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

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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 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.

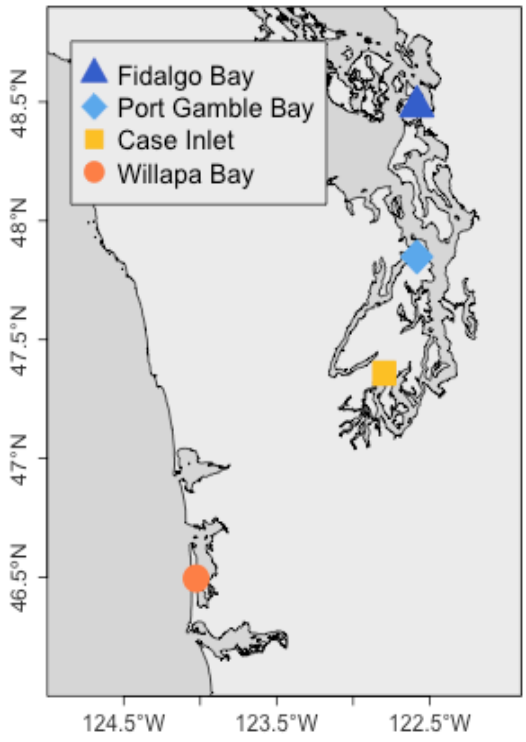

Figure 1: Geoduck juveniles were deployed for 30 days in 2 habitats (eelgrass beds, unvegetated) within 4 bays in Western Washington State

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.

131 # Protein Analysis

132 ## Protein Preparation

133 Relative protein abundance was ultimately assessed in a two-phase proteomics approach using
134 Selected Reaction Monitoring (SRM), with targets identified using Data Independent Acquisition
135 (DIA). Tissues were prepared separately for DIA and SRM, both following the protocol in
136 Timmins-Schiffman et al. (2014) with a few exceptions. For DIA, 8 ctenidia tissue samples were
137 analyzed, one sample from each location and habitat: FB-eelgrass (G048), FB-unvegetated
138 (G058), PGB-eelgrass (G077), PGB-unvegetated (G068), CI-eelgrass (G010), CI-unvegetated
139 (G018), WB-eelgrass (G131), WB-unvegetated (G119). For SRM new ctenidia samples were
140 examined, 12 individuals per bay (Fidalgo Bay, Port Gamble Bay, Case Inlet, Willapa Bay), with
141 6 from each habitat (eelgrass, unvegetated) for 48 samples total. Tissue was homogenized with
142 sterile plastic pestle in 100 µl lysis buffer (50 mM NH_4HCO_3, 6M urea solution) and sonicated
143 with Sonic Dismembrator (Fisher Scientific, Model 120) at 50% amplitude for ten seconds, three
144 times. Protein concentration was quantified via Pierce™ BCA Protein Assay Kit (ThermoFischer
145 Scientific, Waltham, MA USA).
146

147 ## Mini-Trypsin Digestion

148 Aliquots of protein (30 µg for DIA, 100 µg for SRM) were suspended in Lysis Buffer (50 mM
149 NH_4HCO_3 + 6 M urea solution) to a total volume of 100 µl followed by: 1) a 1 hour incubation at
150 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
151 hour at room temperature in dark with 200 mM iodoacetamide (20 µl); 3) 1 hour at room
152 temperature with 200 mM diothiothreitol (20 µl); 4) 1 hour at room temperature with 2 ug/µl Lysyl
153 Endopeptidase (Lys-C, Wako Chemicals) (3.3 µg); 5) overnight at room temperature in 25 mM
154 NH_4HCO_3 (800 µl) + high pressure liquid chromatography grade methanol (200 µl) + Pierce
155 Trypsin Protease, MS Grade (1 ug/µl, Thermo Scientific) at 1:30 enzyme:protein ratio (3.3 µg).
156 Samples were evaporated to near dryness at 4°C using a CentriVap Benchtop Vacuum
157 Concentrator.
158

159 ## Desalting

160 Samples were desalted to isolate peptides using MacroSpin Columns (Nest Group, 50-450 µl,
161 Peptide Protein C18). Peptides were reconstituted in 5% acetonitrile + 0.1% trifluoroacetic acid
162 (TFA) (100 µl), then 10% formic acid (70 µl) was added to achieve pH ≤2. Columns were
163 washed with 60% acetonitrile + 0.1% TFA (Solvent A, 200 µl) four times, then equilibrated with
164 5% acetonitrile + 0.1% TFA (Solvent B, 200 µl) three times. Peptides were bound to the column
165 by running the digest through the column twice, followed by peptide elution with two additions
166 each of Solvent A (100 ul). Columns were spun for 3 minutes at 3000 rpm on VWR Galaxy
167 16DH digital microcentrifuge at each stage. Samples were evaporated to near dryness at 4°C,
168 then reconstituted in the Final Solvent (3% acetonitrile + 0.1% formic acid) (60 µl for 0.5 µg/µl
169 final concentration of protein, and 50 µl for 2 µg/µl final concentration for DIA & SRM,
170 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.

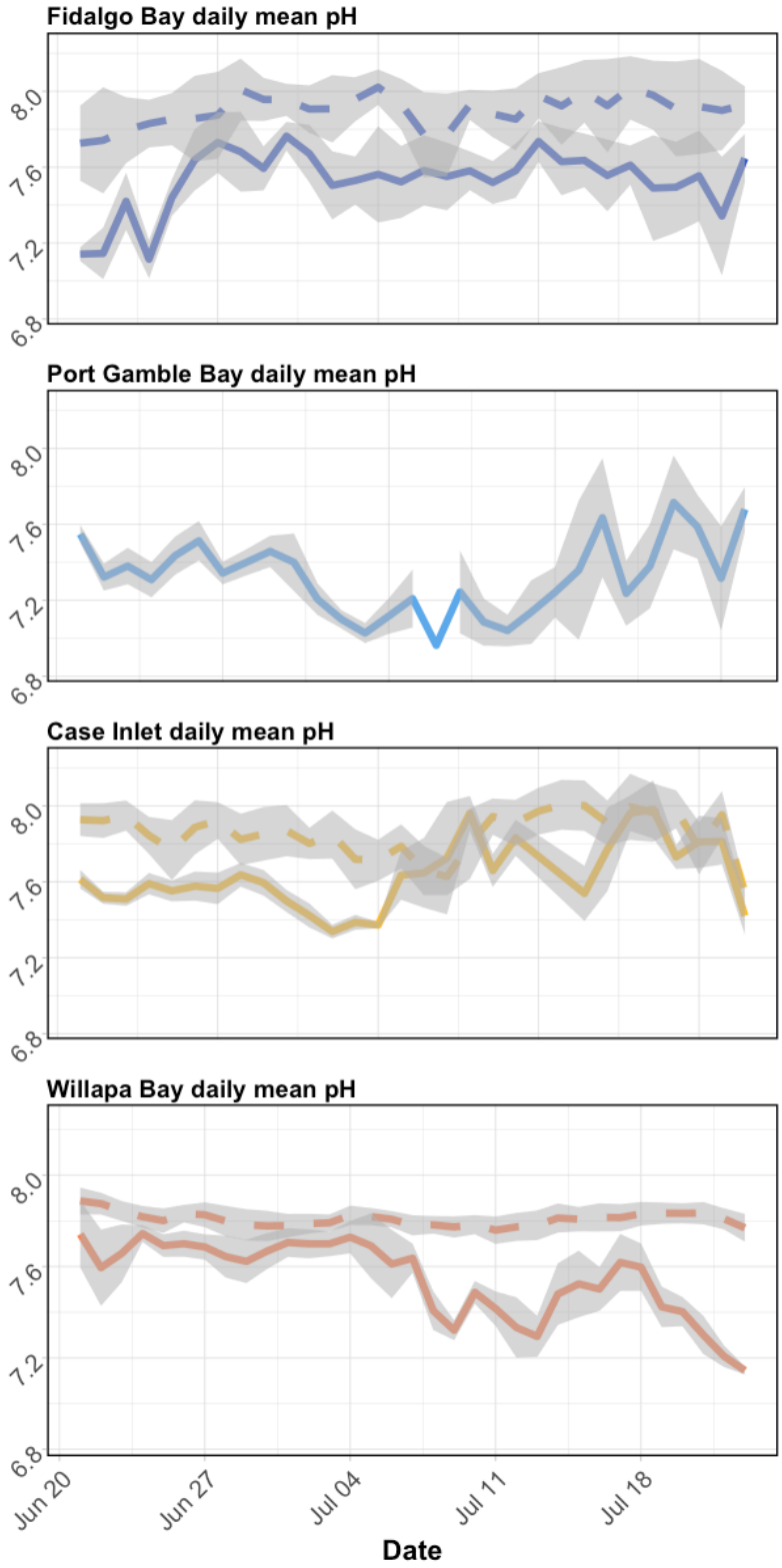

Figure 2: Daily mean pH in eelgrass (dashed lines) and unvegetated (solid lines) across bays during geoduck deployment. Gray ribbons denote daily standard deviation. Data from Port Gamble bay eelgrass are not included due to probe failure.

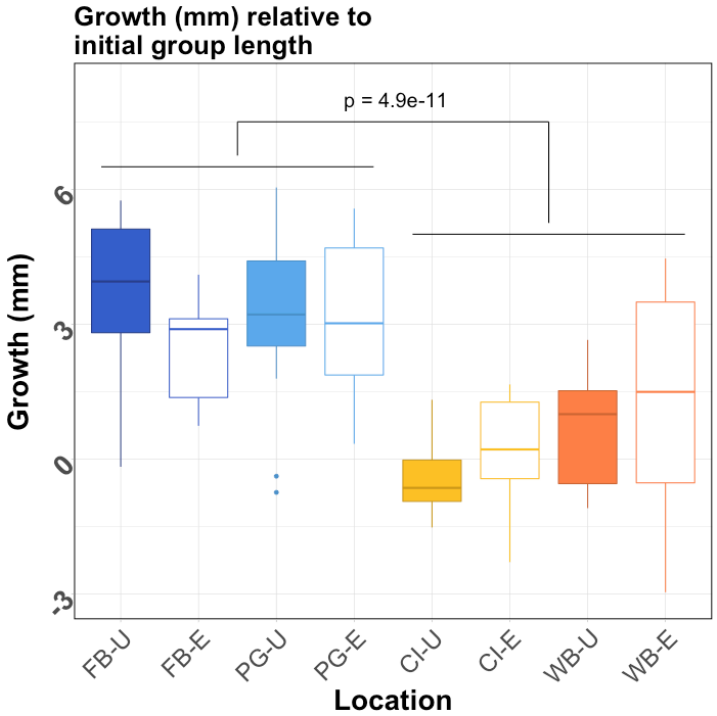

Figure 3: Geoduck shell growth after 30 days across Willapa Bay (WB), Case Inlet (CI), Port Gamble Bay (PG), and Fidalgo Bay (FB), where -U and -E represent unvegetated (filled boxes) and eelgrass habitats (empty boxes), respectively. Growth is relative to the mean initial shell length within deployment 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).

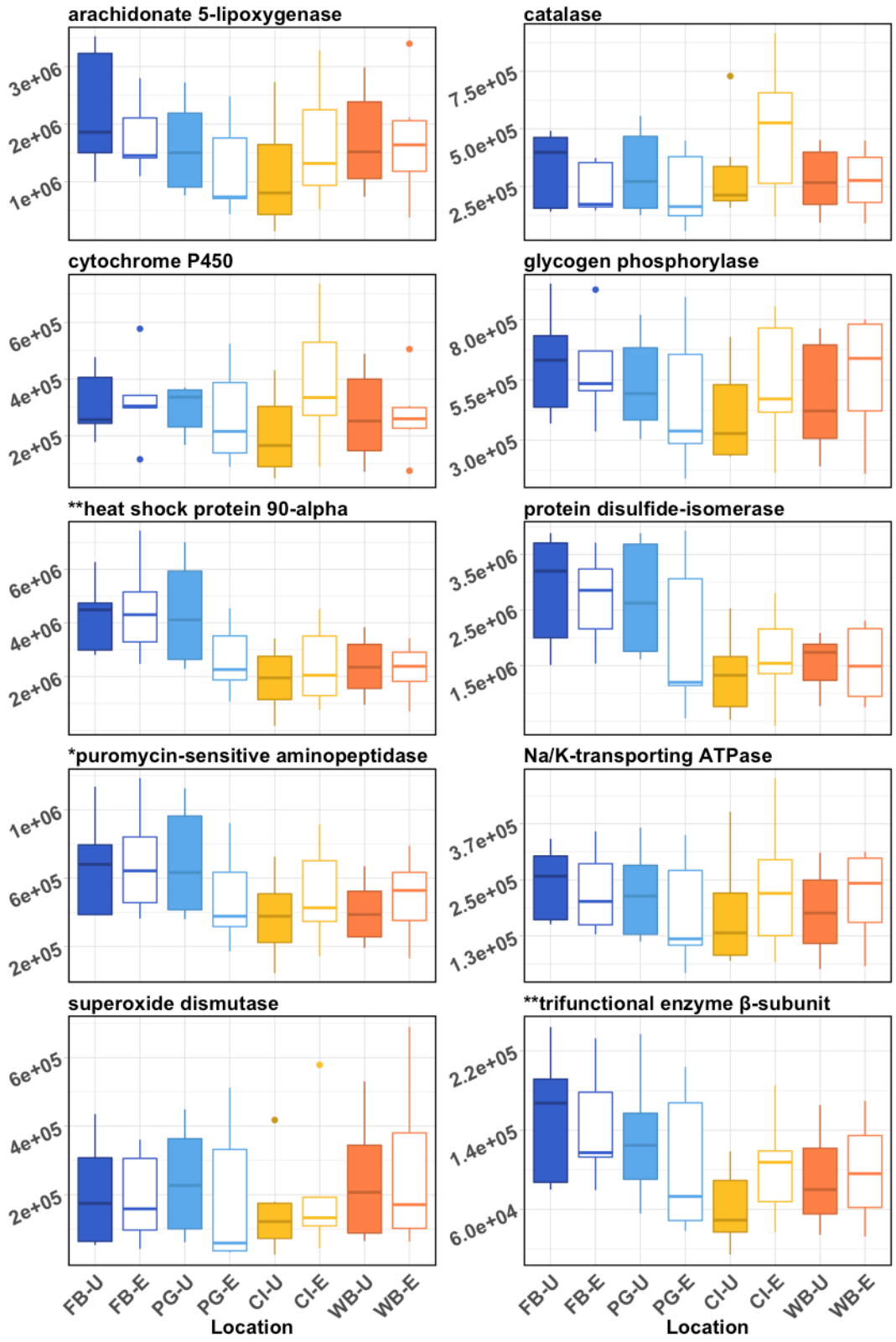
arachidonate 5-lipoxygenase
3e+06
2e+06
1e+06
catalase
7.5e+05
5.0e+05
2.5e+05
cytochrome P450
6e+05
4e+05
2e+05
glycogen phosphorylase
8.0e+05
5.5e+05
3.0e+05
**heat shock protein 90-alpha
6e+06
4e+06
2e+06
protein disulfide-isomerase
3.5e+06
2.5e+06
1.5e+06
*puromycin-sensitive aminopeptidase
1e+06
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.

346 ### Correlation between Environment, Abundance, and Growth

347 Growth positively correlated with peptide abundance for 8 of the 10 targeted proteins, including
348 the three proteins that were differentially abundant between bays (Table 3). Growth also
349 correlated with most environmental parameters (excluding salinity SD). Heat Shock Protein 90
350 correlated positively with dissolved oxygen SD. Mean and SD pH did not correlate with any
351 peptide abundance patterns or growth. None of the environmental parameters, nor growth,
352 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.

353 # Discussion

354 This study tested the effects of varying pH on geoduck, a valuable aquaculture species
355 in a natural setting, and confirmed that *Zostera marina* eelgrass can effectively alter local pH
356 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

acidification research is in its infancy, and these results are a snapshot into geoduck physiology at one developmental stage, with individuals from one genetic pool, and with present-day pH levels in Washington State. To best inform current and future geoduck aquaculture, further foundational studies are needed to elucidate the species' pH limits in conjunction with more acute environmental stressors.

This study also demonstrates applied use of systems such as eelgrass beds in estuaries to test pH effects in a natural system. There is growing interest in using macroalgae as an ocean acidification bioremediation tool, also known as phytoremediation (Greiner et al. 2013; Groner et al. 2018; Hendriks et al. 2014; Washington State Blue Ribbon Panel on Ocean Acidification 2012). Incorporating seagrass into shellfish-pH interaction studies can help evaluate the potential for merging mariculture with shellfish aquaculture to improve growing conditions for vulnerable cultured species.

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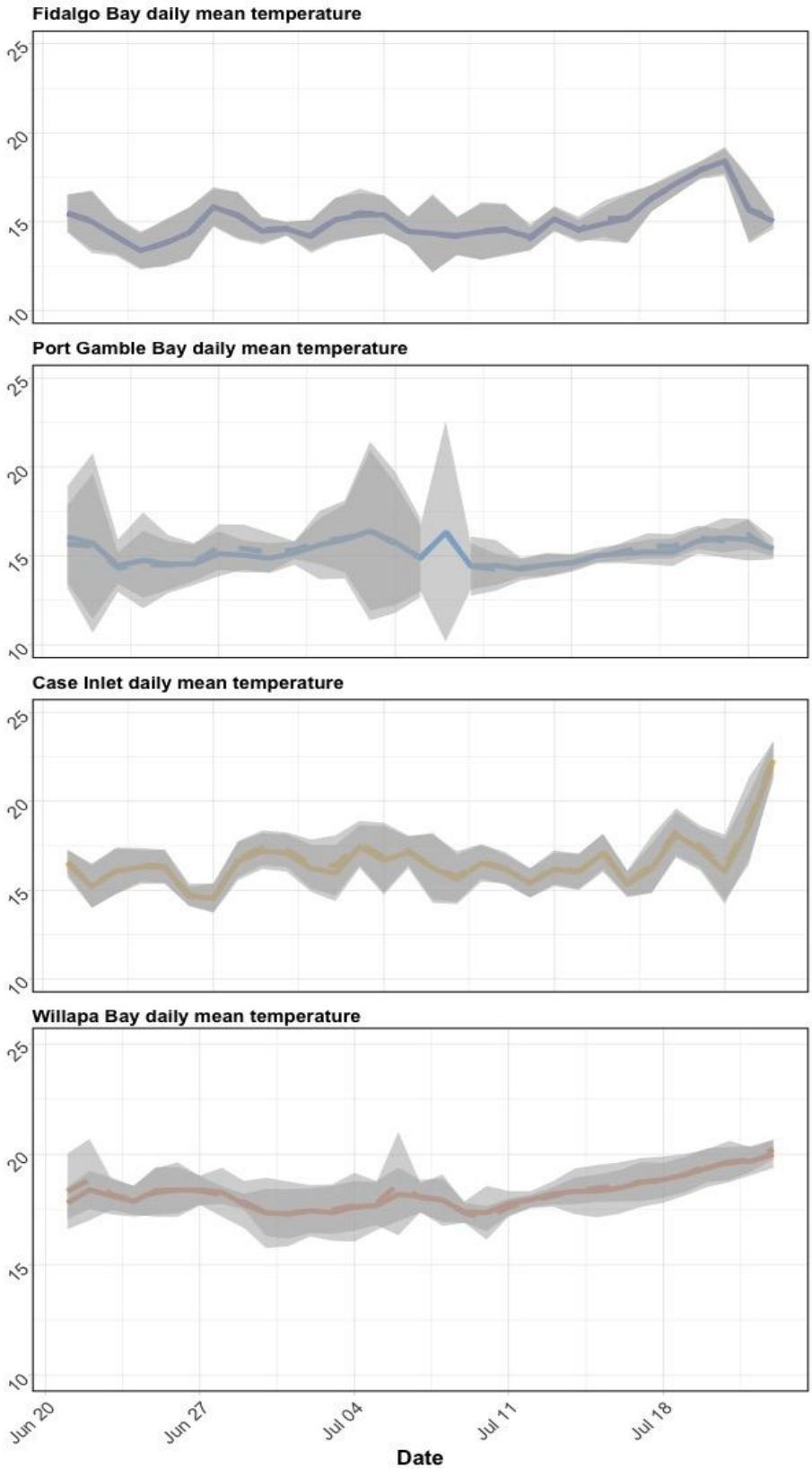
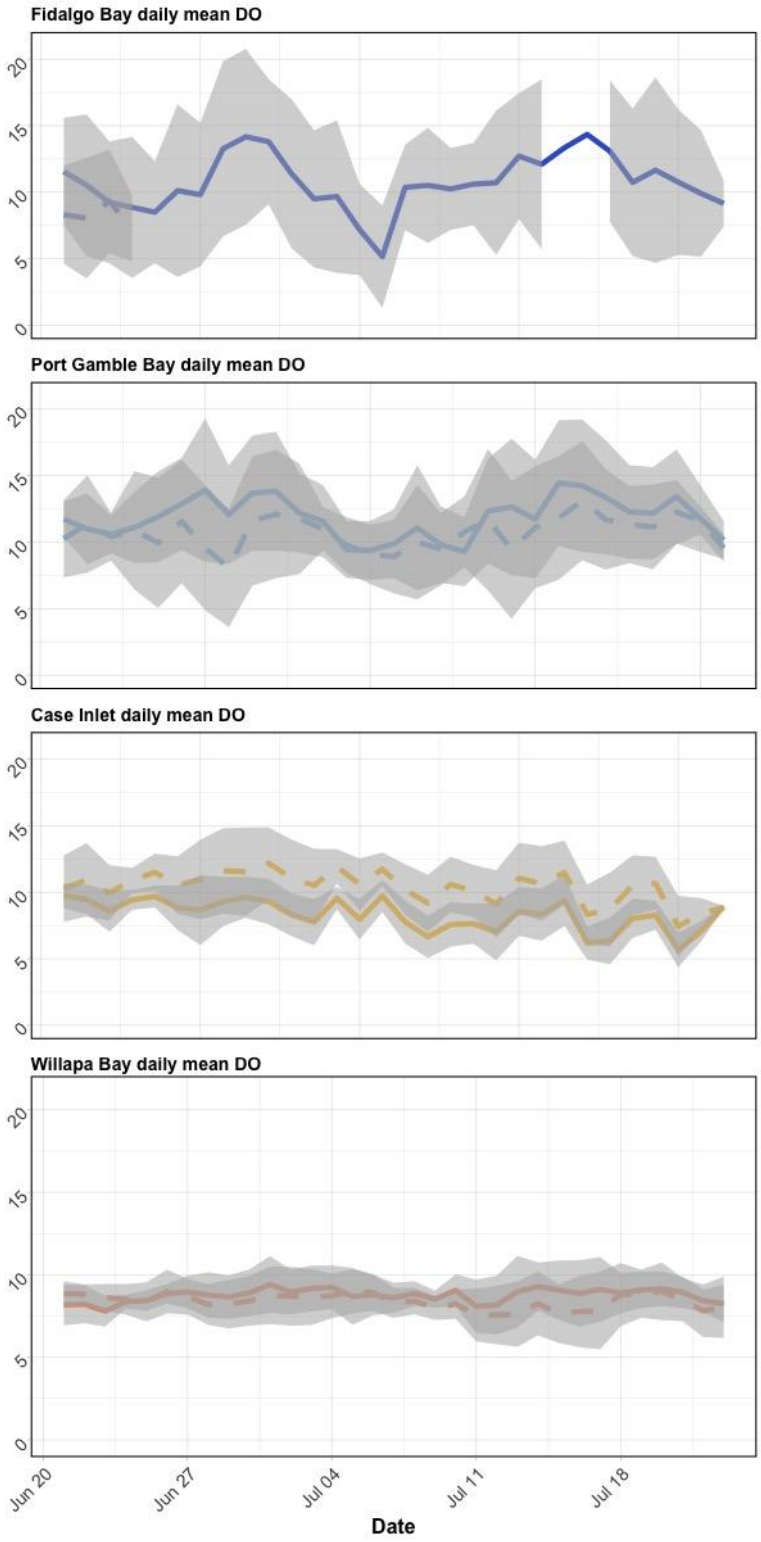
Supplemental Material

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.

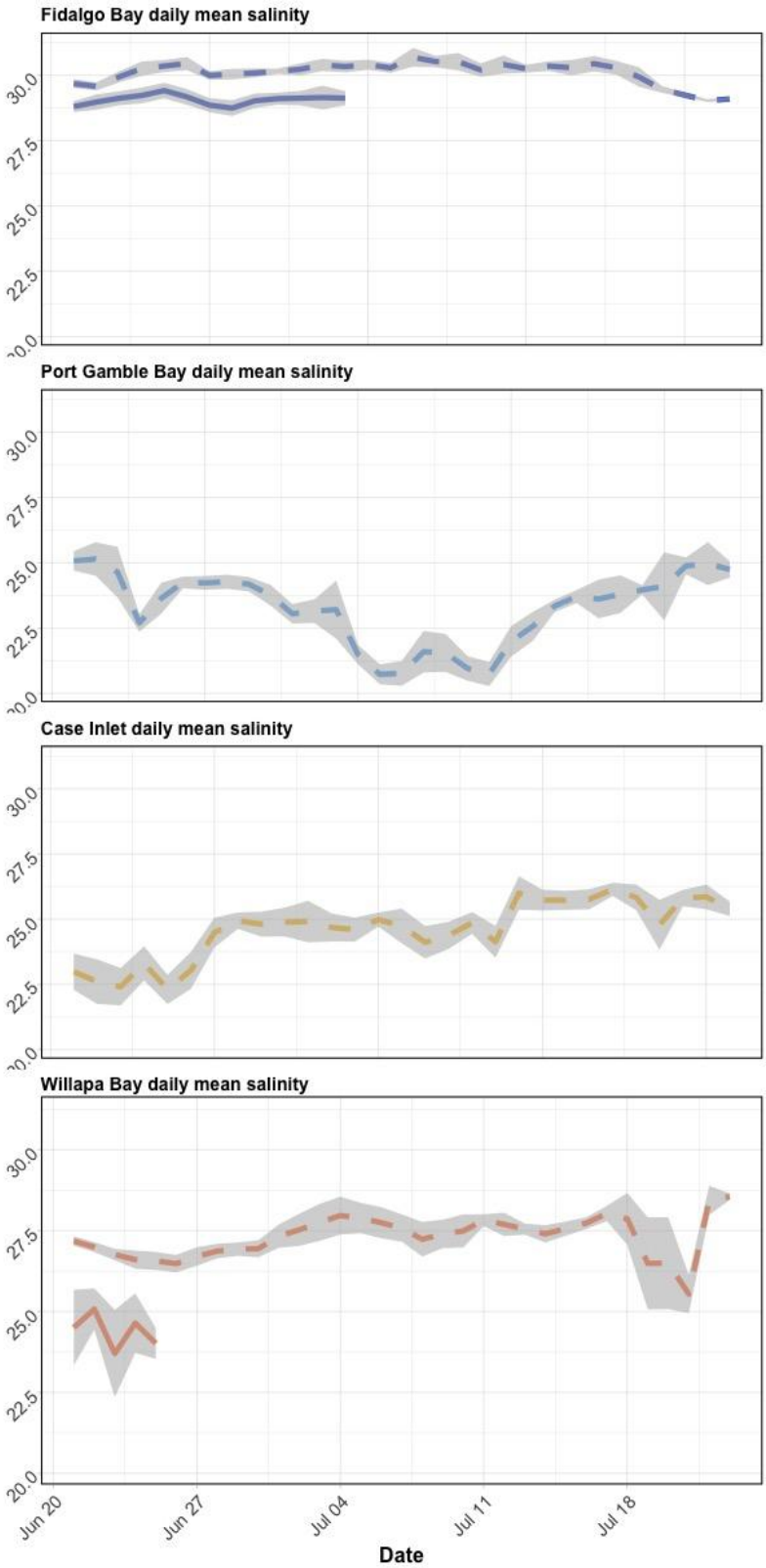
Deployment location	**FB-E**	**FB-U**	**PG-E**	**PG-U**	**CI-E**	**CI-U**	**WB-E**	**WB-U**	**Δ Habitat**	**Δ Bays**
% Survival	87	80	80	93	67	60	93	100	p= 0.88	p=0.25
Mean Relative % Growth (± SD)	16.2 (±7.3)	23.3 (±12.5)	22.2 (±12.5)	21.1 (±13.3)	1.2 (±8.3)	-2.8 (±5.3)	10 (±16.2)	4.5 (±8.3)	p=1	****p= 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.

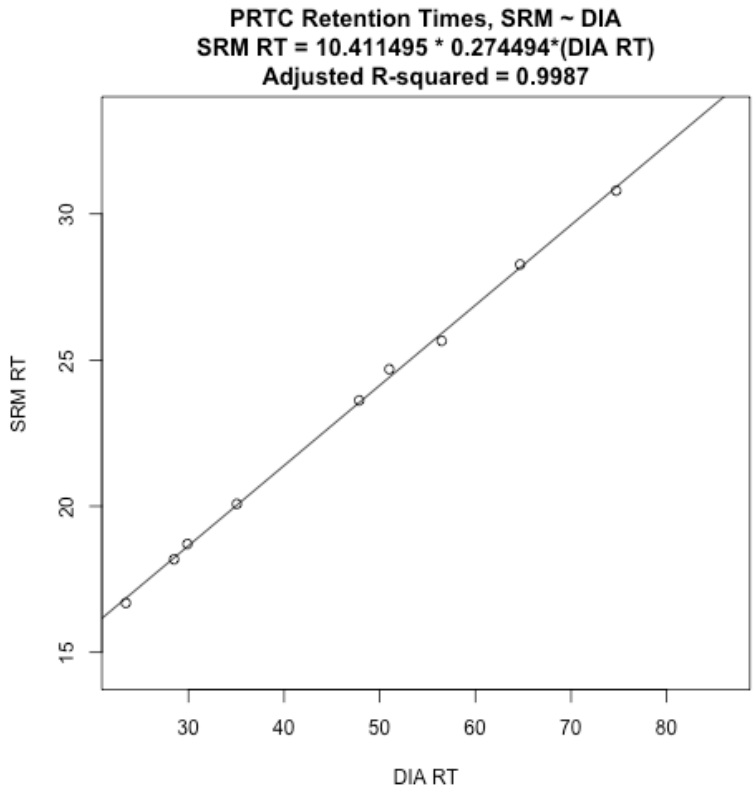
	Comparison	Df	Sum Sq	Mean Sq	F value	Pr(>F)	P.adj
all peptides combined	**North vs. South**	**1**	**18.2**	**18.2**	**10.9**	**0.00010**	**0.0014**
	Bay	**3**	**34.2**	**11.4**	**7.0**	**0.00012**	**0.0017**
	Habitat	1	1.2	1.2	0.75	5.4	14
arachidonate	North vs. South	1,133	7.4	7.4	1.3	0.26	3.6
	Bay	3,131	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

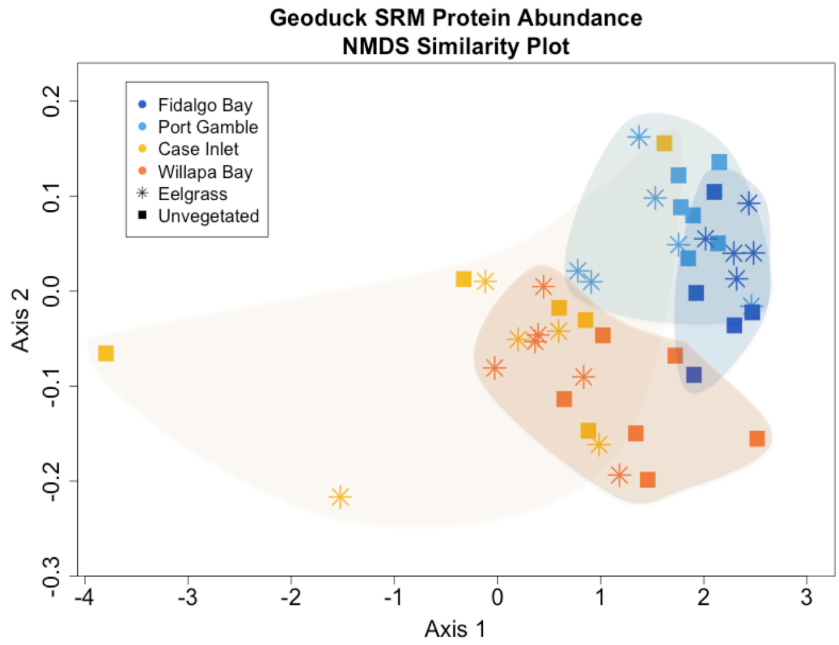
Supplemental Figure 4: Non-metric Multi-Dimensional Scaling plot (NMDS) showing patterns of similarity among DIA peptide abundances between technical replicates (same symbol/color), deployment bay (same color), and north/south region, where each point represents one geoduck. Relative proximity of points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