Characterization of Pacific oyster (*Crassostrea gigas*) proteomic response to natural environmental differences

Running head: Oyster proteomic response to environment

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Abstract

Global climate change is rapidly altering coastal marine ecosystems important for food production. A comprehensive understanding of how organisms will respond to these complex environmental changes can come only from observing and studying species within their natural environment. To this end, the effects of environmental drivers — pH, dissolved oxygen content, salinity, and temperature — on *Crassostrea gigas* physiology were evaluated in an outplant experiment. Sibling juvenile oysters were outplanted to eelgrass and unvegetated habitat at five different estuarine sites within the Acidification Nearshore Monitoring Network in Washington State, USA to evaluate how regional environmental drivers influenced molecular physiology. We tested effects environmental conditions at outplant sites and habitat to determine if macrophyte presence and diurnal cycling buffered pH conditions and changed the oysters' expressed proteome. A novel, two-step, gel-free proteomic approach was used to identify differences in protein abundance in *C. gigas* ctenidia tissue after a 29 day outplant by 1) identifying proteins in a data independent acquisition survey step and 2) comparing relative quantities of targeted environmental response proteins using selected reaction monitoring. While there was no difference in protein abundance detected between habitats or within Puget Sound, *C. gigas* outplanted at Willapa Bay had significantly higher abundances of antioxidant enzymes and carbohydrate metabolism proteins. Environmental factors at Willapa Bay, such as higher average temperature, could have driven this protein abundance pattern. These findings generate a suite of new hypotheses for lab and field experiments to compare the effects of regional conditions on physiological responses of marine invertebrates.

Keywords: proteomics, oysters, in situ measurements, estuarine systems, antioxidant enzymes, carbohydrate metabolism

Introduction

Global climate change will influence estuarine dynamics and impact the organisms that inhabit these environments. Estuaries are already variable across spatial and temporal scales in terms of phytoplankton production (Pennock and Sharp 1986), nutrient availability, (Paerl et al. 2014), heavy metal contamination (Liu et al. 2015), salinity (Banas et al. 2004), and carbonate chemistry (Pelletier et al. 2018; Feely et al. 2010; Ruesink et al. 2015; Baumann and Smith 2018). Since climate change will affect these parameters, it is important to consider how estuarine organisms will respond.

Proteomics, or the study of protein abundance and expression, can be used to shed light on physiological changes on a molecular level. Proteins direct all major cellular functions, thus examining protein abundance provides direct evidence of an organism's physiological response to the estuarine environment (Tomanek 2014). The proteome is dynamic, as it must rapidly respond to perturbation, providing mechanistic information that standard gene expression and mRNA quantification studies cannot (Veldhoen, Ikonomou, and Helbing 2012; Flores-Nunes, Gomes, et al. 2015). As a result of the proteome's dynamic nature, proteins analyzed at the time of collection represent an organism's response to the environment in near real-time. Discovery-based proteomic methods can unbiasedly elucidate responses to environmental drivers (Flores-Nunes, Gomes, et al. 2015). Several studies have connected protein abundances with changes in laboratory-simulated environmental conditions, identifying key proteins and mechanisms involved in specific environmental responses (Timmins-Schiffman et al. 2014; Tomanek 2014; Papakostas et al. 2012; Meng et al. 2017; Parker et al. 2011; Dineshram et al. 2016). While these studies provide insight into organismal

62 adaptation and physiology, laboratory studies alone cannot fully encapsulate the effects
63 of multiple environmental drivers within an ecosystem context (Riebesell and Gattuso
64 2014).

65 Although challenging, *in situ* field studies provide a necessary biological realism
66 when considering variable environments (Cornwall and Hurd 2016; Slattery et al. 2012).
67 Such experiments can be leveraged to study the effects of multiple environmental
68 drivers on organismal physiology and to incorporate realistic variability, as opposed to
69 examining the effect of a single stressor on an organism (Riebesell and Gattuso 2014).
70 Through transcriptomics Chapman et al. (2011) demonstrated the power of an *in situ*
71 experimental design for examining the impacts of regional environmental conditions on
72 Eastern oyster (*Crassostrea virginica*) physiology. Transcript signatures from *C.*
73 *virginica* sampled from various locations in southeastern United States revealed that
74 temperature, pH, salinity, dissolved oxygen and pollutant load at each location impacted
75 gene expression. Furthermore, they were able to disentangle the interactions of these
76 environmental factors on gene expression. RNA and protein abundances can be
77 influenced by several environmental factors, and *in situ* studies can determine which
78 drivers will be more important to consider for organismal physiology.

79 Marine invertebrates have proven to be informative bioindicators in proteomic
80 studies to examine the effects of *in situ* conditions on organismal physiological
81 responses to environmental change. When marine invertebrates have been exposed to
82 varying environmental conditions, proteomics has demonstrated changes in cellular
83 defense, immune responses, and genome function (Veldhoen, Ikonomou, and Helbing
84 2012). Changes in protein abundance in bivalves like blue mussels (*Mytilus edulis spp.*)

85 and the Pacific oyster (*Crassostrea gigas*) have been used to develop biomarkers for
86 environmental contaminants (Beyer et al. 2017; Slattery et al. 2012). Proteomic
87 responses to natural environmental drivers have also been evaluated in bivalves. For
88 example, shotgun proteomic analysis of *M. edulis* ctenidia from Baltic Sea microcosms
89 revealed that low salinity conditions impact abundance of cytoskeleton proteins and
90 signalling and intracellular membrane trafficking pathways (Campos et al. 2016). With a
91 growing wealth of genomic information for these species, and the importance of this
92 information for commercial aquaculture, understanding how these species fare under
93 different environmental conditions is critical.

94 Pacific oyster (*Crassostrea gigas*) rearing in estuarine environments in
95 Washington State, USA provides a perfect system to examine the effect of *in situ*
96 environmental conditions on the expressed proteome. *C. gigas* are extensively farmed
97 in two different estuarine systems that show substantial regional variation: Puget Sound
98 and Willapa Bay. Puget Sound is a complex estuarine system with interconnected sub-
99 basins, each with different freshwater inputs, residence times, and stratification levels
100 (Feely et al. 2010; Pelletier et al. 2018; Bianucci et al. 2018). Willapa Bay is a large
101 shallow estuary on the Pacific coast that exchanges approximately half its water volume
102 with the Pacific Ocean at each tide (Banas et al. 2004, 2007). Seasonality and location
103 within Puget Sound dictates temperature, dissolved oxygen, salinity, and pH conditions,
104 while Willapa Bay conditions are influenced by diurnal fluctuations and proximity to
105 either the ocean or rivers draining into the bay (Pelletier et al. 2018; Feely et al. 2010;
106 Banas et al. 2007; Ruesink et al. 2015). Both Puget Sound and Willapa Bay also host
107 eelgrass beds (*Zostera spp.*) that affect environmental conditions, such as oxygen

concentrations, on diurnal time scales and may provide refuge from ocean acidification or rapid change in temperature through photosynthetic activity. Understanding how different aquaculture grow-out locations and habitats will affect the oyster's ability to persist through environmental change is crucial for the industry and the ecosystem.

The purpose of this study was to use proteomic techniques to uncover the impacts of environmental drivers on Pacific oyster physiological outcomes in estuarine environments in Washington State, USA. Naturally existing environmental variation was harnessed by outplanting *C. gigas* in different locations within Puget Sound and Willapa Bay, and habitat effects were taken into consideration by placing oysters in eelgrass and unvegetated habitats. Gel-free proteomic methods were used to examine the effects of outplant conditions on relative quantities of all expressed proteins in a series of *in situ* experiments in order to identify differentially abundant proteins. We predicted that differences in environmental drivers at each outplant location and within outplant habitats would yield unique protein abundance patterns. Oysters at outplant locations with higher temperatures, lower dissolved oxygen content, lower salinity, or lower pH may have higher abundances of proteins related to environmental response. Eelgrass beds were expected to ameliorate stressful conditions, resulting in lower abundances of environmental stress response proteins than oysters in unvegetated habitats.

Methods

Shellfish deployment

Sibling *C. gigas* (average shell length = 27.2 cm) were outplanted for 29 days starting June 19, 2016 at five locations: Case Inlet (CI), Fidalgo Bay (FB), Port Gamble

Bay (PG), Skokomish River Delta (SK), and Willapa Bay (WB) in Washington State, USA (Table 1; Figure S1). These sites were selected for differences in environmental parameters, as well as for the presence of unvegetated areas and eelgrass beds within each site. All sites were part of the Acidification Nearshore Monitoring Network, a network of sensors placed in various Washington locations to monitor marine chemistry (ANeMoNe; Washington Department of Natural Resources). At each site, fifteen juvenile oysters were placed in bags directly onto the substrate at a tidal height of -1.5 MLLW, both inside and outside of eelgrass beds (n=15 per habitat type), for a total of thirty outplanted oysters per site. Oysters were housed in exclusion cages to prevent predation. Custom-built Durafet-based sensors (Honeywell) were used to monitor pH. Commercially-available MiniDOT loggers (Precision Measurement Engineering) were used to measure dissolved oxygen, and Odyssey loggers were used to measure conductivity. All sensors recorded temperature measurements, and all sensors logged at 10-minute intervals across the outplant period, with the exception of SK, where sensors were installed 21 days into the outplant period.

Juvenile oysters remained at each site for a 29-day exposure period. Oyster shell length was measured before and after the outplant period. Because the ctenidia is the primary site where oysters interact with the environment, ctenidia samples were dissected at the end of the outplant and held on dry ice until storage at -80ºC (Beyer et al. 2017; Meng et al. 2017; Meistertzheim et al. 2007).

Environmental data was treated as follows. Conductivity observations were removed when less than 0, which occurs when the instrument is dry. Remaining observations were converted to salinity measurements using the *swSCTp* function in

153 the *oce* package in R (Kelley and Richards 2018; R Core Team 2018), with temperature
154 at 25ºC and pressure at 10 dbar. For dissolved oxygen, pH, and salinity datasets, data
155 were removed when collected by probes 1) during low tide or 2) when tidal depth was
156 less than one foot to remove readings where the probes may have been exposed.
157 These values were retained for temperature datasets. Outliers were screened using the
158 Tukey method for temperature, dissolved oxygen, pH, and salinity datasets (Hoaglin,
159 Iglewicz, and Tukey 1986). Uniform outplant tidal heights were checked using *prop.test*
160 in R (R Core Team 2018). R Scripts are available in the supplementary Github
161 repository (S2).

162 ***Protein Discovery***

163 To identify select protein targets for characterization across locations and
164 environmental conditions, a subset of tissue samples (2 from each site) were analyzed
165 with data independent acquisition (DIA) mass spectrometry analysis (Table S3).

166 Protein Quantification

167 Tissue samples were homogenized in a solution of 50 mM NH_4HCO_3 with 6M
168 Urea (500µl). Tissues were then sonicated 3 times (Fisher Scientific Sonic
169 Dismembrator Model 100) for 10 seconds each and cooled between sonications in a
170 bath of ethanol and dry ice. Protein quantities were measured with the ThermoScientific
171 BCA Protein Assay Kit microplate assay with a limited quantity of sonicated sample (11
172 µL). The protein concentration was measured via spectroscopy at 540 nm in a
173 Labsystems (Waltham, MA) Multiskan MCC/340 and accompanying Ascent Software

174 Version 2.6. Protein concentrations were calculated based on a standard curve with
175 BSA (Pierce) per manufacturer's instructions.

176 Protein Digestion

177 Protein digestion followed the protocol outlined in Timmins-Schiffman et al.
178 (2013). To each sample of 30 µg protein,1.5 M Tris pH 8.8 buffer (6.6 µL) and 200 mM
179 TCEP (2.5 µL) were added. After solvent additions, each sample's pH was verified to be
180 basic (pH ≥ 8), and placed on a 37ºC heating block for one hour. Iodoacetamide (200
181 mM, 20 µL) was then added to each sample to maximize digestion enzyme access to
182 protein cleavage sites. Samples were covered with aluminum foil to incubate in the dark
183 for 1 hour at room temperature. Afterwards, diothiolthreitol (200 mM, 20 µL) was added
184 and samples were incubated at room temperature for one hour. Lysyl Endopeptidase
185 (Wako Chemicals) was then added to each sample in a 1 µg enzyme:30 µg oyster
186 protein ratio, followed by one hour of incubation at room temperature. Urea was diluted
187 with NH_4HCO_3 (25 mM, 800 µL) and HPLC grade methanol (200 µL). Trypsin
188 (Promega) was added to each sample in a 1 µg trypsin: 30 µg oyster protein ratio for
189 overnight digestion at room temperature.

190 Peptide Isolation

191 After overnight incubation, samples were evaporated to near dryness at 4ºC with
192 a speedvac (CentriVap ® Refrigerated Centrifugal Concentrator Model 7310021).
193 Samples were then reconstituted in 100 µL of a 5% Acetonitrile and 0.1%

194 Trifluoroacetic Acid (Solvent B) to isolate peptides. If samples were not at pH ≤ 2, 10-20
195 μL aliquots of 10% Formic Acid were added until this pH was achieved.

196 Before desalting peptide samples, Macrospin C18 columns (The Nest Group)
197 were prepared by adding 200 μL of a 60% Acetonitrile with 0.1% Trifluoroacetic Acid
198 (Solvent A). The columns were spun for three minutes at 2000 rpm, and flow-through
199 liquid from the column was discarded. The spinning and discarding process was
200 completed a total of four times.To wash columns, 200 μL of Solvent B was added to
201 each column. The columns were once again spun for three minutes at 2000 rpm and
202 liquid was discarded afterwards; the solvent addition, spinning, and discarding process
203 was completed a total of three times.

204 To bind peptides to the columns, digested peptides were added to prepared
205 columns, then the columns were spun at 3000 rpm for three minutes. The filtrate was
206 pipetted back onto the column and spun again at 3000 rpm for three minutes. Solvent B
207 (200 μL) was added to each column three separate times, then the column was spun for
208 three minutes at 3000 rpm to wash salts off the column.

209 Peptides were eluted with two additions of 100 μL Solvent A to each column.
210 Columns were spun at 3000 rpm for three minutes and the peptide fraction (filtrate) was
211 reserved. Samples were placed in a speed vacuum at 4ºC until they were nearly dry
212 (approximately two hours) to dry peptides. Peptides were reconstituted with 60 μL of 3%
213 Acetonitrile + 0.1% Formic Acid, and stored at -80ºC.

214 Internal Standard Addition

215 Peptide Retention Time Calibration (PRTC; Pierce) is a mix of known peptides in
216 known quantities used as an internal standard to ensure consistency of peptides
217 detected and measured throughout a mass spectrometry run. The stock solution of
218 PRTC was diluted to 0.2 pmol/µL PRTC using 3% Acetonitrile with 0.1% Formic Acid. In
219 a clean centrifuge tube, 6 µg of oyster protein and 0.376 pmol of PRTC were mixed
220 together. Sample volume was brought up to 15 µL using a 3% acetonitrile and 0.1%
221 formic acid solution. A quality control solution was also prepared (1 µL PRTC + BSA:3
222 µL 3% Acetonitrile and 0.1% Formic Acid solution).

223 ***Data Independent Acquisition Mass Spectrometry***

224 Peptides were analyzed on an Orbitrap Fusion Lumos mass spectrometer
225 (Thermo Scientific) for Data Independent Acquisition Mass Spectrometry (DIA). DIA
226 analyses were completed as a comprehensive, non-random analytical method for
227 detecting all peptide ions present within a sample. The detection of peptides was then
228 leveraged to develop a targeted proteomics assay for quantification (see Selected
229 Reaction Monitoring Assay). A 30 cm analytical column and 3 cm pre-column were
230 packed in-house with 3 µm C18 beads (Dr. Maisch). Samples were run in a randomized
231 order. A blank injection followed each sample, with procedural blanks run at the very
232 end. Every injection was 3 µL, which included 1 µg of oyster protein and 0.0752 pmol of
233 PRTC. Peptides were analyzed in MS1 over the *m/z* range of 450-950 with 12 *m/z* wide
234 windows with 5 *m/z* overlaps (Egertson et al. 2013). MS1 resolution was 60,000 and
235 AGC target was 400,000 with a three second cycle time. The MS2 loop count was set to

236 20 and MS2 data was collected with a resolution of 15,000 on charge state of 2 with an
237 AGC target of 50,000. No dynamic exclusion was used (Table S4).

238 ***Peptide-Centric Proteomic Analyses***

239 Unknown peptide spectra from mass spectrometry samples were matched with
240 known peptides using Peptide-Centric Analysis in the PECAN software (Ting et al.
241 2015). Raw mass spectrometry files were converted to mzML files, then demultiplexed
242 using the MSConvert command line interface (S5; Chambers et al. 2012). The *C. gigas*
243 proteome was digested with in silico tryptic digest using Protein Digestion Simulator
244 (S6) . All known peptides from the mzML files were identified in an comparison to the
245 digested *C. gigas* proteome (S6).
246
247 The PECAN-generated spectral library (.blib) file was used to detect peptides of interest
248 in raw DIA files in Skyline (MacLean et al. 2010). Skyline identified peptides using
249 chromatogram peak picking, where ions that elute at the same time and mass are
250 detected as a peptide (S7). All PRTC peptides and approximately 100 different oyster
251 proteins and their peptide transitions were manually checked for retention time and
252 peak area ratio consistency to determine a Skyline auto peak picker error rate (24.3% ±
253 25%, range: 0% to 100%).
254 Proteins had to satisfy 4 criteria to be considered appropriate targets for the
255 study. 1) To select proteins for a targeted MS assay, protein data was first evaluated in
256 Skyline to ensure there was no missing data for any peptide or sample. 2) Peaks with
257 significant interference were also not considered. 3) Proteins needed at least two
258 peptides with three transitions per peptide to qualify as a potential target for

259 downstream assays. 4) Proteins with annotated functions related to oxidative stress,
260 hypoxia, heat shock, immune resistance, shell formation growth and cellular
261 maintenance were considered for targets to address hypotheses of response to in situ
262 environmental differences and eelgrass vs. bare habitat. From this list, fifteen proteins
263 (41 peptides and 123 transitions total), with various environmental response and
264 general maintenance functions, were selected as targets (Table 2).

265 ***Selected Reaction Monitoring Assay***

266 Following the protein discovery phase (DIA), proteins were isolated as described
267 above from an additional five randomly selected samples per site and habitat
268 combination (for a total of 5 oysters per group) and analyzed with Selected Reaction
269 Monitoring (SRM). Samples were prepared as described for DIA, except tissue samples
270 were homogenized in 100 µL, and peptide samples were evaporated at 25ºC after
271 peptide isolation.

272 Proteins of interest identified from the DIA analysis were used as targets in a
273 SRM assay following the workflow and informatic pipeline of Timmins-Schiffman et al.
274 2016; 2017. Target peptide transitions were monitored using SRM on a Thermo TSQ
275 Vantage. SRM data were collected during a gradient of 2-60% acetonitrile over 40
276 minutes. All samples were run in technical duplicates in a randomized order with a 1 µg
277 oyster peptide and 0.0752 pmol PRTC injection. A quality control injection and blank
278 injection were run after every five sample injections, and PRTC peptides were
279 monitored throughout the experiment.

280 ## Target Peptide Specificity

281 To ensure SRM assay specificity to oyster peptides of interest, oyster peptides
282 were diluted in a background matrix of similar complexity (Pacific geoduck — *Panopea*
283 *generosa* — peptides), then analyzed using the oyster SRM assay. An oyster-specific
284 SRM target would decrease in abundance with a decreasing abundance of oyster
285 peptides in a mixture. Non-specific peptides — more likely to be found in background
286 matrix of similar complexity — or peptides susceptible to interference would not
287 correlate with oyster peptide abundance, and therefore, would be uninformative. Five *C.*
288 *gigas* samples used for SRM were randomly selected and pooled in equal quantities. A
289 ten-sample oyster:geoduck dilution series was prepared and run using the same
290 methods as other SRM samples (Table S8).

291 ## Target Analysis

292 Raw SRM files, a background *C. gigas* proteome, and the PECAN spectral
293 library file from DIA were used to create a Skyline document (S9, S10). Correct
294 transition peaks were selected based on predicted retention times from DIA results by
295 comparing the relative retention times between identical PRTC peptides in the DIA and
296 SRM datasets (R^2 = 0.99431). Based on peptide specificity analyses, heat shock protein
297 70 B2 and one constituent peptide of
298 glucose-6-phosphate 1-dehydrogenase were removed from analyses (S11).
299 Coefficients of variation were calculated between technical replicates for each
300 peptide transition. Any sample missing data for more than 50% of peptide transitions
301 was deemed poor quality for downstream analyses and excluded. Abundance data was
302 normalized using total ion current (TIC) values from the mass spectrometer.

303 Consistency between technical replicates was verified in remaining samples using a
304 non-metric multidimensional scaling plot (NMDS) with TIC-normalized data and a
305 euclidean dissimilarity matrix, and technical replicates were averaged after verification
306 with the NMDS. Analysis of Variance and post-hoc Tukey's Honest Significant
307 Difference tests were used to determine significant differences in peptide abundance
308 among sites for average abundances. The Benjamini-Hochberg method with a false
309 discovery rate (FDR) of 10% was used to correct for multiple comparisons.

310 **Results**

311 ***Conditions at Outplant Locations***

312 Over the course of the outplant, Willapa Bay (WB) had the highest average
313 temperatures (Table 3, Figure S12). There were no large differences in mean pH
314 between locations (Table 3). Oysters at Case Inlet spent more time at low tide than
315 those at the other four sites ($\chi 2 = 25.29$, df = 4, p = 4.408e-05).

316 ***Data Independent Acquisition Mass Spectrometry***

317 Out of 39,816 predicted proteins in the *C. gigas* FASTA proteome, 9,047 proteins
318 were detected in *C. gigas* across five sites and two habitats using DIA (Skyline auto
319 peak picker error rate 24.3% ± 25% , range: 0% to 100%). Proteins detected included,
320 but were not limited to, those annotated from processes such as responses to hypoxia
321 and oxidative stress, removal of superoxide radicals, protein folding, muscle organ
322 development and negative regulation of apoptosis.

323 ***Selected Reaction Monitoring Assay***

324 Differential abundance of protein targets was evaluated at the peptide level after
325 combining technical replicates (Figure S16, Figure S17). Proteins were considered
326 differentially abundant if at least one monitored peptide was differentially abundant.
327 There was no significant difference in SRM peptides between unvegetated and eelgrass
328 habitats across sites (Figure S18). Abundance data from both habitats were pooled for
329 downstream analyses comparing protein abundances among sites.

330 Peroxiredoxin-5 (PRX), thioredoxin reductase 3 (TrxR), catalase (EC 1.11.1.6)
331 (CAT), NAD(P) transhydrogenase (NADPt), glucose-6-phosphate 1-dehydrogenase
332 (G6PD), protein phosphatase 1B (PP1), and glycogen phosphorylase (GPH) had
333 differentially abundant peptides between Willapa Bay and the other four locations (Table
334 S19; ANOVA and Tukey's Honest Significant Difference, FDR = 0.1). These proteins
335 are either involved in oxidative stress response or related to carbohydrate metabolism
336 (Table 4).

337 All differentially abundant proteins were detected at higher levels in the WB
338 oysters than in oysters from the other four PS locations (CI, FB, PG, and SR),
339 regardless of protein function (Figure 1). No differences in protein abundance were
340 detected among the PS sites (Table S19**;** ANOVA and Tukey's Honest Significant
341 Difference).

342 **Discussion**

343 Among sibling Pacific oysters placed at five sites in Washington State, we found
344 higher abundances of antioxidant enzymes and carbohydrate metabolism proteins in

345 oysters outplanted at Willapa Bay (WB). Significant differences in protein abundances
346 were detected between WB and all four PS sites; no differences were observed within
347 PS. Outplanted individuals at WB had higher abundances of antioxidant enzymes and
348 proteins involved in carbohydrate metabolism. Increased antioxidant activity and
349 changes in carbohydrate metabolism have been consistently documented as responses
350 to extreme temperatures, hyposalinity, low pH and air exposure (Tomanek 2014; Zhang
351 et al. 2015). Higher average temperatures may have driven differential protein
352 abundances (Table 3, Figures S12).

353 To our knowledge, this is the first time combined use of DIA and SRM analytical
354 methods have been applied in an ecological context. Previously, both gel-based
355 (Campos et al. 2016; Tomanek 2014; Zhang et al. 2015; Parker et al. 2011) and gel-free
356 (Timmins-Schiffman et al. 2014; Timmins-Schiffman et al. 2017; Campos et al. 2016;
357 Dineshram et al. 2016; Müller et al. 2018) proteomic methods have been used in lab
358 settings to evaluate the effects of environmental conditions on organismal physiology,
359 with gel-based methods having a limited dynamic range. Similar gel-free methods has
360 also been used to examine physiology of other non-model marine species (Kültz et al.
361 2015; Plumel et al. 2013). Our methods allowed for increased acquisition of proteomic
362 data for a non-model organism. We detected over 9,000 proteins — far more than
363 previous studies have detected. The application of a novel, two step, gel-free proteomic
364 approach broadened the scope of proteins detected in the DIA phase, thus revealing
365 more possibilities for SRM target design.The SRM assay allowed for increased
366 detection when protein abundance was relatively low.

367 ***Antioxidant enzymes***

368 Higher antioxidant enzyme abundances can be a direct response to an increase
369 in reactive oxygen species (ROS) that arise from changes in oxygen conditions (Limón-
370 Pacheco and Gonsebatt 2009; Zhang et al. 2015). During electron transport, oysters
371 can produce ROS that induce oxidative stress if not neutralized. (Abele et al. 2007;
372 Limón-Pacheco and Gonsebatt 2009). Peroxiredoxin-5 (PRX), thioredoxin reductase
373 (TrxR), and catalase (CAT) scavenge these ROS and degrade them before they cause
374 cellular harm (Tomanek 2014; Sussarellu et al. 2012; Limón-Pacheco and Gonsebatt
375 2009; Flores-Nunes, Mattos, et al. 2015; Sussarellu et al. 2010), while NAD(P)
376 transhydrogenase (NADPt) maintains the cellular redox state. Higher abundances of
377 antioxidant enzymes in WB oysters suggest the need for ROS scavenging to
378 acclimatize to local conditions.

379 The shallow bathymetry of WB may have also contributed to elevated ROS and
380 the increased need for antioxidant enzymes. At high tide, shallow waters would warm
381 much faster, leading to the observed higher temperatures at WB (Table 1). Warmer
382 waters at low tide may prompt oysters to spend more times with their shells closed,
383 producing higher levels of ROS that would need to be neutralized. Lack of oxygen and
384 higher pCO_2 while the oyster is closed would lead to lower hemolymph pH, causing
385 higher levels of ROS. Oysters would respond through increased abundance of
386 antioxidant enzymes (PRX, CAT, TrxR, and NADPt).

387 ROS are produced in response to many environmental changes, thus biomarkers
388 of ROS scavenging are difficult to link to a single environmental difference in a variable
389 and complex setting. In particular, reduction of ROS species was found to be a common

390 response to both increased temperatures and aerial exposure in another experiment
391 with *C. gigas* (Zhang et al. 2015). Because ROS mediation is a conserved response to
392 several stressors, it is possible that environmental parameters we did not measure (e.g.,
393 contaminants, microbiota abundance, trace metals) could explain the observed variation
394 in antioxidant enzyme abundance. Future work at these locations should take these
395 variables into account.

396 ***Carbohydrate metabolism***

397 Higher abundance of carbohydrate metabolism proteins at WB — protein
398 phosphatase 1B (PP1), glucose-6-phosphate 1-dehydrogenase (G6PD), and glycogen
399 phosphorylase (GPH) — indicate increased glycogen catabolism at this location (Table
400 3). Increased glycogen catabolism (PP1 and GPH) has been reported with respect to
401 environmental drivers (Fuhrmann et al. 2018; Papakostas et al. 2012; Bacca et al. 2005;
402 Serrano et al. 1993; David et al. 2005). For example, enhanced GPH activity was found
403 in *M. galloprovincialis* responding to thermal stress to provide more energy during high
404 temperature acclimation (Serrano et al. 1993). *C. gigas* outplanted at WB could be
405 using the same process to acclimatize to higher average temperatures. Warmer WB
406 temperatures could have also sped up basal metabolism, necessitating igher
407 abundances of PP1 and GPH to catabolize glycogen stores at a faster rate (Wu et al.
408 1998; She et al. 2015; Bacca et al. 2005). Increasing the amount of energy available to
409 bivalves by catabolizing glycogen could explain higher abundance of PP1 and GPH in
410 WB oysters.

411 Increased glycogen catabolism at WB may also be explained by decreased rates
412 of food capture due to less time spent filter feeding or less food availability at this

413 location. With closed shells, *C. gigas* are unable to filter-feed and would need to
414 catabolize glycogen for energy, facilitated by increased abundances of PP1 and GPH.
415 The WB oysters also had lower total fatty acid content when compared to oysters
416 outplanted at PS locations, indicative of less access to food (less food availability or less
417 time with valves open for filtration) or poorer quality food (Lowe et al. *in prep*). Whether
418 due to more time spent with their shells closed or decreased caloric intake, the WB
419 oysters had to rely on energy stores of glycogen more than oysters throughout Puget
420 Sound.

421 Higher G6PD abundance in WB oysters indicate that oysters at WB were still
422 metabolically active. G6PD catalyzes the oxidative portion of the pentose phosphate
423 pathway, and products from this pathway are often precursors for nucleic and aromatic
424 amino acids ((L., n.d.)). Additionally, G6PD activity generates NADPH and can indirectly
425 ameliorate effects of ROS ((L., n.d.)), as demonstrated in *C. gigas*: after exposure to
426 linear alklybenzenes and other pollutants, *C. gigas* ctenidia expressed higher G6PD
427 activity to regulate the redox environment (Flores-Nunes, Mattos, et al. 2015). Increased
428 abundance of G6PD in WB not only could have maintained transcription and translation
429 processes, but also levels of cellular and metabolic activity by indirectly dealing with
430 ROS. Increased G6PD abundance, therefore, provides a link between carbohydrate
431 metabolism and oxidative stress response in oysters outplanted at Willapa Bay.

432 ***Proteomic Responses in Puget Sound and Between Habitats***

433 Due to the observed differences across environmental parameters between
434 Willapa Bay and Puget Sound locations, similar abundances for proteins involved in
435 various environmental responses may be evidence of physiological plasticity. One

436 particular protein that we expected to be differentially abundant was heat shock 70 kDA
437 protein 12A (HSP 12A), since Willapa Bay had higher average temperatures (Table 3).
438 Higher abundances of heat shock proteins (HSPs) are generally induced when
439 organisms are exposed to thermal stress (Hamdoun, Cheney, and Cherr 2003; Zhang
440 et al. 2015; Meistertzheim et al. 2007). In our experiment, average temperatures *C.*
441 *gigas* experienced were lower than the threshold to induce elevated HSP expression
442 (Figure 1; Table 2) (Hamdoun, Cheney, and Cherr 2003). While HSP12A is involved in
443 a general response to environmental drivers, differentially expressed antioxidant
444 enzymes and carbohydrate metabolism proteins could be more indicative of molecular
445 processes required for acclimatization.

446 The lack of differential abundance for protein targets — both among Puget
447 Sound sites and between unvegetated and eelgrass habitats — was unexpected. These
448 similar proteomic profiles could be due to three factors. First, it is possible that a
449 different suite of environmental response proteins in the SRM assay could have yielded
450 a different view of acclimatization to the various outplant sites; however, the targets we
451 chose have proven to yield insight into a range of environmental responses in previous
452 studies. Second, outplant duration could have been too short to capture varied
453 physiological response within Puget Sound, or oysters could have also acclimatized to
454 conditions in their outplant locations Finally, is that it is possible that the proteomic
455 response was not different because the environmental conditions that would elicit up- or
456 down-regulation of monitored proteins were similar across these five locations (Table 3).
457 While eelgrass beds are touted as a potential refuge for organisms from the negative
458 effects of environmental drivers like pH, they can also drive more extreme carbonate

chemistry conditions (Pacella et al. 2018). It is possible that the refuge effects and extreme effects cancelled each other out over the course of our outplant, effectively making the eelgrass beds just like the unvegetated sites for outplanted oysters. If this is the case, it would explain why there was no detected difference in protein abundance between unvegetated and eelgrass habitats.

Conclusion

The differential protein abundance observed between sibling oysters placed for 29 days in Willapa Bay or Puget Sound indicates that environmental factors at Willapa Bay lend themselves to increased antioxidant enzyme and carbohydrate metabolism protein abundance. Because the observed proteomic responses can be triggered by a variety of drivers, from a field trial it is difficult to discern if high temperatures or other environmental factors at WB led to these responses. However, the observed responses generate a suite of new hypotheses for lab and field experiments comparing the effects of environmental conditions on physiological responses of marine invertebrates. This is the first to link regional environmental conditions to physiological responses in Puget Sound, and one of the first to compare responses between Puget Sound and Willapa Bay. Linking environmental drivers to other performance metrics, such as growth and fatty acid composition, is crucial for contextualizing these protein abundance findings (Lowe et al. *in prep*).

Understanding the difference between these two estuaries is important for the persistence of oyster reefs and aquaculture in the face of climate change. Since aquaculture species are outplanted in the same location for many months to years, the

481 lack of differential protein abundance among Puget Sound outplants could mean that
482 environmental response should not be heavily considered when selecting grow-out
483 locations in this region. Future experiments with a longer outplant duration and multiple
484 sampling points may provide a clearer connection between environmental drivers,
485 protein expression, and performance, as well as assist with developing a practical assay
486 for elucidating stress responses in the field.
487

488 **Figures and Tables**

489 **Table 1.** Latitude and longitude of *C. gigas* outplants. Oysters were placed at five
490 locations sites: Case Inlet (CI), Fidalgo Bay (FB), Port Gamble Bay (PG), Skokomish
491 River Delta (SK), and Willapa Bay (WB).

Location	Latitude	Longitude
CI	47.3579367	-122.7957627
FB	48.481691	-122.58353
PG	47.842676	-122.583832
SK	47.35523	-123.1572
WB	46.4944789	-124.0261356

492

493 **Table 2.** Proteins used as targets for a Selected Reaction Monitoring Assay (SRM).
494 Targets were identified based on differential abundance and stress-related annotations.
495 At least two peptides and six transitions were included in the assy for each protein. The
496 protein Catalase had two isoforms under separate proteome IDs —
497 CHOYP_CATA.1.3|m.11120 and CHOYP_CATA.3.3|m.21642 — and target peptides
498 were chosen from both IDs. A total of four peptides and twelve associated transitions
499 were used as SRM targets.

Protein	Proteome ID	Number of Peptides	Number of Transitions
3-ketoacyl-CoA thiolase	CHOYP_ACAA2.1.1|m.30666	3	9

Peroxiredoxin-5	CHOYP_BRAFLDRAFT_119799.1.1|m.23765	2	6
Thioredoxin reductase 3	CHOYP_BRAFLDRAFT_122807.1.1|m.3729	3	9
Protein phosphatase 1B	CHOYP_BRAFLDRAFT_275870.1.1|m.12895	3	9
Carbonic anyhdrase 2	CHOYP_CAH2.1.1|m.42306	3	9
Catalase 1	CHOYP_CATA.1.3|m.11120	3	9
Catalase 2	CHOYP_CATA.3.3|m.21642	1	3
Glucose-6-phosphate 1-dehydrogenase	CHOYP_G6PD.2.2|m.46923	3	9
Heat shock 70 kDa protein	CHOYP_HS12A.25.33|m.60352	2	6

Heat shock protein 70 B2	CHOYP_HSP74.1.1|m.13095	2	6
NAD(P) transhydrogenase	CHOYP_LOC100633041.1.1|m.35428	2	6
Glycogen phosphorylase	CHOYP_LOC100883864.1.1|m.41791	3	9
Multidrug resistance-associated protein	CHOYP_MRP1.5.10|m.34368	2	6
Protein disulfide-isomerase	CHOYP_PDIA1.1.1|m.5297	3	9
Protein disulfide-isomerase	CHOYP_PDIA3.1.1|m.60223	3	9
Puromysin-sensitive amirase	CHOYP_PSA.1.1|m.27259	3	9

500

501 **Table 3.** Environmental conditions (mean ± standard deviation) at outplant locations
502 (Case Inlet (CI), Fidalgo Bay (FB), Port Gamble Bay (PG), Skokomish River Delta (SK),
503 Willapa Bay (WB).

Metric	CI	FB	PG	SK	WB
Mean Temperature (ºC)	16.1 ± 1.7	14.8 ± 1.8	15.1 ± 2.9	15.1± 2.2	18.0 ± 1.3
Mean Salinity (PSU)	24.47 ± 1.35	30.20 ± 0.38	23.25 ± 1.56	13.43 ± 1.04	27.28 ± 0.73
Mean Dissolved Oxygen Content (mg/L)	8.27 ± 1.91	9.63 ± 4.59	11.70 ± 3.70	9.99 ± 4.58	8.76 ± 1.78
Mean pH	7.63 ± 0.19	7.54 ± 0.23	7.33 ± 0.25	7.37 ± 0.24	7.56 ± 0.18

504

505 **Table 4.** Functions of differentially abundant proteins

Protein	Function	Citations
Peroxiredoxin-5 (PRX)	Scavenges ROS located in mitochondria using cysteine residuals to reduce substrates	Tomanek 2014; Sussarellu et al. 2012
Thioredoxin reductase	Thiol-specific antioxidant	Limón-Pacheco and

(TrxR)	reducing agent	Gonsebatt 2009
Catalase (CAT)	Degrades hydrogen peroxide into water and oxygen	Flores-Nunes, Mattos, et al. 2015; Sussarellu et al. 2012
NAD(P) transhydrogenase (NADPt)	Involved in maintenance of cellular redox state	Sussarellu et al. 2010, 2012
Glucose-6-phosphate 1-dehydrogenase (G6PD)	Catalyzes first step in the pentose phosphate pathway and controls rate of pathway's oxidative portion	Livingstone., 1982
Protein phosphatase 1B (PP1)	Regulation of glygcogen metabolism by dephosphorylating glycogen synthase	Wu et al. 1998
Glycogen phosphorylase (GPH)	Key glycogen catabolism enzyme. Removes glucose from glycogen molecules to aid in glycogen breakdown	David et al. 2005; Oyster and Gigas 1993; She et al. 2015

506

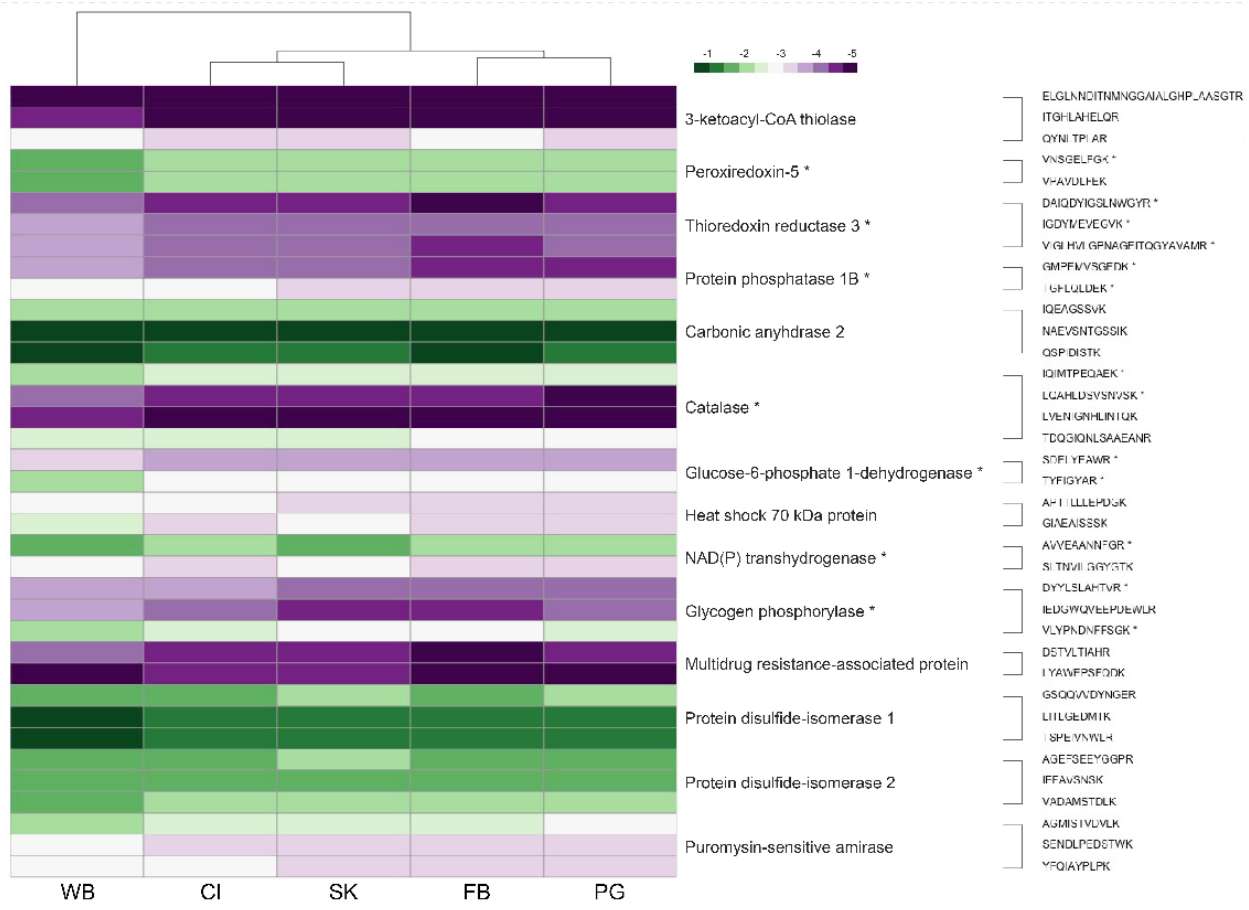

Figure 1. Average protein abundance by constituent peptides across experimental sites from SRM. Peptide abundance data at each site was averaged, then log transformed. Proteins were considered differentially abundant if at least one constituent peptide was significantly different (indicated by an asterisk). There were no significant differences in protein abundance among the Puget Sound locations, or between unvegetated and eelgrass habitats.

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