiology. In addition this study contributes to the understanding of how eelgrass patches influences water chemistry.

Key words

Aquaculture, comparative physiology, ocean acidification, Panopea generosa, proteomics

Introduction

The Pacific geoduck, *Panopea generosa,* is native to the North American Pacific Coast and is a burgeoning aquaculture species with strong overseas demand as a luxury commodity (Coan et al. 2000; Shamshak and King 2015; Vadopalas et al. 2010). The largest burrowing clam in the world, cultured geoduck reach upwards of 180mm and are harvested after growing approximately 6-7 years in sub- or intertidal sediment (Vadopalas et al. 2015; WA DNR website 2018; Washington Sea Grant 2013). The long grow-out period and high per-animal value highlights the importance of site selection for farmers to maximize investment, however there remains a paucity of data on the optimal environmental conditions for geoduck aquaculture.

As marine calcifiers, geoduck may be vulnerable to ocean acidification due to their reliance on calcite and aragonite (forms of calcium carbonate) for shell secretion (Orr et al. 2005; Weiss et al. 2002), both of which become less biologically available as seawater pH declines with pCO_2 enrichment (Feely et al. 2008). A broadening body of research on marine calcifiers indicates that, generally, projected low pH will shift organisms' physiology to the detriment of species-wide abundances and distributions (Boyd et al. 2016; DeBiasse and Kelly 2016; Kurihara 2008; Kroeker et al. 2010; Pörtner 2008; Pörtner and Farrell 2008; Ries et al. 2009). However, broad generalizations of how ocean acidification affects calcifiers are few due

to varying pH sensitivity between taxa (Clark et al. 2009; Gazeau et al. 2007; Miller et al. 2009; Ries et al. 2009; Talmage and Gobler 2009; Waldbusser et al. 2016) and life stage (Kurihara 2008; Kroeker et al. 2010), thus lessons learned from other bivalve species cannot directly be applied to geoduck without investigation.

The effect of low pH on cultured geoduck needs to be explored to help the aquaculture industry make informed site selection, selective breeding, and investment decisions. For practical application, geoduck ocean acidification studies should best replicate the natural environment in which they are grown, which is primarily Washington State (90% of global production) in the Puget Sound estuary where environmental drivers vary between subbasin, season, and diurnal cycle (Moore et al. 2008; Shamshak and King 2015). Lessons from more advanced ocean acidification research on other taxa can also be leveraged. For example a significant conclusion is that low pH is not occurring in isolation, but rather in conjunction with changes in other environmental drivers (for example temperature, dissolved oxygen, salinity), and thus single-stressor studies are limited in their predictive capacity (Ban et al. 2014; Byrne and Przeslawski 2013; DeBiasse and Kelly 2016; Gobler et al. 2014; Harvey et al. 2013; Kroeker et al. 2013; Padilla-Gamiño et al. 2013; Przeslawski et al. 2015). Another consideration is the incorporation of naturally-occuring diurnal pH variability into ocean acidification studies, as variable pH can have differing effects on marine calcifiers compared to persistent low pH (Baumann et al. 2015; Boyd et al. 2016; Cornwall et al. 2013; Frieder et al. 2014; Gunderson et al. 2016; Paganini et al. 2014; Reum et al. 2016).

To best predict the effect of ocean acidification on geoduck aquaculture this project deployed geoduck in variable environmental conditions and leveraged the natural pH differences between eelgrass and unvegetated habitats in Washington State estuaries. Ocean acidification studies are increasingly exploiting naturally low pH systems to monitor the environmental heterogeneity alongside test organisms (for example, hydrothermal vents, shallow CO_2 seeps, coastal upwelling regions, and eutrophic estuaries) (Duquette et al. 2017; Hofmann et al. 2011; Howarth et al. 2011; Thomsen et al. 2013; Tunnicliffe et al. 2009; Kerrison et al. 2011). Compared to controlled laboratory studies, these deployment studies can uniquely incorporate daily cycles in air exposure, temperature, pH, dissolved oxygen, salinity, and food availability (Chapman et al. 2011; Groner et al. 2018; Middelboe and Hansen 2007; Ringwood and Keppler 2002).

Estuaries along the United States Pacific Coast are ideal, natural mesocosms for examining the effect of ocean acidification on commercially vital calcifiers, as they contain dense macroalgae beds (Bulthuis 1995), environmental conditions that vary considerably between subbasins (Babson et al. 2006; Banas et al. 2004; Dethier et al. 2010; Moore et al. 2008; Yang and Khangaonkar 2010), and have rich communities of native and cultured shellfish (Dethier et al. 2006; Miller et al. 2009; Washington Sea Grant 2015). Furthermore, coastal estuaries have already shifted towards lower pH, warmer averages, and are projected to continue along this trend (Abatzoglou et al. 2013; Busch et al. 2013; Doney et al. 2007; Feely et al. 2012, 2010, 2008; Mote and Salathé 2010). The buffering capacity of macroalgae (seagrass meadows, kelp forests) allows for block-designed experiments to examine the effect of pH, while controlling for varying background environments and maintaining diurnal fluctuations (Middelboe and Hansen 2007; Palacios and Zimmerman 2007; Wahl et al. 2018).

In order to better inform geoduck aquaculture practices, we set out to examine how low pH and other natural variation in environmental conditions influence geoduck growth and physiology, using eelgrass as a primary determinant of water chemistry. Physiology was evaluated with a two-phase proteomics approach using Selected Reaction Monitoring, with targets identified using Data Independent Acquisition. This novel application demonstrates the advances in proteomic research and the potential it has to improve aquaculture production.

Methods

Experimental Design

Panopea generosa juveniles (14.0 +/- 0.85 mm) from the same hatchery-produced cohort were out-planted in four bays throughout Western Washington State from June 21 to July 21, 2016: Fidalgo Bay (FB), Port Gamble Bay (PG), and Case Inlet (CI) in Puget Sound, and Willapa Bay (WB) located off the southwest Pacific Coast of Washington (Table 1, Figure 1). All locations were selected based on the criteria that both eelgrass beds ("eelgrass"), and unvegetated sediment ("unvegetated") habitats were present. Clams were placed in 10 cm diameter polymerizing vinyl chloride (PVC) pipes buried in sediment with 5 cm exposed; this method replicates aquaculture techniques. Five clams were placed in each of the 3 tubes in both the eelgrass and unvegetated habitat, with a total of 30 clams across 6 tubes per bay. Pipes were covered with a protective mesh exclosure to limit predation. The replicate structures surrounded and were equidistant to a suite of water quality sensors capturing pH, salinity, dissolved oxygen, and temperature (via pH and DO probes) for the duration of the 30-day outplant. Sensors were modified for submersible, autonomous data collection.

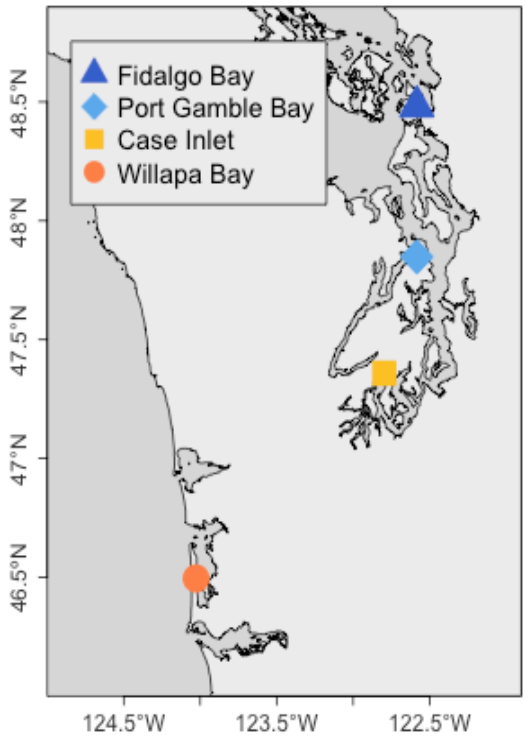


Figure 1: Geoduck juveniles were deployed for 30 days in 2 habitats (eelgrass beds, unvegetated) within 4 bays in Western Washington State

Bay	Eelgrass Habitat	Unvegetated Habitat
Fidalgo Bay	48° 28' 52.8312" N 122° 35' 0.7044" W	48° 28' 54.0876" N 122° 35' 0.708" W
Port Gamble Bay	47° 50' 52.7388" N 122° 34' 58.5084" W	47° 50' 33.6336" N 122° 35' 1.7952" W
Case Inlet	47° 21' 30.3808" N 122° 47' 47.2182" W	47° 21' 28.5721" N 122° 47' 44.7457" W
Willapa Bay	46° 29' 42.288" N 124° 1' 35.472" W	46° 29' 40.124" N 124° 1' 34.0882" W

Table 1: Coordinates for geoduck placement.

Collection and Sampling

Animals were collected during low tide and transferred on wet ice to shore where mortality and size were recorded. Live animals were dissected, ctenidia tissue was isolated and flash-frozen in an ethanol-dry ice bath. During sampling all instruments were sterilized between samples via bleach then ethanol, and rinsed with nanopure water. Samples were held on dry ice while transported back to the lab and stored at -80℃.

Environmental and Growth Data

Temperature, pH, salinity and dissolved oxygen data were compared between outplant locations. Outliers for all environmental parameters were removed, as determined using Tukey Interquartile Range (IQR) method (Tukey 1977), excluding data outside the inner fence (1.5*IQR). Tidal charts from WWW Tide/Current Predictor and salinity data (<20ppt) were also used to remove DO and pH data corresponding to periods of tidal exposure. Four probe failures occurred during deployment (Supplemental Table 1, *failed*) and these data were not included in the analysis (pH at Port Gamble-eelgrass, salinity at Port-Gamble unvegetated & Case-Inlet eelgrass, DO in Fidalgo Bay eelgrass). Salinity data from two additional locations was not reliably measured (Willapa Bay-unvegetated, Fidalgo Bay-unvegetated), so habitat comparisons were not performed for salinity data. For each parameter at each location, daily mean and daily standard deviation time series were calculated. Relative growth for each animal was determined as (L_f-L_i) , where L_f= final geoduck shell length, L_i = average initial geoduck shell length within each exclosure (n=5). Differences in growth and environmental parameters between habitat were compared using 2-way analysis of variance (ANOVA) applied to regression models (value ~ habitat*bay). Bays and ad-hoc regions (north vs. south bays) were tested using 1-way ANOVA. Pairwise comparisons were tested with the t-statistic. Significance for all tests was defined as $P < 0.05$, corrected for multiple comparisons using the Bonferroni correction.

Protein Analysis

Protein Preparation

Relative protein abundance was ultimately assessed in a two-phase proteomics approach using Selected Reaction Monitoring (SRM), with targets identified using Data Independent Acquisition (DIA). Tissues were prepared separately for DIA and SRM, both following the protocol in Timmins-Schiffman et al. (2014) with a few exceptions. For DIA, 8 ctenidia tissue samples were analyzed, one sample from each location and habitat: FB-eelgrass (G048), FB-unvegetated (G058), PGB-eelgrass (G077), PGB-unvegetated (G068), CI-eelgrass (G010), CI-unvegetated (G018), WB-eelgrass (G131), WB-unvegetated (G119). For SRM new ctenidia samples were examined, 12 individuals per bay (Fidalgo Bay, Port Gamble Bay, Case Inlet, Willapa Bay), with 6 from each habitat (eelgrass, unvegetated) for 48 samples total. Tissue was homogenized with sterile plastic pestle in 100 µl lysis buffer (50 mM NH_4HCO_3, 6M urea solution) and sonicated with Sonic Dismembrator (Fisher Scientific, Model 120) at 50% amplitude for ten seconds, three times. Protein concentration was quantified via Pierce™ BCA Protein Assay Kit (ThermoFischer Scientific, Waltham, MA USA).

Mini-Trypsin Digestion

Aliquots of protein (30 µg for DIA, 100 µg for SRM) were suspended in Lysis Buffer (50 mM NH_4HCO_3 + 6 M urea solution) to a total volume of 100 µl followed by: 1) a 1 hour incubation at 37°C with 200 mM Tris(2-carboxyethyl)phosphine (2.5µl) and 1.5 M Tris at pH 8.8 (6.6 µl); 2) 1 hour at room temperature in dark with 200 mM iodoacetamide (20 µl); 3) 1 hour at room temperature with 200 mM diothiothreitol (20 µl); 4) 1 hour at room temperature with 2 ug/µl Lysyl Endopeptidase (Lys-C, Wako Chemicals) (3.3 µg); 5) overnight at room temperature in 25 mM NH_4HCO_3 (800 µl) + high pressure liquid chromatography grade methanol (200 µl) + Pierce Trypsin Protease, MS Grade (1 ug/µl, Thermo Scientific) at 1:30 enzyme:protein ratio (3.3 µg). Samples were evaporated to near dryness at 4°C using a CentriVap Benchtop Vacuum Concentrator.

Desalting

Samples were desalted to isolate peptides using MacroSpin Columns (Nest Group, 50-450 µl, Peptide Protein C18). Peptides were reconstituted in 5% acetonitrile + 0.1% trifluoroacetic acid (TFA) (100 µl), then 10% formic acid (70 µl) was added to achieve pH ≤2. Columns were washed with 60% acetonitrile + 0.1% TFA (Solvent A, 200 µl) four times, then equilibrated with 5% acetonitrile + 0.1% TFA (Solvent B, 200 µl) three times. Peptides were bound to the column by running the digest through the column twice, followed by peptide elution with two additions each of Solvent A (100 ul). Columns were spun for 3 minutes at 3000 rpm on VWR Galaxy 16DH digital microcentrifuge at each stage. Samples were evaporated to near dryness at 4°C, then reconstituted in the Final Solvent (3% acetonitrile + 0.1% formic acid) (60 µl for 0.5 µg/µl final concentration of protein, and 50 µl for 2 µg/µl final concentration for DIA & SRM, respectively).

Autosampler preparation and internal standard

Final mixtures for mass spectrometry included 3.13 fmol/µl Peptide Retention Time Calibration mixture (PRTC), 0.33 µg/µl and 0.5 µg/µl protein for DIA and SRM, respectively, in Final Solvent for 15µl total volume. To confirm that peptides were quantified correctly in SRM, 10 µg from 5 randomly selected geoduck peptide samples were pooled, and 8 dilutions were prepared by diluting with oyster peptides at known concentrations (1x, 1.3x, 2x, 4x, 6x, 8x, 8.7x, 9x) and analyzed alongside other samples.

Data Independent Acquisition

Data acquisition

Data Independent Acquisition (DIA) was performed to assess global protein abundance patterns and to identify consistently detectable peptides for SRM targets. Eight samples, one per deployment location, were analyzed in technical duplicates via liquid chromatography tandem mass spectrometry (LC-MS/MS) with the Thermo Scientific™ Orbitrap Fusion Lumos™ Tribrid™. Prior to sample analysis, the 30 cm analytical column and 3 cm trap were packed in-house with with C18 beads (Dr. Maisch HPLC, Germany, 0.3 µm). For each sample, 3 µl of protein + PRTC solution (1.0 µg of geoduck protein) was injected and analyzed in MS1 over 400-900 *m/z* range, in 5 *m/z* overlapping isolation windows from 450-850 *m/z* with 15K resolution in MS2. Final Solvent blanks were run between each geoduck peptide injection to ensure against peptide carry-over. Lumos MS/MS method and sequence files are available in the project repository (GitHub).

Protein identification and analysis

Proteins were inferred using an assembled, translated, and annotated *P. generosa* gonad transcriptome (combined male and female) (Timmins-Schiffman et al. 2017). Transcriptome peptides were queried against those detected by the Lumos MS/MS using PEptide-Centric Analysis (PECAN) (Ting 2017) to create a peptide spectral library (.blib type file). DIA raw files were first demultiplexed using MSConvert (ProteoWizard version 3.0.10738, 2017-04-12) (Chambers et al. 2012) with filters set to vendor centroiding for msLevels [2,3] (--”peakPicking true 1-2”), and optimizing overlapping spectra (“demultiplex optimization-overlap only”). The transcriptome fasta file was tryptic digested *in silico* in Protein Digestion Simulator (version 2.2.6471.25262), set to Fully Tryptic from 400-6000 fragment mass range, 5 minimum residues allowed, 3 maximum missed cleavages and peak matching thresholds set to 5 ppm mass tolerance, and 0.05 ppm NET tolerance. Skyline version 3.7.0.11317 (MacLean et al. 2010) automatically selected transition peaks and quantified peptide abundances using peak area integration. All PRTC peptide peak selections were manually verified and corrected. Skyline peak selection error rate was calculated by manually checking chromatograms from 100 proteins across all DIA samples. Auto-selected peaks were assigned correct or incorrect selection based on transition retention time alignment across replicates, using PRTC peptides as a reference. Transition peak area, defined henceforth as abundance, was exported from Skyline for analysis in R version 2.4-3 (R Core Team 2016). Abundance was normalized by the total ion current (TIC) for each injection. Technical replicate, bay and habitat differences were assessed to inform SRM analysis via non-metric multidimensional scaling (NMDS) analysis

using `metaNMDS` in the vegan package (Oksanen et al. n.d.) on log(x+1) transformed abundances using a Bray-Curtis dissimilarity matrix. Technical replicate spectral abundances clustered together on NMDS plots, thus were averaged across each sample. Bay and habitat differences in global abundance were visually but not quantitatively analyzed (Supplemental Figure 4).

Selected Reaction Monitoring

Target selection

Thirteen proteins were selected for SRM targets (Table 2). First, candidate proteins (~200) were selected based on biological function listed in the Universal Protein Knowledgebase (Apweiler et al. 2004) and scientific literature, emphasizing proteins involved in the cellular response to oxidative stress (Matoo et al. 2013, Tomanek 2011, 2014, 2016). Candidates were screened for detectability using DIA results. Selected proteins were required to have ≥3 high quality peptides, each with ≥3 transitions, present in all DIA biological and technical replicate data. Quality peptides had uniform peak morphology and retention time in Skyline across replicates. A total of 49 peptides were selected for SRM: 39 to quantify 13 target proteins (116 transitions), and 10 for internal standard (30 transitions). A full list of transition targets are published on panoramaweb.org and available in the project repository (GitHub).

SRM Protein Targets	Top Blast Hit Uniprot SpID (E-value)	Peptide sequences
heat shock protein 90-alpha	P30946 (0)	GVVDSEDLPLNISR EVVQSSAFVER DSSTMGYMAAK
heat shock protein 70	Q91233 (0)	TTPSYVAFNDTER NAVVTVPAYFNDAQR IINEPTAAALAYGLDK
superoxide dismutase	P28757 (1e-57)	THGAPTDEER ISLTGPHSIIGR TIVVHADVDDLGK
catalase	P00432 (0)	AGELGGSDPDYAMR LYSYSDTHR LTANIAGHLIGAQEFIQK
peroxiredoxin-1	Q6B4U9 (2e-95)	ALFIIDDK QITMNDLPVGR LVQAFQFTDK
puromycin-sensitive aminopeptidase	Q11011 (0)	LNSGSVGVYR SLTENFVTEEQAK SIQQSVENIR
protein disulfide-isomerase	P07237 (0)	NNKPSDYQGGR DNVVVIGFFK MDSTANEVEDVK
ras-related protein rab-11B	O35509 (1e-85)	VVLVGDSGVGK STIGVEFATR AQLWDTAGQER
sodium/potassium-transporting ATPase subunit alpha	Q13733 (9e-62)	TVIEPMAGDGLR MVTGDNVNTAR LLDQVWPDLR

glycogen phosphorylase (muscle form)	Q9WUB3 (0)	APNSFNLR VLYPNDNFFEGK TSFDAFPDK
trifunctional enzyme β-Subunit (mitochondrial)	O46629 (0)	AAQDNGLLTDVLAYK ALELGLKPK FNLWGGSLSLGHPFGATGV R
cytochrome P450	P00185 (7e-39)	IITRPFNVNGLLAYDSR WLDESGVFLPEEHPSR QSLLPFGATGPR
arachidonate 5-lipoxygenase	P09917 (2e-94)	APGLPAQIK MDVEGTLPEDLK GLGLGGVPGQNGK

Table 2: SRM proteins targets selected based on biological function and detectability across DIA samples

Data acquisition

SRM samples were analyzed on a Vantage Triple-Stage Quadrupole Mass Spectrometer (Thermo Scientific, San Jose, CA, USA), and injected by a nanoACQUITY UPLC® system (Waters, Milford, MA, USA) at random in two technical replicates. For each sample, 2 µl of protein + PRTC solution (1.0 µg of geoduck protein) was injected, trapped on a 3 cm pre-column and separated on a 30 cm analytical column using a chromatography gradient of 2-60% acetonitrile over 60 minutes. Columns were prepared as in DIA (above). Samples were injected in randomized groups of 5, followed by a Peptide Retention Time Calibration (PRTC) plus bovine serum albumin peptides (BSA) standard, then Final Solvent blank. Vantage MS sequence and method files are available in the project repository (GitHub).

Protein identification and quality assurance

Peptides were identified and quantified via Skyline-daily version 3.7.1.11357 (MacLean et al. 2010). Raw SRM files were imported into a Skyline-daily project along with the target protein transitions, and the spectral library (.blib file) created previously in the DIA Protein Identification step. SRM peptides were verified by regressing PRTC peptide retention time (RT) in SRM against retention time in DIA. A fitted model from PRTC peptides predicted RT of protein target peptides. Where necessary, peak selection and boundaries were manually adjusted for SRM peptide chromatograms, and actual RT were regressed against predict RT to confirm correct selection ($F(1,38)$: 5768, p-value: < 2.2e-16, Adjusted R-squared: 0.9933) (Supplemental Figure 5). Transition peak area, defined henceforth as abundance, was exported from Skyline for further analysis in R (R Core Team 2016). Abundance results from the separate serial dilution samples were used to remove peptides that did not adhere to the dilution curve. Briefly, dilution abundances (exported from Skyline) for each transition were normalized by the most dilute sample abundance, then regressed against predicted ratios. Peptides with slope coefficient $0.2<x<1.5$ and adjusted R^2 >0.7 were included in analysis. Ten of the 39 peptides were discarded from the dataset based on dilution standards results (Supplemental Figure 6). To determine and remove disparate technical replicates, NMDS analysis was performed as described above. Technical replicates with ordination distance >0.2 were removed, and only samples with two technical replicates were preserved for analysis (Supplemental Figure 7). Thirteen technical replicates from different samples and all replicates from three sample were

discarded, for 84% technical replicate and 94% biological replicate retention. Within samples, transitions with coefficients of variation (CV) > 40% between technical replicates were also discarded (2% of all transitions across 21 samples). In final dataset for differential analysis, 10 proteins, 26 peptides, and 77 transitions were retained. Mean transition abundance was calculated for replicates, with zero in the place of n/a values, which Skyline generates for replicates without peaks. Transition abundances within each peptide were summed for a total peptide abundance before analyzing for differential abundance.

Differential protein analysis

After data quality screening, peptide abundance was analyzed for differences between locations and habitats. NMDS plots visualized patterns in peptide abundances by bay and habitat as described above. Global peptide abundance was compared between bay and habitats using two-way ANOVA on log-transformed abundances. For protein-specific comparisons, peptide abundances were grouped by protein, box-cox transformed (Box and Cox 1964) and normality confirmed via qqplot (Wickham 2017). Two-way ANOVA tested abundances for each protein between eelgrass and unvegetated habitats within and between bays. Pairwise comparisons for differentially abundant proteins were tested with the t-statistic. Peptides within proteins were regressed against each other to confirm stable abundance patterns. For all statistical analyses, significance was defined as alpha ≤ 0.05, corrected for multiple comparisons using the Bonferroni correction.

Correlative analysis

To understand how environmental and biometric parameters covaried, Pearson's product-moment correlation and scatter plots were assessed between protein abundances, growth, and environmental summary statistics (mean and variance). Each protein was assessed independently. Due to salinity probe malfunction, salinity data were not included in correlation tests.

All analyses were performed in RStudio version 1.1.383 (R Core Team 2016). Data and R scripts are available in the project repository, currently located at github.com/RobertsLab/Paper-geoduck-eelgrass-OA, and Skyline project files are published to panoramaweb.org.

Results

Environmental & Growth Data

Mean pH differed significantly between habitats across all bays ($F(1,346)=325.1$, $p=1.6e-50$) (Figure 2). During the deployment pH was recorded from 6.71 to 8.34, with mean pH 7.86±0.15 in eelgrass, and 7.51±0.25 in unvegetated habitats. Variability in pH was significantly different between bays ($F(3,346=52.7$, $p=8.4e-27$), but variability did not differ between habitats. On average across all locations, pH fluctuated daily by 0.46±0.23 pH units. Considerable heterogeneity between bays was observed in the other environmental parameters. Mean temperature was significantly different between all bays ($F(3,396) =192.7$, $p=7.6e-76$), and temperature decreased with latitude (coldest in Fidalgo Bay, warmest in Willapa bay). Temperature did not differ between habitats within bays (Supplemental Figure 1). Dissolved

oxygen (DO) varied between bays in both daily standard deviation ($F(3,370)=170.4$, $p=1.9e-71$) and mean ($F(3,370)=97.3$, $p=3.1e-45$). DO variability was substantially higher in the two northern bays (SD was 5.6 and 3.9 mg/L in FB, PGB), as compared to the southern bays (2.5 and 1.4mg/L in CI, WB), but variability did not differ between habitats (Supplemental Figure 2). Mean salinity differed by bay ($F(3,253)=456.9$, $p=1.4e-100$), with the largest differences between Fidalgo Bay (mean 29.9 ppt) and the other three bays (mean 23.4-27.0 ppt) (Supplemental Figure 3). Growth significantly differed between northern and southern bays ($F(1,97)=54.8$, $P=4.9-11$), but not between habitats either within or across all bays. Geoduck in Fidalgo Bay and Port Gamble Bay grew larger compared to Willapa Bay, and Case Inlet (Figure 3). Survival did not differ among locations.

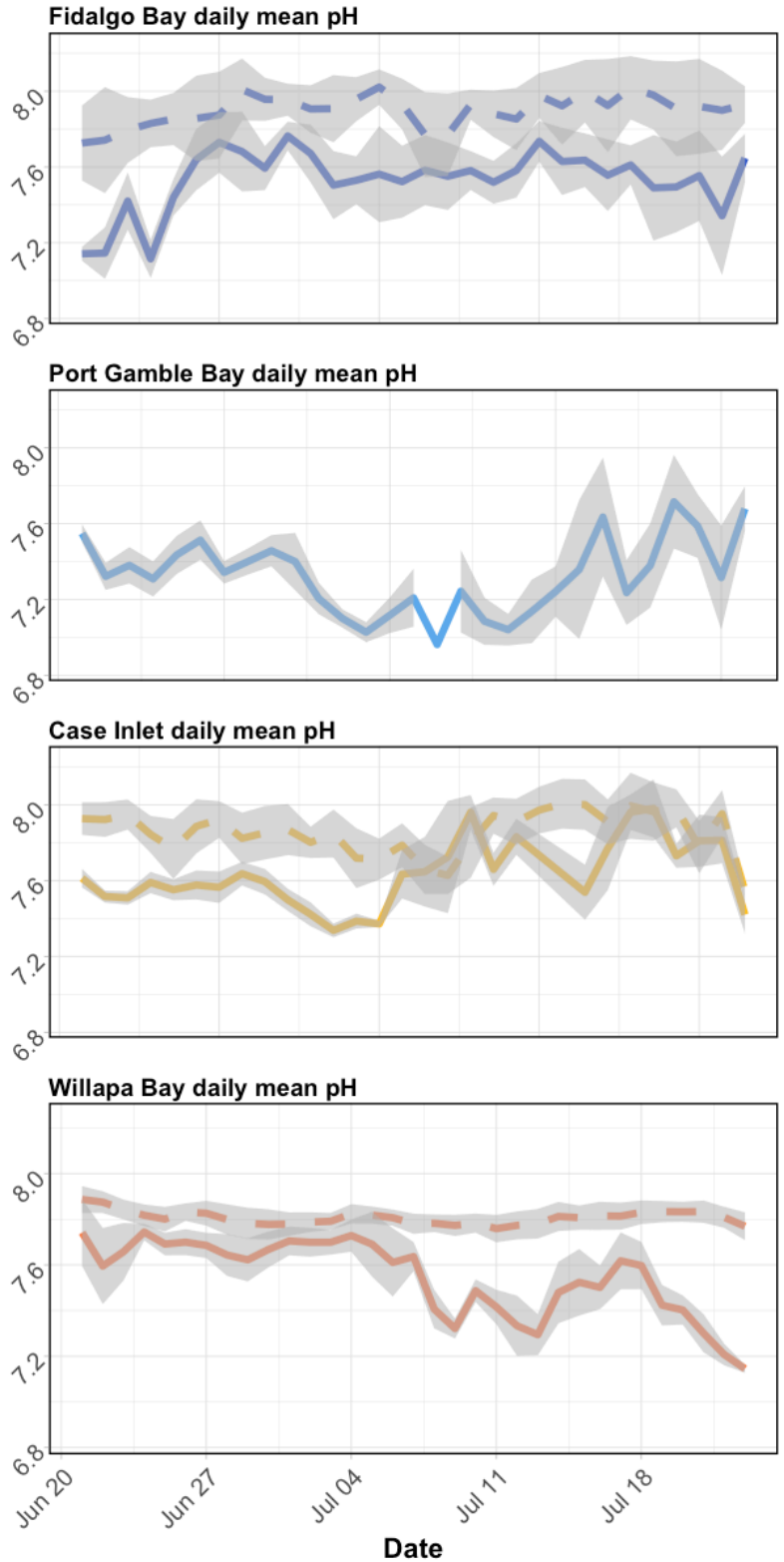


Figure 2: Daily mean pH in eelgrass (dashed lines) and unvegetated (solid lines) across bays during geoduck deployment. Gray ribbons denote daily standard deviation. Data from Port Gamble bay eelgrass are not included due to probe failure.

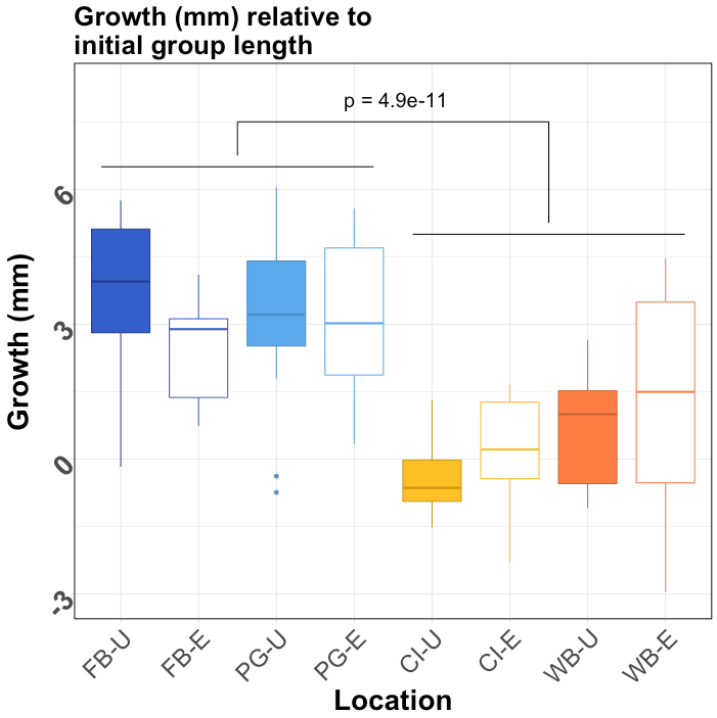


Figure 3: Geoduck shell growth after 30 days across Willapa Bay (WB), Case Inlet (CI), Port Gamble Bay (PG), and Fidalgo Bay (FB), where -U and -E represent unvegetated (filled boxes) and eelgrass habitats (empty boxes), respectively. Growth is relative to the mean initial shell length within deployment groups (n=5 per group, 3 groups per location). Geoduck that did not survive deployment are not included. Growth differed significantly between southern bays (WB, CI) and northern bays (PG, FB).

Protein Detection and Variance

In DIA, a total of 298,345 peptide transitions were detected from 30,659 distinct peptides across the 8 samples (one sample per habitat from each bay). These peptides were associated with 8,077 proteins, and more than half of the proteins (4,252) were annotated using Universal Protein Resource database (UniProt). Automated peak selection (Skyline) success rate was 71%.

In SRM, final dataset after screening included 10 proteins, 26 peptides, and 77 transitions. The 3 proteins fully screened from the dataset were heat shock protein 70, peroxiredoxin-1, and ras-related rab. The SRM mean coefficients of variation (CV) of technical replicate abundances across all transitions decreased from 18.2% to 9.6% after screening. Transition abundance CV within bays ranged from 24.9% to 83.2% with mean 50.1%, and within deployment locations CV ranged from 11.6% to 93.0% with mean 48.9% (Supplemental Table 3). Within proteins, regression analysis indicated that peptide abundances from the same protein differed slightly, however the relative abundances across samples was consistent. This indicates a small degree of background variability in peptide detection, digestion, or stability within proteins that applied to all samples (Pep1xPep2: R^2_A=0.985, coefficient=0.682; Pep1xPep3: R^2_A=0.990, coefficient=0.954; Pep2xPep3: R^2_A=0.990, coefficient=0.954).

Protein Abundance Differences

None of the 10 targeted proteins were significantly different between habitats within or across bays (Figure 4, Supplemental Table 2). NMDS plots of all transitions in DIA and those targeted in SRM revealed clustering of overall proteomic response by bay (Supplemental Figures 4 & 8). In SRM, Fidalgo Bay and Port Gamble Bay samples clustered together (henceforth “northern bays”), and some overlap between Case Inlet and Willapa Bay (“southern bays”) indicated similar protein abundances within these ad-hoc regions (Supplemental Figure 8). This was verified from the ANOVA results, which detected significant differences between northern and southern bays in three proteins: HSP90-α (HSP90) ($F(1,133)=20.5$, p-adj=1.8e-4), trifunctional-enzyme subunit β-subunit (TEβ) ($F(1,88)=11.1$, p-adj=0.018), and puromycin-sensitive aminopeptidase (PSA) ($F(1,130)=9.11$, p-adj=0.043). HSP90 and TEβ were also significantly different between bays (respectively: $F(3,131)= 7.80$, p-adj=0.0011; $F(3,345)= 5.19$, p-adj=0.034). For these three proteins, abundance was lowest in Case Inlet (southernmost in Puget Sound) followed by WIllapa Bay (southernmost overall), then Port Gamble Bay, and highest in Fidalgo Bay (northernmost).

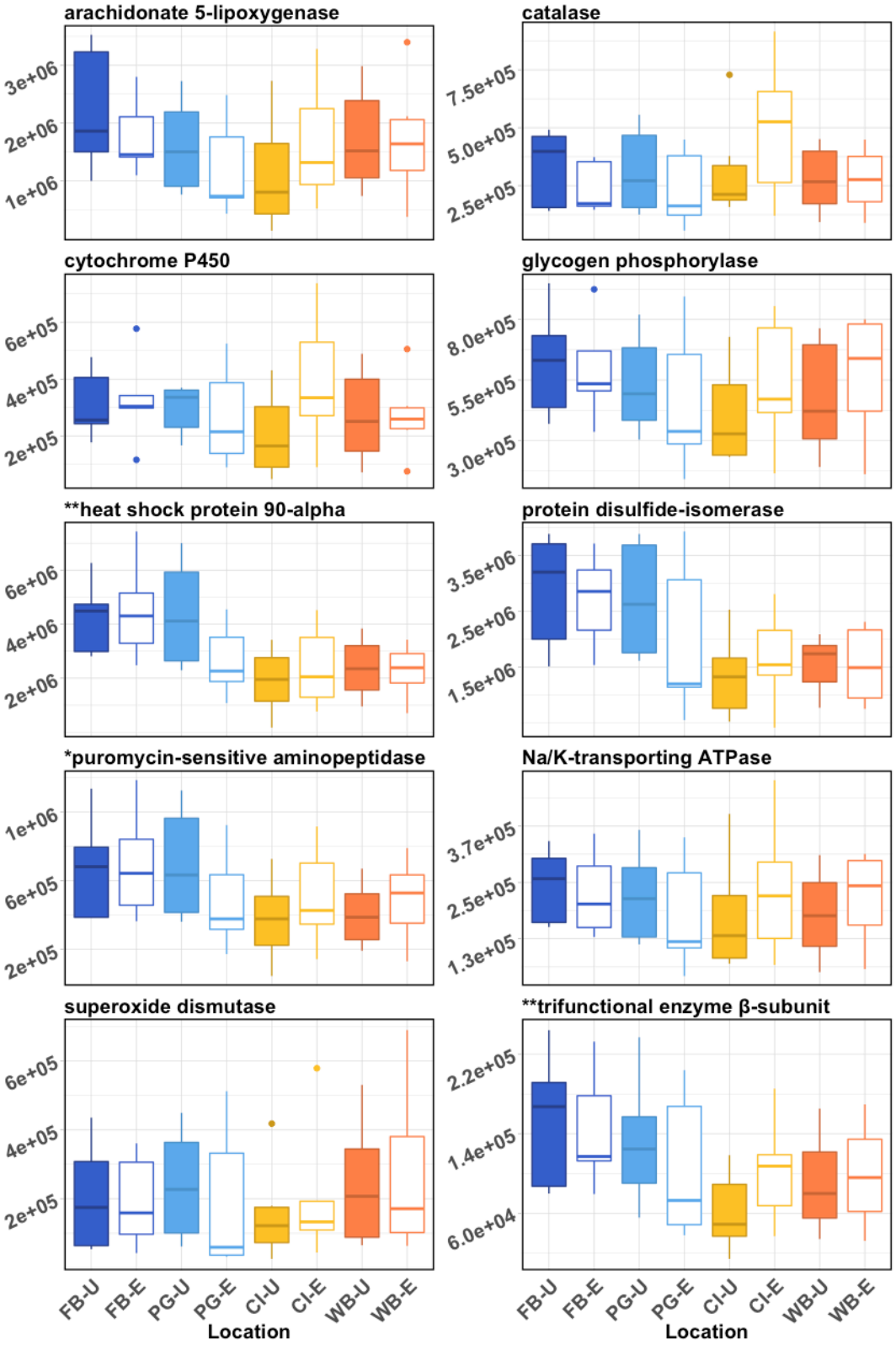

arachidonate 5-lipoxygenase
3e+06
2e+06
1e+06
catalase
7.5e+05
5.0e+05
2.5e+05
cytochrome P450
6e+05
4e+05
2e+05
glycogen phosphorylase
8.0e+05
5.5e+05
3.0e+05
**heat shock protein 90-alpha
6e+06
4e+06
2e+06
protein disulfide-isomerase
3.5e+06
2.5e+06
1.5e+06
*puromycin-sensitive aminopeptidase
1e+06
6e+05
2e+05
Na/K-transporting ATPase
3.7e+05
2.5e+05
1.3e+05
superoxide dismutase
6e+05
4e+05
2e+05
**trifunctional enzyme β-subunit
2.2e+05
1.4e+05
6.0e+04
FB-U
FB-E
PG-U
PG-E
CI-U
CI-E
WB-U
WB-E
Location

Figure 4: Protein mean spectral abundances (mean of 2 or 3 peptides targeted for each protein). For each location, n=5 or 6 geoduck. Note varying abundance ranges between proteins. Fidalgo Bay (FB), Port Gamble Bay (PG), Case Inlet (CI), and Willapa Bay (WB), where -U and -E represent unvegetated (filled boxes) and eelgrass (white boxes) habitats, respectively. Differentially abundant proteins between region and bay are indicated by (**), and bay only by (*). No proteins were significantly different between habitats.

Correlation between Environment, Abundance, and Growth

Growth positively correlated with peptide abundance for 8 of the 10 targeted proteins, including the three proteins that were differentially abundant between bays (Table 3). Growth also correlated with most environmental parameters (excluding salinity SD). Heat Shock Protein 90 correlated positively with dissolved oxygen SD. Mean and SD pH did not correlate with any peptide abundance patterns or growth. None of the environmental parameters, nor growth, correlated significantly with peptide abundances pooled across all proteins.

	Growth	**heat shock protein 90**	**puromycin sensitive aminopeptidase**	**trifunctional enzyme β-subunit**
Growth	**---**	**r=0.55 p=2.15e-11**	**r=0.48 p=6.67e-9**	**r=0.41 p=8.59e-6**
T_{mean}	**r= -0.39 p=8.11e-21**	r= -0.36 p=0.060	r= -0.24 p=0.48	r= -0.21 p=0.70
T_{sd}	**r= 0.39 p=2.10e-20**	r= 0.24 p=0.42	r= 0.15 p=1	r= 0.11 p= 1
DO_{mean}	**r= 0.45 p=2.12e-28**	r= 0.26 p=0.34	r=0.18 p=0.91	r=0.21 p=0.66
DO_{sd}	**r= 0.48 p=2.64e-32**	**r=0.41 p=0.021**	r=0.31 p=0.16	r=0.31 p=0.15
pH_{mean}	**r= -0.32 p=1.08e-12**	r= -0.09 p=1	r=-0.02 p=1	r=-0.03 p=1
pH_{sd}	**r= 0.30 p=8.72e-11**	r= 0.22 p=0.73	r=0.06 p=1	r=0.04 p=1

Table 3: Correlation analysis results between growth, environmental parameters, and peptide abundance (z-transformed). Correlation coefficient r shown with p-values adjusted via Bonferroni correction (number of comparisons). Correlations deemed significant are in bold.

Discussion

This study tested the effects of varying pH on geoduck, a valuable aquaculture species in a natural setting, and confirmed that *Zostera marina* eelgrass can effectively alter local pH during warm summer months (June and July). With this design, future ocean acidification

research on cultured shellfish can augment findings from controlled laboratory studies with field deployments to incorporate natural variability associated with an organism's habitat. Global and targeted proteomics was assessed alongside growth and environmental data for an integrated view of how geoduck respond to varying environmental conditions. Proteomics is a powerful approach suitable for comparative physiological studies of non-model, cultured fishes. Using a two step method, this study detected substantially more proteins (8,077) compared to the previous geoduck study using Data Dependent Analysis (3,651) (Timmins-Schiffman et al. 2017). This produced a valuable protein catalogue for future projects, as researchers can now skip directly to the targeted SRM phase to greatly reduce the cost and time associated with a discovery analysis.

Geoduck exhibited no phenotypic differences between pH conditions, counter to our predictions. We predicted that within eelgrass habitats pH would be higher, creating a refuge against the less alkaline surrounding waters and reducing oxidative stress. Concordantly, proteins involved in the oxidative stress response would be less abundant inside eelgrass habitats (such as superoxide dismutase, peroxiredoxin-1, catalase, and HSP), possibly translating to differential growth. While pH in eelgrass habitats was found to be consistently higher in this study, no differences in protein abundance, growth or survival were found between habitats across all four bays. This suggests that juvenile geoduck may tolerate a wide pH range in the context of the natural environment in which they are cultured.

Earlier studies on other clam species point to some degree of pH tolerance, but also describe complex responses to low pH that vary between metrics, species, and when secondary stressors are applied (Fernández-Reiriz et al. 2011; Ivanina et al. 2013; Matoo et al. 2013; Range et al. 2011; Ries et al. 2009; Ringwood and Keppler 2002; Waldbusser et al. 2010). For example, juvenile carpet shell clams (*Ruditapes decussatus*) under ambient (8.2) and reduced pH (7.8, 7.5) for 75 days displayed no difference in size, weight, or calcification rate (Range et al. 2011), but other physiological parameters (clearance, ingestion, respiration, ammonia excretion) differed at day 87 (Fernández-Reiriz et al. 2011). In the hard shell clam *Mercenaria mercenaria,* protein oxidation, biomineralization, and standard metabolic rate (SMR) in adults were largely unaffected by hypercapnia alone, but when combined with elevated temperature SMR increased and shell strength decreased (Ivanina et al. 2013; Matoo et al. 2013). Interestingly, the baltic clam (*Macoma balthica*) grew significantly larger in low pH (7.35 vs. 7.85 for 29 days), and were largest when combined with low dissolved oxygen (3.0 mg/L vs. 8.5 mg/L) (Jansson et al. 2015). Geoduck metrics examined in this study were not affected by varying pH, but other physiological parameters may be affected (metabolic rate, biomineralization, reproductive development, cytoskeleton), and should be examined in future studies.

The complex, mixed responses exhibited in clam species may, in part, be a function of local adaptation to varying environmental drivers. Pacific geoduck are native to the Puget Sound, a region that experiences regular episodes of low pH in certain areas (Busch et al. 2013; Feely et al. 2010, 2008), and thus the species may have evolved under selective pressure to withstand periods of low pH. The native Northeast Pacific Coast oyster, *Ostrea lurida,* also shows signs of pH tolerance as veliger larvae compared to non-native *Crassostrea gigas* (Waldbusser et al. 2016), a stage primarily found to be vulnerable in other calcifying species (for reviews see Byrne and Przeslawski 2013; Kurihara 2008). Geoduck are also infaunal

organisms, extending their long siphons into the water column for feeding and retreating to deep burrows during low tide or when disturbed (Goodwin and Pease 1987). Sediment and burrow chemistry, while influenced by the overlying water column, can have lower pH due to aerobic microbial activity (Gattuso and Hansson 2011), another potential source of selective pressure shaping this giant clam's pH tolerance (Gattuso and Hansson 2011; Widdicombe and Spicer 2008). An important future step is to assess the relative influence of sediment pH and overlying water column pH on burrowing calcifiers. This is particularly applicable when comparing habitats that likely have varying bacterial communities and activity.

While pH was not a universal predictor of geoduck phenotype in this study, mean temperature and dissolved oxygen variability correlated significantly with biometric parameters and separated into two groups: northern bays (Fidalgo and Port Gamble Bays), and southern bays (Case Inlet, and Willapa Bay). Geoduck grew less (or not at all, in Case Inlet) with lower protein levels in the southern bays, which were warmer with less variable dissolved oxygen content.

Temperatures in the southern bays (16-18°C) during the deployment dates may have been thermally stressful, depleting energy reserves due to elevated metabolism and denatured protein repair, particularly if food availability was limited (Lesser et al. 2010; Newell and Branch 1980; Thomsen et al. 2013). Proteomics data from the southern bays did not suggest acute stress as key stress-response proteins, HSP90 and HSP70, were inversely related to temperature, however tissues were collected at day 30 and a stress signal could have been captured with earlier or more frequent samples. Conversely, ectothermic organisms acclimatized to lower temperature can compensate for depressed enzymatic reaction rates by increasing concentrations of proteins (Lesser and Kruse 2004), and this may be at play in the protein data from geoduck placed in northern, colder bays. Temperature also tends to control microbial communities in the ocean, which could have been a factor influencing geoduck growth (Sunagawa et al. 2015).

Whether dissolved oxygen (DO) variability could have influenced geoduck in this study is less clear. Less DO fluctuation in the southern bays could be an indication of less phytoplankton biomass, translating to lower food availability (Anderson and Taylor 2001; Bergondo et al. 2005; Winter et al. 1975). While we were unable to monitor chlorophyll during the outplant, both southern bays, Willapa Bay and Case Inlet, may have phytoplankton populations that are controlled by shellfish grazers due to long residence times and aquaculture activity (Banas et al. 2007; Washington Sea Grant 2015). It is possible that food availability was different between northern and southern locations during the outplant period (June-July), and could be the underlying cause of higher growth and protein abundances in the northern sites (Carmichael et al. 2004; Liu et al. 2016; Loosanoff and Davis 1963), although this warrants additional data collection.

Conclusion

This is the first study to investigate geoduck performance alongside varying pH conditions, and contributes a geoduck peptide database useful for quantifying multiple proteins simultaneously. The primary finding is that geoduck aquaculture may be less impacted by ocean acidification compared to other environmental stressors, for example ocean warming. Geoduck ocean

443 acidification research is in its infancy, and these results are a snapshot into geoduck physiology
444 at one developmental stage, with individuals from one genetic pool, and with present-day pH
445 levels in Washington State. To best inform current and future geoduck aquaculture, further
446 foundational studies are needed to elucidate the species' pH limits in conjunction with more
447 acute environmental stressors.
448 This study also demonstrates applied use of systems such as eelgrass beds in estuaries
449 to test pH effects in a natural system. There is growing interest in using macroalgae as an
450 ocean acidification bioremediation tool, also known as phytoremediation (Greiner et al. 2013;
451 Groner et al. 2018; Hendriks et al. 2014; Washington State Blue Ribbon Panel on Ocean
452 Acidification 2012). Incorporating seagrass into shellfish-pH interaction studies can help
453 evaluate the potential for merging mariculture with shellfish aquaculture to improve growing
454 conditions for vulnerable cultured species.

455 Acknowledgements

456 Our gratitude to the following people who assisted with this project: Grace Crandall, Kaitlyn
457 Mitchell and Jose Guzman assisted with protein extraction. Jarrett Egertson contributed to DIA
458 acquisition design. Austin Keller adapted MSConvert to correctly demultiplex and convert DIA
459 files. Han-Yin Yang, Brian Searle and Sean Bennett assisted with running PECAN.
460
461 This work was supported by the National Science Foundation Graduate Research Internship
462 Program, the NOAA Ocean Acidification Program and in part by the University of Washington's
463 Proteomics Resource (UWPR95794) and the Washington Department of Natural Resources.

Reference List

464 Abatzoglou, John T., David E. Rupp, and Philip W. Mote. 2013. "Seasonal Climate Variability
465 and Change in the Pacific Northwest of the United States." *Journal of Climate* 27 (5): 2125–
466 42. https://doi.org/10.1175/JCLI-D-13-00218.1.
467 Anderson, Timothy H., and Gordon T. Taylor. 2001. "Nutrient Pulses, Plankton Blooms, and
468 Seasonal Hypoxia in Western Long Island Sound." *Estuaries* 24 (2): 228–43.
469 https://doi.org/10.2307/1352947.
470 Apweiler, Rolf, Amos Bairoch, Cathy H. Wu, Winona C. Barker, Brigitte Boeckmann, Serenella
471 Ferro, Elisabeth Gasteiger, et al. 2004. "UniProt: The Universal Protein Knowledgebase."
472 *Nucleic Acids Research* 32 (Database issue): D115–19. https://doi.org/10.1093/nar/gkh131.
473 Babson, A. L., M. Kawase, and P. MacCready. 2006. "Seasonal and Interannual Variability in
474 the Circulation of Puget Sound, Washington: A Box Model Study." *Atmosphere-Ocean* 44
475 (1): 29–45. https://doi.org/10.3137/ao.440103.
476 Banas, N. S., B. M. Hickey, P. MacCready, and J. A. Newton. 2004. "Dynamics of Willapa Bay,
477 Washington: A Highly Unsteady, Partially Mixed Estuary." *Journal of Physical*
478 *Oceanography* 34 (11): 2413–27. https://doi.org/10.1175/JPO2637.1.
479 Banas, N. S., B. M. Hickey, J. A. Newton, and J. L. Ruesink. 2007. "Tidal Exchange, Bivalve
480 Grazing, and Patterns of Primary Production in Willapa Bay, Washington, USA." *Marine*
481 *Ecology Progress Series* 341: 123–39. https://doi.org/10.3354/meps341123.

482 Ban, Stephen S., Nicholas A. J. Graham, and Sean R. Connolly. 2014. “Evidence for Multiple
483 Stressor Interactions and Effects on Coral Reefs.” *Global Change Biology* 20 (3): 681–97.
484 https://doi.org/10.1111/gcb.12453.
485 Baumann, Hannes, Ryan B. Wallace, Tristen Tagliaferri, and Christopher J. Gobler. 2015.
486 “Large Natural pH, CO2 and O2 Fluctuations in a Temperate Tidal Salt Marsh on Diel,
487 Seasonal, and Interannual Time Scales.” *Estuaries and Coasts* 38 (1): 220–31.
488 https://doi.org/10.1007/s12237-014-9800-y.
489 Bergondo, Deanna L., Dana R. Kester, Heather E. Stoffel, and Wendy L. Woods. 2005. “Time-
490 Series Observations during the Low Sub-Surface Oxygen Events in Narragansett Bay
491 during Summer 2001.” *Marine Chemistry* 97 (1): 90–103.
492 https://doi.org/10.1016/j.marchem.2005.01.006.
493 Box, G. E. P., and D. R. Cox. 1964. “An Analysis of Transformations.” *Journal of the Royal*
494 *Statistical Society. Series B, Statistical Methodology* 26 (2): 211–52.
495 http://www.jstor.org/stable/2984418.
496 Boyd, Philip W., Christopher E. Cornwall, Andrew Davison, Scott C. Doney, Marion Fourquez,
497 Catriona L. Hurd, Ivan D. Lima, and Andrew McMinn. 2016. “Biological Responses to
498 Environmental Heterogeneity under Future Ocean Conditions.” *Global Change Biology* 22
499 (8): 2633–50. https://doi.org/10.1111/gcb.13287.
500 Bulthuis, Douglas A. 1995. “Distribution of Seagrasses in a North Puget Sound Estuary: Padilla
501 Bay, Washington, USA.” *Aquatic Botany* 50 (1): 99–105. https://doi.org/10.1016/0304-
502 3770(94)00443-P.
503 Busch, D. Shallin, Chris J. Harvey, and Paul McElhany. 2013. “Potential Impacts of Ocean
504 Acidification on the Puget Sound Food Web.” *ICES Journal of Marine Science: Journal Du*
505 *Conseil* 70 (4): 823–33. https://doi.org/10.1093/icesjms/fst061.
506 Byrne, Maria, and Rachel Przeslawski. 2013. “Multistressor Impacts of Warming and
507 Acidification of the Ocean on Marine Invertebrates’ Life Histories.” *Integrative and*
508 *Comparative Biology* 53 (4): 582–96. https://doi.org/10.1093/icb/ict049.
509 Carmichael, R. H., Andrea C. Shriver, and I. Valiela. 2004. “Changes in Shell and Soft Tissue
510 Growth, Tissue Composition, and Survival of Quahogs, Mercenaria Mercenaria, and
511 Softshell Clams, Mya Arenaria, in Response to Eutrophic-Driven Changes in Food Supply
512 and Habitat.” *Journal of Experimental Marine Biology and Ecology* 313 (1): 75–104.
513 https://doi.org/10.1016/j.jembe.2004.08.006.
514 Chambers, Matthew C., Brendan Maclean, Robert Burke, Dario Amodei, Daniel L. Ruderman,
515 Steffen Neumann, Laurent Gatto, et al. 2012. “A Cross-Platform Toolkit for Mass
516 Spectrometry and Proteomics.” *Nature Biotechnology* 30 (10): 918–20.
517 https://doi.org/10.1038/nbt.2377.
518 Chapman, Robert W., Annalaura Mancia, Marion Beal, Artur Veloso, Charles Rathburn, Anne
519 Blair, A. F. Holland, et al. 2011. “The Transcriptomic Responses of the Eastern Oyster,
520 Crassostrea Virginica, to Environmental Conditions.” *Molecular Ecology* 20 (7): 1431–49.
521 https://doi.org/10.1111/j.1365-294X.2011.05018.x.
522 Clark, Dana, Miles Lamare, and Mike Barker. 2009. “Response of Sea Urchin Pluteus Larvae
523 (Echinodermata: Echinoidea) to Reduced Seawater pH: A Comparison among a Tropical,
524 Temperate, and a Polar Species.” *Marine Biology* 156 (6): 1125–37.
525 https://doi.org/10.1007/s00227-009-1155-8.

526 Coan, Eugene V., Paul Valentich Scott, and Frank R. Bernard. 2000. *Bivalve Seashells of*
527 *Western North America: Marine Bivalve Mollusks from Arctic Alaska to Baja California*.
528 Santa Barbara Museum of Natural History.
529 Cornwall, Christopher E., Christopher D. Hepburn, Christina M. McGraw, Kim I. Currie, Conrad
530 A. Pilditch, Keith A. Hunter, Philip W. Boyd, and Catriona L. Hurd. 2013. "Diurnal
531 Fluctuations in Seawater pH Influence the Response of a Calcifying Macroalga to Ocean
532 Acidification." *Proc. R. Soc. B* 280 (1772): 20132201.
533 https://doi.org/10.1098/rspb.2013.2201.
534 DeBiasse, Melissa B., and Morgan W. Kelly. 2016. "Plastic and Evolved Responses to Global
535 Change: What Can We Learn from Comparative Transcriptomics?" *The Journal of Heredity*
536 107 (1): 71–81. https://doi.org/10.1093/jhered/esv073.
537 Dethier, Megan N., Tom Mumford, Tom Leschine, Kurt Presh, Si Simenstad, Hugh Shipman,
538 Doug Myers, et al. 2006. "Native Shellfish in Nearshore Ecosystems of Puget Sound."
539 WASHINGTON UNIV SEATTLE. http://www.dtic.mil/docs/citations/ADA477852.
540 Dethier, Megan N., Jennifer Ruesink, Helen Berry, Amy G. Sprenger, and Blain Reeves. 2010.
541 "Restricted Ranges in Physical Factors May Constitute Subtle Stressors for Estuarine Biota."
542 *Marine Environmental Research* 69 (4): 240–47.
543 https://doi.org/10.1016/j.marenvres.2009.10.015.
544 Doney, Scott C., Natalie Mahowald, Ivan Lima, Richard A. Feely, Fred T. Mackenzie, Jean-
545 Francois Lamarque, and Phil J. Rasch. 2007. "Impact of Anthropogenic Atmospheric
546 Nitrogen and Sulfur Deposition on Ocean Acidification and the Inorganic Carbon System."
547 *Proceedings of the National Academy of Sciences of the United States of America* 104 (37):
548 14580–85. https://doi.org/10.1073/pnas.0702218104.
549 Duquette, Ashley, James B. McClintock, Charles D. Amsler, Alberto Pérez-Huerta, Marco
550 Milazzo, and Jason M. Hall-Spencer. 2017. "Effects of Ocean Acidification on the Shells of
551 Four Mediterranean Gastropod Species near a CO2 Seep." *Marine Pollution Bulletin* 124
552 (2): 917–28. https://doi.org/10.1016/j.marpolbul.2017.08.007.
553 Feely, Richard A., Simone R. Alin, Jan Newton, Christopher L. Sabine, Mark Warner, Allan
554 Devol, Christopher Krembs, and Carol Maloy. 2010. "The Combined Effects of Ocean
555 Acidification, Mixing, and Respiration on pH and Carbonate Saturation in an Urbanized
556 Estuary." *Estuarine, Coastal and Shelf Science* 88 (4): 442–49.
557 https://doi.org/10.1016/j.ecss.2010.05.004.
558 Feely, Richard A., Terrie Klinger, Jan A. Newton, and Meg Chadsey. 2012. *Scientific Summary*
559 *of Ocean Acidification in Washington State Marine Waters*. US Department of Commerce,
560 National Oceanic and Atmospheric Administration, Office of Oceanic and Atmospheric
561 Research.
562 https://pdfs.semanticscholar.org/5964/ffc14ae680fc855864a55b10351ed86e5935.pdf.
563 Feely, Richard A., Christopher L. Sabine, J. Martin Hernandez-Ayon, Debby Ianson, and Burke
564 Hales. 2008. "Evidence for Upwelling of Corrosive' Acidified' Water onto the Continental
565 Shelf." *Science* 320 (5882): 1490–92.
566 http://science.sciencemag.org/content/320/5882/1490.short.
567 Fernández-Reiriz, Ma José, Pedro Range, Xosé Antón Álvarez-Salgado, and Uxio Labarta.
568 2011. "Physiological Energetics of Juvenile Clams Ruditapes Decussatus in a High CO_2
569 Coastal Ocean." *Marine Ecology Progress Series* 433: 97–105.

570 http://www.jstor.org/stable/24875417.
571 Frieder, Christina A., Jennifer P. Gonzalez, Emily E. Bockmon, Michael O. Navarro, and Lisa A.
572 Levin. 2014. “Can Variable pH and Low Oxygen Moderate Ocean Acidification Outcomes for
573 Mussel Larvae?” *Global Change Biology* 20 (3): 754–64. https://doi.org/10.1111/gcb.12485.
574 Gattuso, Jean-Pierre, and Lina Hansson. 2011. *Ocean Acidification*. OUP Oxford.
575 https://market.android.com/details?id=book-eoxpAgAAQBAJ.
576 Gazeau, Frédéric, Christophe Quiblier, Jeroen M. Jansen, Jean-Pierre Gattuso, Jack J.
577 Middelburg, and Carlo H. R. Heip. 2007. “Impact of Elevated CO2 on Shellfish Calcification.”
578 *Geophysical Research Letters* 34 (7): L07603. https://doi.org/10.1029/2006GL028554.
579 Gobler, Christopher J., Elizabeth L. DePasquale, Andrew W. Griffith, and Hannes Baumann.
580 2014. “Hypoxia and Acidification Have Additive and Synergistic Negative Effects on the
581 Growth, Survival, and Metamorphosis of Early Life Stage Bivalves.” *PloS One* 9 (1): e83648.
582 https://doi.org/10.1371/journal.pone.0083648.
583 Goodwin, C. Lynn, and Bruce Pease. 1987. *The Distribution of Geoduck (Panope Abrupta)*
584 *Size, Density, and Quality in Relation to Habitat Characteristics such as Geographic Area,*
585 *Water Depth, Sediment Type, and Associated Flora and Fauna in Puget Sound,*
586 *Washington*. State of Washington, Department of Fisheries, Shellfish Division.
587 Grant, Washington Sea. 2013. “Final Report: Geoduck Aquaculture Research Program.” *Report*
588 *to the Washington State Legislature. Washington Sea Grant Technical Report WSG-TR*,
589 13–03.
590 Greiner, Jill T., Karen J. McGlathery, John Gunnell, and Brent A. McKee. 2013. “Seagrass
591 Restoration Enhances ‘Blue Carbon’ Sequestration in Coastal Waters.” *PloS One* 8 (8):
592 e72469. https://doi.org/10.1371/journal.pone.0072469.
593 Groner, Maya L., Colleen A. Burge, Ruth Cox, Natalie Rivlin, Mo Turner, Kathryn L. Van
594 Alstyne, Sandy Wyllie-Echeverria, John Bucci, Philip Staudigel, and Carolyn S. Friedman.
595 2018. “Oysters and Eelgrass: Potential Partners in a High pCO2 Ocean.” *Ecology*, May.
596 https://doi.org/10.1002/ecy.2393.
597 Gunderson, Alex R., Eric J. Armstrong, and Jonathon H. Stillman. 2016. “Multiple Stressors in a
598 Changing World: The Need for an Improved Perspective on Physiological Responses to the
599 Dynamic Marine Environment.” *Annual Review of Marine Science* 8: 357–78.
600 https://doi.org/10.1146/annurev-marine-122414-033953.
601 Harvey, Ben P., Dylan Gwynn-Jones, and Pippa J. Moore. 2013. “Meta-Analysis Reveals
602 Complex Marine Biological Responses to the Interactive Effects of Ocean Acidification and
603 Warming.” *Ecology and Evolution* 3 (4): 1016–30. https://doi.org/10.1002/ece3.516.
604 Hendriks, Iris E., Y. S. Olsen, L. Ramajo, L. Basso, A. Steckbauer, T. S. Moore, J. Howard, and
605 C. M. Duarte. 2014. “Photosynthetic Activity Buffers Ocean Acidification in Seagrass
606 Meadows.” *Biogeosciences* 11 (2): 333.
607 http://search.proquest.com/openview/dd8bd20d2b762c317796efc36e6dd278/1?pq-
608 origsite=gscholar&cbl=105740.
609 Hofmann, Gretchen E., Jennifer E. Smith, Kenneth S. Johnson, Uwe Send, Lisa A. Levin,
610 Fiorenza Micheli, Adina Paytan, et al. 2011. “High-Frequency Dynamics of Ocean pH: A
611 Multi-Ecosystem Comparison.” *PloS One* 6 (12): e28983.
612 https://doi.org/10.1371/journal.pone.0028983.
613 Howarth, Robert, Francis Chan, Daniel J. Conley, Josette Garnier, Scott C. Doney, Roxanne

614 Marino, and Gilles Billen. 2011. “Coupled Biogeochemical Cycles: Eutrophication and
615 Hypoxia in Temperate Estuaries and Coastal Marine Ecosystems.” *Frontiers in Ecology and*
616 *the Environment* 9 (1): 18–26. https://doi.org/10.1890/100008.
617 Ivanina, Anna V., Gary H. Dickinson, Omera B. Matoo, Rita Bagwe, Ashley Dickinson, Elia
618 Beniash, and Inna M. Sokolova. 2013. “Interactive Effects of Elevated Temperature and
619 CO2 Levels on Energy Metabolism and Biomineralization of Marine Bivalves Crassostrea
620 Virginica and Mercenaria Mercenaria.” *Comparative Biochemistry and Physiology. Part A,*
621 *Molecular & Integrative Physiology* 166 (1): 101–11.
622 https://doi.org/10.1016/j.cbpa.2013.05.016.
623 Jansson, Anna, Joanna Norkko, Sam Dupont, and Alf Norkko. 2015. “Growth and Survival in a
624 Changing Environment: Combined Effects of Moderate Hypoxia and Low pH on Juvenile
625 Bivalve Macoma Balthica.” *Journal of Sea Research* 102 (August): 41–47.
626 https://doi.org/10.1016/j.seares.2015.04.006.
627 Kerrison, Philip, Jason M. Hall-Spencer, David J. Suggett, Leanne J. Hepburn, and Michael
628 Steinke. 2011. “Assessment of pH Variability at a Coastal CO2 Vent for Ocean Acidification
629 Studies.” *Estuarine, Coastal and Shelf Science* 94 (2): 129–37.
630 https://doi.org/10.1016/j.ecss.2011.05.025.
631 Kroeker, Kristy J., Rebecca L. Kordas, Ryan Crim, Iris E. Hendriks, Laura Ramajo, Gerald S.
632 Singh, Carlos M. Duarte, and Jean-Pierre Gattuso. 2013. “Impacts of Ocean Acidification on
633 Marine Organisms: Quantifying Sensitivities and Interaction with Warming.” *Global Change*
634 *Biology* 19 (6): 1884–96. https://doi.org/10.1111/gcb.12179.
635 Kroeker, Kristy J., Rebecca L. Kordas, Ryan N. Crim, and Gerald G. Singh. 2010. “Meta-
636 Analysis Reveals Negative yet Variable Effects of Ocean Acidification on Marine
637 Organisms.” *Ecology Letters* 13 (11): 1419–34. https://doi.org/10.1111/j.1461-
638 0248.2010.01518.x.
639 Kurihara, H. 2008. “Effects of CO2-Driven Ocean Acidification on the Early Developmental
640 Stages of Invertebrates.” *Marine Ecology Progress Series* 373 (December): 275–84.
641 https://doi.org/10.3354/meps07802.
642 Lesser, Michael P., Meredith A. Bailey, Daniel G. Merselis, and John R. Morrison. 2010.
643 “Physiological Response of the Blue Mussel Mytilus Edulis to Differences in Food and
644 Temperature in the Gulf of Maine.” *Comparative Biochemistry and Physiology. Part A,*
645 *Molecular & Integrative Physiology* 156 (4): 541–51.
646 https://doi.org/10.1016/j.cbpa.2010.04.012.
647 Lesser, Michael P., and Valerie A. Kruse. 2004. “Seasonal Temperature Compensation in the
648 Horse Mussel, Modiolus Modiolus: Metabolic Enzymes, Oxidative Stress and Heat Shock
649 Proteins.” *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative*
650 *Physiology* 137 (3): 495–504. https://doi.org/10.1016/j.cbpb.2003.10.022.
651 Liu, W., C. M. Pearce, R. S. McKinley, and I. P. Forster. 2016. “Nutritional Value of Selected
652 Species of Microalgae for Larvae and Early Post-Set Juveniles of the Pacific Geoduck Clam,
653 Panopea Generosa.” *Aquaculture* 452 (Supplement C): 326–41.
654 https://doi.org/10.1016/j.aquaculture.2015.10.019.
655 Loosanoff, Victor L., and Harry C. Davis. 1963. “Rearing of Bivalve Mollusks.” In *Advances in*
656 *Marine Biology*, edited by F. S. Russell, 1:1–136. Academic Press.
657 https://doi.org/10.1016/S0065-2881(08)60257-6.

658 MacLean, Brendan, Daniela M. Tomazela, Nicholas Shulman, Matthew Chambers, Gregory L.
659 Finney, Barbara Frewen, Randall Kern, David L. Tabb, Daniel C. Liebler, and Michael J.
660 MacCoss. 2010. “Skyline: An Open Source Document Editor for Creating and Analyzing
661 Targeted Proteomics Experiments.” *Bioinformatics* 26 (7): 966–68.
662 https://doi.org/10.1093/bioinformatics/btq054.
663 Matoo, Omera B., Anna V. Ivanina, Claus Ullstad, Elia Beniash, and Inna M. Sokolova. 2013.
664 “Interactive Effects of Elevated Temperature and CO2 Levels on Metabolism and Oxidative
665 Stress in Two Common Marine Bivalves (Crassostrea Virginica and Mercenaria
666 Mercenaria).” *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative*
667 *Physiology* 164 (4): 545–53. https://doi.org/10.1016/j.cbpa.2012.12.025.
668 Middelboe, Anne Lise, and Per Juel Hansen. 2007. “High pH in Shallow-Water Macroalgal
669 Habitats.” *Marine Ecology Progress Series* 338: 107–17.
670 http://www.jstor.org/stable/24871746.
671 Miller, A. Whitman, Amanda C. Reynolds, Cristina Sobrino, and Gerhardt F. Riedel. 2009.
672 “Shellfish Face Uncertain Future in High CO2 World: Influence of Acidification on Oyster
673 Larvae Calcification and Growth in Estuaries.” *PloS One* 4 (5): e5661.
674 https://doi.org/10.1371/journal.pone.0005661.
675 Moore, Stephanie K., Nathan J. Mantua, Jan A. Newton, Mitsuhiro Kawase, Mark J. Warner,
676 and Jonathan P. Kellogg. 2008. “A Descriptive Analysis of Temporal and Spatial Patterns of
677 Variability in Puget Sound Oceanographic Properties.” *Estuarine, Coastal and Shelf Science*
678 80 (4): 545–54. https://doi.org/10.1016/j.ecss.2008.09.016.
679 Mote, Philip W., and Eric P. Salathé. 2010. “Future Climate in the Pacific Northwest.” *Climatic*
680 *Change* 102 (1-2): 29–50. https://doi.org/10.1007/s10584-010-9848-z.
681 Newell, R. C., and G. M. Branch. 1980. “The Influence of Temperature on the Maintenance of
682 Metabolic Energy Balance in Marine Invertebrates.” In *Advances in Marine Biology*, edited
683 by J. H. S. Blaxter, Frederick S. Russell, and Maurice Yonge, 17:329–96. Academic Press.
684 https://doi.org/10.1016/S0065-2881(08)60304-1.
685 Oksanen, J., F. Guillaume Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P. R.
686 Minchin, et al. n.d. *Vegan: Community Ecology Package* (version R package version 2.4-1,
687 2016). Accessed December 18, 2017. https:// CRAN.R-project.org/package=vegan.
688 Orr, James C., Victoria J. Fabry, Olivier Aumont, Laurent Bopp, Scott C. Doney, Richard A.
689 Feely, Anand Gnanadesikan, et al. 2005. “Anthropogenic Ocean Acidification over the
690 Twenty-First Century and Its Impact on Calcifying Organisms.” *Nature* 437 (September):
691 681. https://doi.org/10.1038/nature04095.
692 Padilla-Gamiño, Jacqueline L., Morgan W. Kelly, Tyler G. Evans, and Gretchen E. Hofmann.
693 2013. “Temperature and CO2 Additively Regulate Physiology, Morphology and Genomic
694 Responses of Larval Sea Urchins, Strongylocentrotus Purpuratus.” *Proceedings of the*
695 *Royal Society of London B: Biological Sciences* 280 (1759): 20130155.
696 https://doi.org/10.1098/rspb.2013.0155.
697 Paganini, Adam W., Nathan A. Miller, and Jonathon H. Stillman. 2014. “Temperature and
698 Acidification Variability Reduce Physiological Performance in the Intertidal Zone Porcelain
699 Crab Petrolisthes Cinctipes.” *The Journal of Experimental Biology* 217 (22): 3974–80.
700 https://doi.org/10.1242/jeb.109801.
701 Palacios, Sherry L., and Richard C. Zimmerman. 2007. “Response of Eelgrass Zostera Marina

to CO^2 Enrichment: Possible Impacts of Climate Change and Potential for Remediation of Coastal Habitats." *Marine Ecology Progress Series* 344: 1–13. http://www.jstor.org/stable/24871448.

Pörtner, Hans O. 2008. "Ecosystem Effects of Ocean Acidification in Times of Ocean Warming: A Physiologist's View." *Marine Ecology Progress Series* 373: 203–18. http://www.jstor.org/stable/24872925.

Pörtner, Hans O., and Anthony P. Farrell. 2008. "Physiology and Climate Change." *Science* 322 (5902): 690–92. http://www.jstor.org/stable/20145158.

Przeslawski, Rachel, Maria Byrne, and Camille Mellin. 2015. "A Review and Meta-Analysis of the Effects of Multiple Abiotic Stressors on Marine Embryos and Larvae." *Global Change Biology* 21 (6): 2122–40. https://doi.org/10.1111/gcb.12833.

Range, P., M. A. Chícharo, R. Ben-Hamadou, D. Piló, D. Matias, S. Joaquim, A. P. Oliveira, and L. Chícharo. 2011. "Calcification, Growth and Mortality of Juvenile Clams Ruditapes Decussatus under Increased pCO2 and Reduced pH: Variable Responses to Ocean Acidification at Local Scales?" *Journal of Experimental Marine Biology and Ecology* 396 (2): 177–84. https://doi.org/10.1016/j.jembe.2010.10.020.

R Core Team. 2016. *R: A Language and Environment for Statistical Computing* (version 1.1.383). https://www.R-project.org/.

Reum, Jonathan C. P., Simone R. Alin, Chris J. Harvey, Nina Bednaršek, Wiley Evans, Richard A. Feely, Burke Hales, et al. 2016. "Interpretation and Design of Ocean Acidification Experiments in Upwelling Systems in the Context of Carbonate Chemistry Co-Variation with Temperature and Oxygen." *ICES Journal of Marine Science: Journal Du Conseil* 73 (3): 582–95. https://doi.org/10.1093/icesjms/fsu231.

Ries, Justin B., Anne L. Cohen, and Daniel C. McCorkle. 2009. "Marine Calcifiers Exhibit Mixed Responses to CO2-Induced Ocean Acidification." *Geology* 37 (12): 1131–34. https://doi.org/10.1130/G30210A.1.

Ringwood, Amy H., and Charles J. Keppler. 2002. "Water Quality Variation and Clam Growth: Is pH Really a Non-Issue in Estuaries?" *Estuaries* 25 (5): 901–7. https://doi.org/10.1007/BF02691338.

Shamshak, Gina Louise, and Jonathan R. King. 2015. "From Cannery to Culinary Luxury: The Evolution of the Global Geoduck Market." *Marine Policy* 55 (May): 81–89. https://doi.org/10.1016/j.marpol.2015.01.014.

Sunagawa, Shinichi, Luis Pedro Coelho, Samuel Chaffron, Jens Roat Kultima, Karine Labadie, Guillem Salazar, Bardya Djahanschiri, et al. 2015. "Ocean Plankton. Structure and Function of the Global Ocean Microbiome." *Science* 348 (6237): 1261359. https://doi.org/10.1126/science.1261359.

Talmage, Stephanie C., and Christopher J. Gobler. 2009. "The Effects of Elevated Carbon Dioxide Concentrations on the Metamorphosis, Size, and Survival of Larval Hard Clams (Mercenaria Mercenaria), Bay Scallops (Argopecten Irradians), and Eastern Oysters (Crassostrea Virginica)." *Limnology and Oceanography*.

Thomsen, Jörn, Isabel Casties, Christian Pansch, Arne Körtzinger, and Frank Melzner. 2013. "Food Availability Outweighs Ocean Acidification Effects in Juvenile Mytilus Edulis: Laboratory and Field Experiments." *Global Change Biology* 19 (4): 1017–27. https://doi.org/10.1111/gcb.12109.

746 Timmins-Schiffman, Emma B., Grace A. Crandall, Brent Vadopalas, Michael E. Riffle, Brook L.
747 Nunn, and Steven B. Roberts. 2017. “Integrating Discovery-Driven Proteomics and Selected
748 Reaction Monitoring To Develop a Noninvasive Assay for Geoduck Reproductive
749 Maturation.” *Journal of Proteome Research* 16 (9): 3298–3309.
750 https://doi.org/10.1021/acs.jproteome.7b00288.
751 Ting, Ying Sonia. 2017. “Shifting the Paradigm: Peptide-Centric Analysis of Systematically
752 Sampled Mass Spectrometry Data.”
753 https://digital.lib.washington.edu/researchworks/bitstream/handle/1773/38147/Ting_washing
754 ton_0250E_16676.pdf?sequence=1&isAllowed=y.
755 Tomanek, Lars. 2014. “Proteomics to Study Adaptations in Marine Organisms to Environmental
756 Stress.” Journal of Proteomics 105 (Supplement C): 92–106.
757 Tomanek, Lars. 2015. “Proteomic Responses to Environmentally Induced Oxidative Stress.”
758 The Journal of Experimental Biology 218 (Pt 12): 1867–79.
759 Tomanek, Lars, Marcus J. Zuzow, Anna V. Ivanina, Elia Beniash, and Inna M. Sokolova. 2011.
760 “Proteomic Response to Elevated PCO2 Level in Eastern Oysters, Crassostrea Virginica:
761 Evidence for Oxidative Stress.” The Journal of Experimental Biology 214 (Pt 11): 1836–44.
762 Tukey, John W. 1977. “Exploratory Data Analysis.”
763 https://pdfs.semanticscholar.org/2f40/fd06bc9fd00a27437e14ed171e96e4fd9326.pdf.
764 Tunnicliffe, Verena, Kimberley T. A. Davies, David A. Butterfield, Robert W. Embley, Jonathan
765 M. Rose, and William W. Chadwick Jr. 2009. “Survival of Mussels in Extremely Acidic
766 Waters on a Submarine Volcano.” *Nature Geoscience* 2 (April): 344.
767 https://doi.org/10.1038/ngeo500.
768 Vadopalas, Brent, T. W. Pietsch, C. S. Friedman - Malacologia, and 2010. 2010. “… Proper
769 Name for the Geoduck: Resurrection of Panopea Generosa Gould, 1850, from the
770 Synonymy of Panopea Abrupta (Conrad, 1849)(Bivalvia: Myoida: Hiatellidae).” *BioOne*.
771 http://www.bioone.org/doi/abs/10.4002/040.052.0111.
772 Vadopalas, Brent, Jonathan P. Davis, and Carolyn S. Friedman. 2015. “Maturation, Spawning,
773 and Fecundity of the Farmed Pacific Geoduck Panopea Generosa in Puget Sound,
774 Washington.” *Journal of Shellfish Research* 34 (1): 31–37.
775 https://doi.org/10.2983/035.034.0106.
776 Wahl, M., S. Schneider Covachã, V. Saderne, C. Hiebenthal, J. D. Müller, C. Pansch, and Y.
777 Sawall. 2018. “Macroalgae May Mitigate Ocean Acidification Effects on Mussel Calcification
778 by Increasing pH and Its Fluctuations: Biogenic Fluctuations Mitigate OA Effects.” *Limnology
779 and Oceanography* 63 (1): 3–21. https://doi.org/10.1002/lno.10608.
780 Waldbusser, George G., Matthew W. Gray, Burke Hales, Chris J. Langdon, Brian A. Haley, Iria
781 Gimenez, Stephanie R. Smith, Elizabeth L. Brunner, and Greg Hutchinson. 2016. “Slow
782 Shell Building, a Possible Trait for Resistance to the Effects of Acute Ocean Acidification.”
783 *Limnology and Oceanography* 61 (6): 1969–83. https://doi.org/10.1002/lno.10348.
784 Waldbusser, G. G., H. Bergschneider, and M. A. Green. 2010. “Size-Dependent pH Effect on
785 Calcification in Post-Larval Hard Clam Mercenaria Spp.” *Marine Ecology Progress Series*
786 417 (November): 171–82. https://doi.org/10.3354/meps08809.
787 Washington Sea Grant. 2015. “Shellfish Aquaculture in Washington State.” *Final Report to the
788 Washington State Legislature* 84.
789 https://pdfs.semanticscholar.org/b833/e0fcb8a0459697f94fd86b9848ee0e59c0a2.pdf.

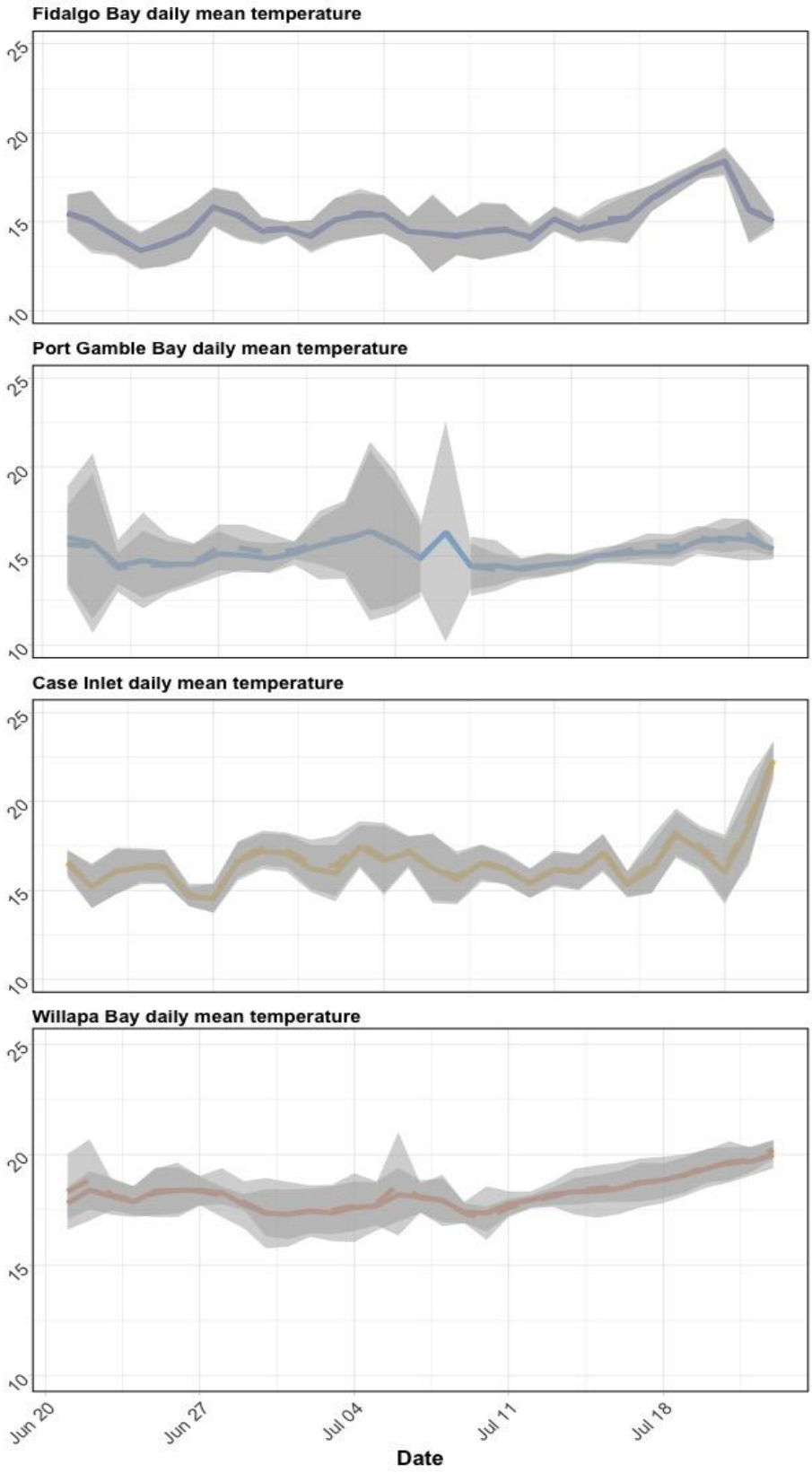
790 Washington State Blue Ribbon Panel on Ocean Acidification. 2012. “Ocean Acidification: From
791 Knowledge to Action, Washington State’s Strategic Response.” 12-01-015 . Washington
792 Department of Ecology, Olympia, Washington.
793 https://fortress.wa.gov/ecy/publications/documents/1201015.pdf.
794 “Washington’s Wild Geoduck Fishery | WA - DNR.” n.d. Accessed November 20, 2017.
795 https://www.dnr.wa.gov/programs-and-services/aquatics/shellfish/washingtons-wild-
796 geoduck-fishery.
797 Weiss, Ingrid Maria, Noreen Tuross, Lia Addadi, and Steve Weiner. 2002. “Mollusc Larval Shell
798 Formation: Amorphous Calcium Carbonate Is a Precursor Phase for Aragonite.” *The Journal*
799 *of Experimental Zoology* 293 (5): 478–91. https://doi.org/10.1002/jez.90004.
800 Wickham, Hadley. 2017. “ggplot2 - Elegant Graphics for Data Analysis (2nd Edition).” *Journal of*
801 *Statistical Software, Book Reviews* 77 (2): 1–3. https://doi.org/10.18637/jss.v077.b02.
802 Widdicombe, Stephen, and John I. Spicer. 2008. “Predicting the Impact of Ocean Acidification
803 on Benthic Biodiversity: What Can Animal Physiology Tell Us?” *Journal of Experimental*
804 *Marine Biology and Ecology* 366 (1): 187–97. https://doi.org/10.1016/j.jembe.2008.07.024.
805 Winter, D. F., K. Banse, and G. C. Anderson. 1975. “The Dynamics of Phytoplankton Blooms in
806 Puget Sound a Fjord in the Northwestern United States.” *Marine Biology* 29 (2): 139–76.
807 https://doi.org/10.1007/BF00388986.
808 “WWW Tide/Current Predictor - Site Selection.” n.d. Accessed December 7, 2017.
809 http://tbone.biol.sc.edu/tide/.
810 Yang, Zhaoqing, and Tarang Khangaonkar. 2010. “Multi-Scale Modeling of Puget Sound Using
811 an Unstructured-Grid Coastal Ocean Model: From Tide Flats to Estuaries and Coastal Waters.”
812 *Ocean Dynamics* 60 (6): 1621–37. https://doi.org/10.1007/s10236-010-0348-5.

Supplemental Material

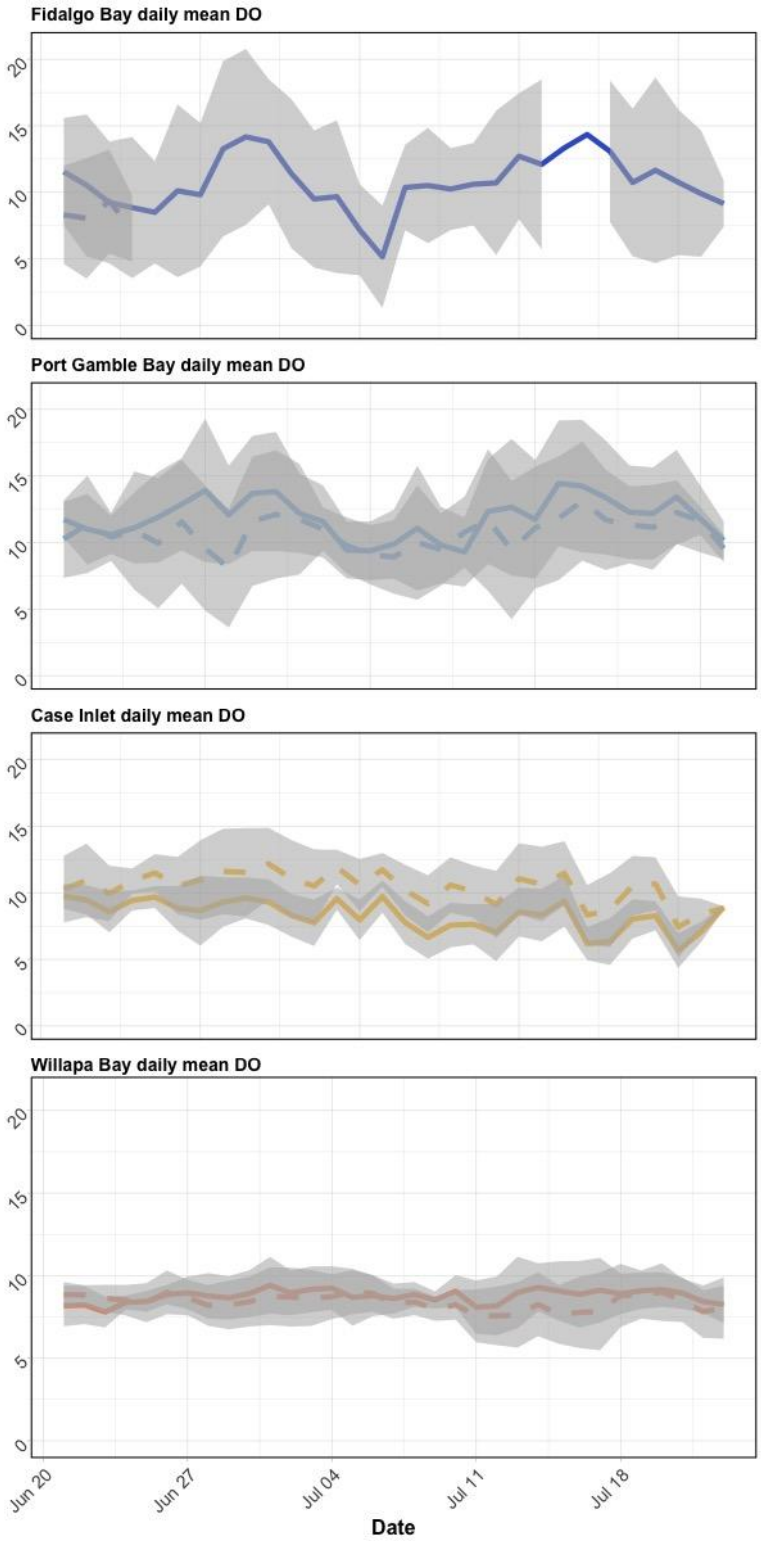
Supplemental Table 1. Outplant location metrics; FB=Fidalgo Bay, PG=Port Gamble, SR=Skokomish River Delta, CI=Case Inlet, WB=Willapa Bay; -E=Eelgrass, -U=Unvegetated.
Δ Habitats & Δ Bays: F-statistic and p-adjusted from 2-way ANOVA testing differences between habitats and bays.
**Growth did not differ within ad-hoc regions (south=CI & WB, north=FB & PG).
*Salinity probes failed fully or intermittently at 4 of the 8 locations; habitat salinities were not compared.

Deployment location	FB-E	FB-U	PG-E	PG-U	CI-E	CI-U	WB-E	WB-U	Δ Habitat	Δ Bays
% Survival	87	80	80	93	67	60	93	100	p= 0.88	p=0.25
Mean Relative % Growth (± SD)	16.2 (±7.3)	23.3 (±12.5)	22.2 (±12.5)	21.1 (±13.3)	1.2 (±8.3)	-2.8 (±5.3)	10 (±16.2)	4.5 (±8.3)	p=1	****p= 2.4e-10**
Mean pH (TS)	7.90	7.54	*failed*	7.32	7.87	7.63	7.81	7.55	**p= 1.6e-50**	**p= 7.3e-35**
pH Standard Deviation (TS)	0.19	0.23	*failed*	0.25	0.16	0.20	0.06	0.18	p=1	**p= 8.4e-27**
Mean DO (mg/L)	*failed*	10.9	10.8	11.9	10.4	8.3	8.4	8.8	p=1	**p= 3.1e-45**

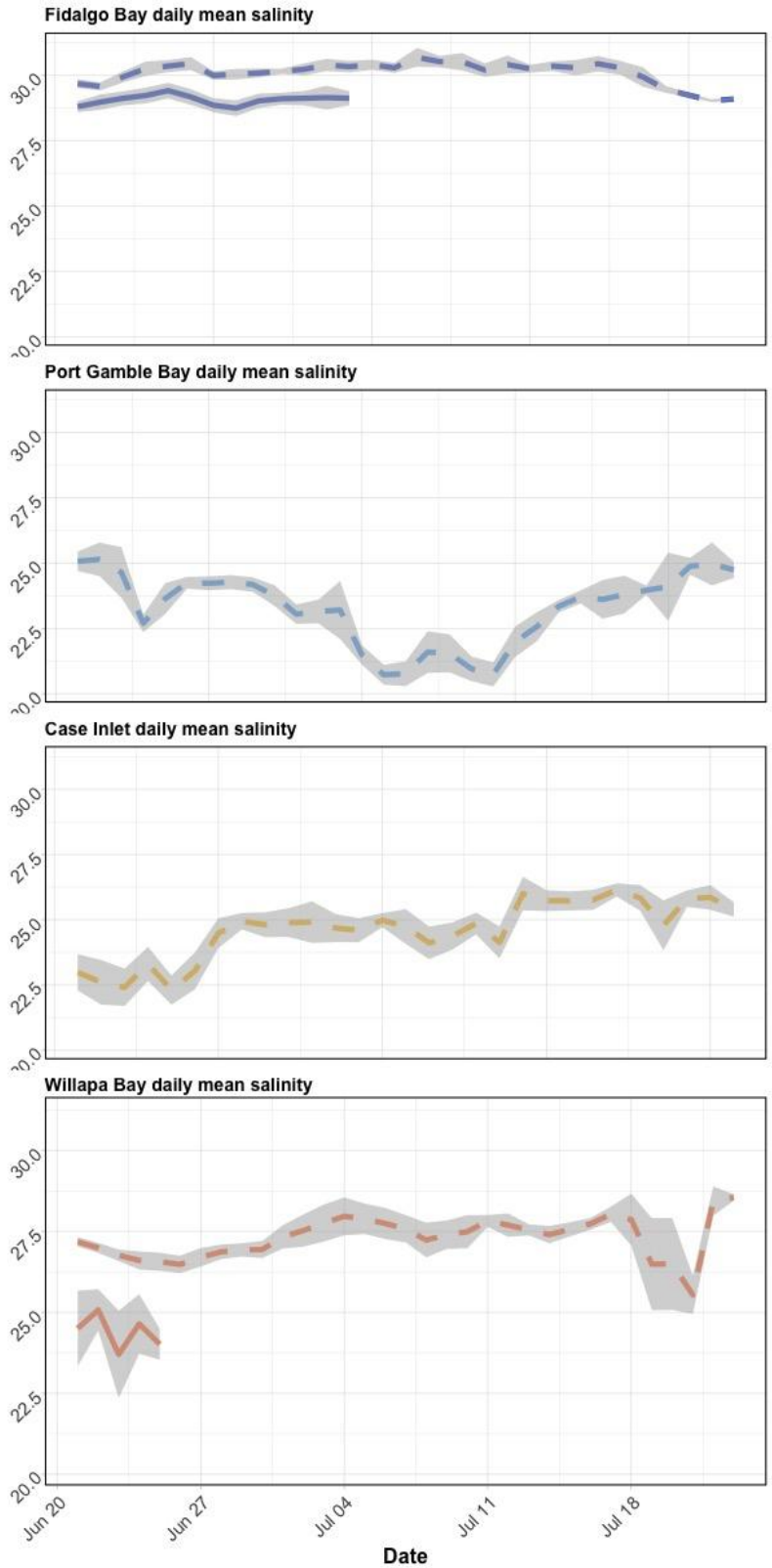
DO Standard Deviation (mg/L)	*failed*	5.7	3.9	3.9	2.6	1.9	1.5	1.3	p=1	**p= 1.9e-71**
Mean Temp (°C)	15.1	15.1	15.3	15.2	16.6	16.4	18.2	18.2	p=1	**p= 7.6e-76**
Temp Standard Deviation (°C)	1.6	1.6	2.1	2.4	1.7	1.6	1.2	1.2	p=1	p=1
Mean Salinity (ppt)	30.2	*29.1	23.4	*failed*	*failed*	24.7	27.3	*24.4	*Not tested	***p= 1.4e-100**
Salinity Standard Deviation (ppt)	0.4	*0.34	1.3	*failed*	*failed*	1.2	0.70	*1.1	*Not tested	***p= 1.9e-6**



Supplemental Figure 1: Daily mean temperature in eelgrass (dashed lines) and unvegetated (solid lines) across bays during geoduck deployment. Gray ribbons denote standard deviations per day.



Supplemental Figure 2: Daily mean DO in eelgrass (dashed lines) and unvegetated (solid lines) across bays during geoduck deployment. Gray ribbons denote standard deviations per day. Fidalgo Bay eelgrass probe failed towards the beginning of the outplant period.



Supplemental Figure 3: Daily mean salinity in eelgrass (dashed lines) and unvegetated (solid lines) across bays during geoduck deployment. Gray ribbons denote standard deviations per day. Salinity probes failed at several sites; no habitat comparisons were made.

Supplemental Table 2: SRM protein ANOVA results by Region (FB/PGB vs. CI/WB), Bay, and Habitat for all proteins combined, then each protein individually, with Pr(>F)-adjusted calculated via the Bonferroni Correction. Bold = significantly different abundance. Habitat was tested using 2-way ANOVA with abundance ~ Bay*Habitat.

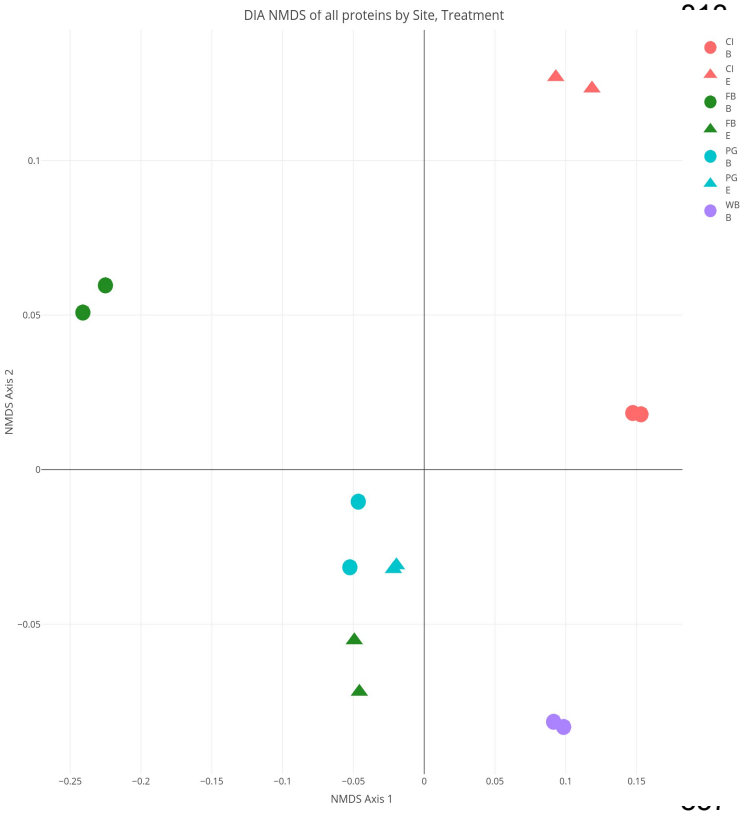
	Comparison	Df	Sum Sq	Mean Sq	F value	Pr(>F)	P.adj
all peptides combined	**North vs. South**	**1**	**18.2**	**18.2**	**10.9**	**0.00010**	**0.0014**
	Bay	**3**	**34.2**	**11.4**	**7.0**	**0.00012**	**0.0017**
	Habitat	1	1.2	1.2	0.75	5.4	14
arachidonate	North vs. South	1,133	7.4	7.4	1.3	0.26	3.6
	Bay	3,131	60	20	3.7	0.014	0.20
	Habitat	1,130	2.2	2.2	0.40	0.53	7.4
catalase	North vs. South	1,128	8.6E-05	8.6E-05	0.46	0.50	7.0
	Bay	3,126	0.00019	6.4E-05	0.34	0.79	11
	Habitat	1,125	1.4E-08	1.4E-08	7.4E-05	0.99	14
cytochrome P450	North vs. South	1,133	0.0065	0.0065	0.96	0.33	4.6
	Bay	3,131	0.015	0.0051	0.76	0.52	7.3
	Habitat	1,130	0.0085	0.0085	1.3	0.26	3.7
glycogen phosphorylase	North vs. South	1,133	94	94	1.05	0.31	4.3
	Bay	3,131	459	153	1.7	0.16	2.3
	Habitat	1,130	114	114	1.3	0.26	3.6
HSP90-alpha	**North vs. South**	**1,133**	**421,443**	**421,443**	**21**	**1.3E-05**	**0.00018**
	Bay	**3,131**	**477,740**	**159,247**	**7.8**	**7.83E-05**	**0.0011**
	Habitat	1,130	567	567	0.028	0.87	12
protein disulfide isomerase	North vs. South	1,84	3.8	3.8	5.55	0.021	0.29
	Bay	3,82	4.6	1.5	2.2	0.10	1.4
	Habitat	1,81	0.01	0.006	0.0078	0.93	13

puromycin-sensitive	**North vs. South**	**1,130**	**0.94**	**0.94**	**9.1**	**0.0031**	**0.043**
	Bay	3,128	1.2	0.40	3.87	0.011	0.15
	Habitat	1,127	0.09	0.089	0.87	0.35	4.9
sodium/potassium transporting ATPase	North vs. South	1,88	0.65	0.65	0.42	0.52	7.2
	Bay	3,86	3.2	1.06	0.68	0.56	7.9
	Habitat	1,85	0.85	0.85	0.55	0.46	6.5
superoxide dismutase	North vs. South	1,66	0.0012	0.0012	1.8	0.18	2.5
	Bay	3,64	0.0020	0.00067	1.02	0.39	5.5
	Habitat	1,63	5.4E-05	5.4E-05	0.081	0.78	11
trifunctional enzyme β-subunit	**North vs. South**	**1,88**	**252**	**252**	**11.1**	**0.0013**	**0.018**
	Bay	**3,86**	**345**	**115**	**5.19**	**0.0024**	**0.034**
	Habitat	1,85	22	22	1.0	0.32	4.5

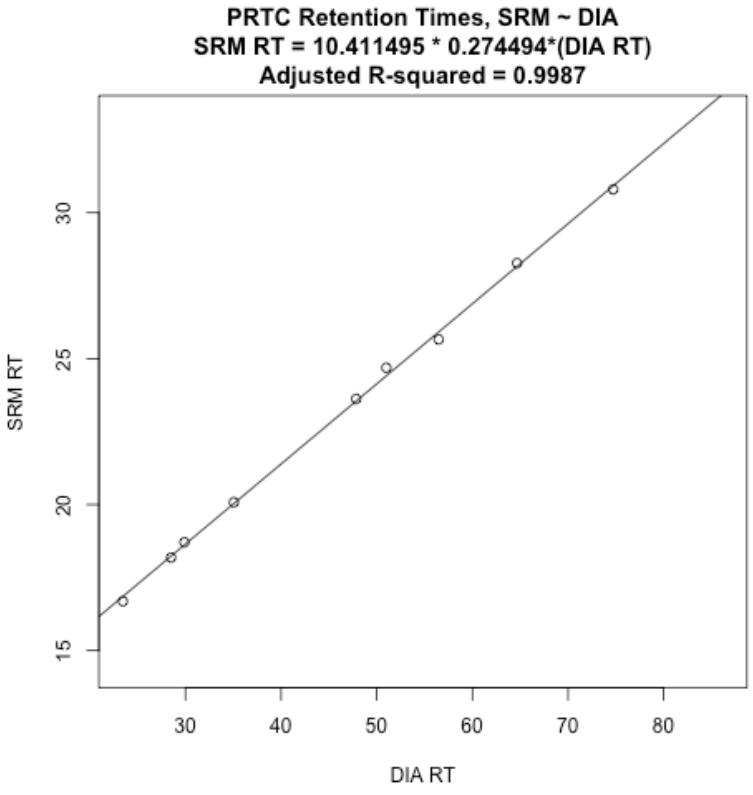
Supplemental Table 3: Mean coefficients of variation of SRM transition abundance for each protein, location

Protein	**CI-B**	**CI-E**	**FB-B**	**FB-E**	**PG-B**	**PG-E**	**WB-B**	**WB-E**
arachidonate 5-lipoxygenase	0.84	0.57	0.44	0.33	0.46	0.64	0.48	0.58
catalase	0.61	0.52	0.47	0.42	0.50	0.66	0.50	0.43
cytochrome P450	0.74	0.57	0.38	0.43	0.26	0.58	0.56	0.46
glycogen phosphorylase (muscle form)	0.55	0.49	0.35	0.35	0.37	0.61	0.51	0.47
heat shock protein 70	0.59	0.61	0.20	0.33	0.37	0.44	0.45	0.38
heat shock protein 90-alpha	0.70	0.62	0.32	0.40	0.45	0.52	0.50	0.49
protein disulfide isomerase	0.63	0.50	0.38	0.31	0.38	0.70	0.41	0.43
peroxiredoxin-1	0.56	0.46	0.31	0.33	0.42	0.46	0.40	0.36
puromycin-sensitive aminopeptidase	0.69	0.52	0.39	0.40	0.42	0.51	0.44	0.42
ras-related protein rab-11B	0.61	0.43	0.30	0.33	0.42	0.63	0.57	0.54
Na/K transporting ATPase	0.70	0.58	0.30	0.36	0.40	0.62	0.51	0.44

Superoxide dismutase	0.49	0.53	0.49	0.57	0.30	0.71	0.46	0.44
Trifunctional Enzyme β-subunit	0.61	0.48	0.41	0.38	0.46	0.63	0.52	0.53



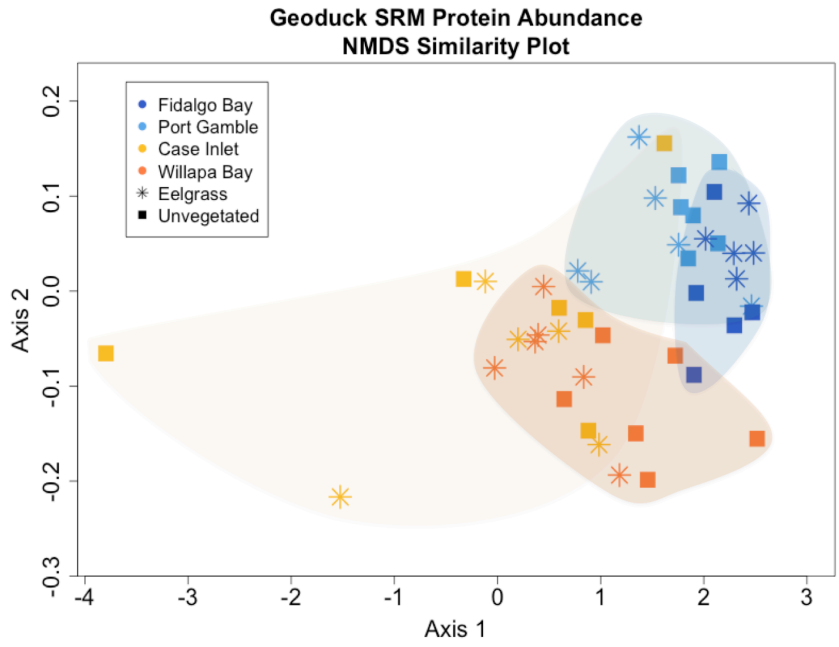
Supplemental Figure 4: Non-metric Multi-Dimensional Scaling plot (NMDS) showing patterns of similarity among DIA peptide abundances between technical replicates (same symbol/color), deployment bay (same color), and north/south region, where each point represents one geoduck. Relative proximity of points represents overall degree of peptide abundance similarity.



Supplemental Figure 5: Linear regression model fit of PRTC internal standard peptides in SRM against DIA, used to confirm identity of targeted peptides in SRm by calculating adjustment in retention time between SRM and DIA.

Supplemental Figure 6 (Interactive, online): Dilution curve peptide abundance ratios regressed against predicted ratios from serial sample dilutions. Peptides with slope coefficient 0.2<x<1.5 and adjusted R2 >0.7 were included in analysis. Link to interactive figure: http://owl.fish.washington.edu/generosa/Generosa_DNR/Dilution-Curve-Transitions.html

Supplemental Figure 7 (Interactive, online): Non-metric Multi-Dimensional Scaling plot (NMDS) of SRM technical replicates. Link to interactive figure: http://owl.fish.washington.edu/generosa/Generosa_DNR/NMDS-technical-replicate.html



Supplemental Figure 8: Non-metric Multi-Dimensional Scaling plot (NMDS) showing patterns of similarity among targeted SRM peptide abundances between deployment locations, where each point represents one geoduck. Relative proximity of points represents overall degree of peptide abundance similarity.