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

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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 Pacific oyster (*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 influence molecular physiology. Within each site, we also determined if eelgrass presence that buffered pH conditions 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 among sites within Puget Sound, *C. gigas* outplanted at Willapa Bay had significantly higher abundances of antioxidant enzymes and molecular chaperones. Environmental factors at Willapa Bay, such as higher average temperature, may 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, molecular chaperones

Abbreviations: PS: Puget Sound, CI: Case Inlet, FB: Fidalgo Bay, PG: Port Gamble Bay, SK: Skokomish River Delta, WB: Willapa Bay, DIA: Data Independent Acquisition Mass Spectrometry, SRM: Selected Reaction Monitoring Assay

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 & Sharp 1986), nutrient availability (Paerl et al. 2014), heavy metal contamination (Liu et al. 2015), salinity (Banas et al. 2004), and carbonate chemistry (Feely et al. 2010; Pelletier et al. 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 et al. 2012; Flores-Nunes 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. Long-term exposure to environmental conditions, as well as natural organismal aging, are also reflected in the proteome (Hercus et al. 2003). Discovery-based proteomic methods can elucidate responses to environmental drivers (Flores-Nunes 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; Dineshram et al. 2016; Meng et al. 2017). While these studies provide insight

65 into organismal adaptation and physiology, laboratory studies alone cannot fully
66 encapsulate the effects of multiple environmental drivers within an ecosystem context
67 (Riebesell & Gattuso 2014).

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

82 Marine invertebrates have proven to be informative bioindicators in proteomic
83 studies to examine the effects of *in situ* conditions on organismal physiological
84 responses to environmental change. When marine invertebrates have been exposed to
85 varying environmental conditions, proteomics have demonstrated changes in cellular
86 defense, immune responses, and genome function (Veldhoen et al. 2012). Changes in
87 protein abundance in bivalves like the Pacific oyster (*Crassostrea gigas*) and blue

88 mussels (*Mytilus edulis spp.*) have been used to develop biomarkers for environmental
89 contaminants (Slattery et al. 2012; Beyer et al. 2017). Proteomic responses to natural
90 environmental drivers have also been evaluated in bivalves. For example, shotgun
91 proteomic analysis of *M. edulis* ctenidia from Baltic Sea microcosms revealed that low
92 salinity conditions lead to decreased abundance of cytoskeleton proteins, as well as
93 calcium-binding messenger calmodulin, which plays an important role in signalling and
94 intracellular membrane trafficking pathways (Campos et al. 2016). Using a growing
95 wealth of genomic information to understand how these species fare under differential
96 environmental conditions is critical for monitoring natural populations and commercial
97 aquaculture.

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

111 Both Puget Sound and Willapa Bay also host eelgrass beds (*Zostera spp.*) that
112 affect environmental conditions, such as oxygen concentrations, on diurnal time scales.
113 The 2012 Washington State Blue Ribbon Panel on Ocean Acidification outlines key
114 early actions, which include the examination of "vegetation-based systems of
115 remediation" (Action 6.1.1) to improve local pH through photosynthetic drawdown of
116 carbon dioxide. This experiment set out to test whether protein abundance patterns
117 reflect reduced stress within vegetation. For example, eelgrass beds may reduce
118 emersion stress relative to unvegetated areas through shading, the retention of water,
119 and increased evaporative cooling at low tide. They can also ameliorate effects of
120 ocean acidification through photosynthetic activity. Reduced pathogen prevalence has
121 also been documented in seagrass beds, but not specifically in eelgrass (Lamb et al.
122 2017). In contrast, eelgrass beds may drive more extreme carbonate chemistry
123 conditions (Pacella et al. 2018). Lowe et al. (2018) also found that *C. gigas* shell
124 strength and survival was significantly lower in eelgrass habitats in WA. Understanding
125 how different aquaculture grow-out locations and habitats will affect the oyster's ability
126 to persist through environmental change is crucial for the industry and the ecosystem.
127 The purpose of this study was to use proteomic techniques to uncover the
128 impacts of environmental drivers on Pacific oyster physiological outcomes in estuarine
129 environments in WA. Naturally existing environmental variation was harnessed by
130 outplanting *C. gigas* in different locations within Puget Sound and Willapa Bay, and
131 habitat effects were taken into consideration by placing oysters in eelgrass and
132 unvegetated habitats. Gel-free proteomic methods were used to examine the effects of
133 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 warmer water temperatures, more variable water 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 juvenile *C. gigas* (average shell length = 27.2 mm, age = 2 months) 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). 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 WA locations to monitor marine chemistry (ANeMoNe; Washington Department of Natural Resources). Prior to the outplant, oysters were reared in a controlled hatchery setting. At each site and habitat combination, 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 two days into the outplant period. At each site, juvenile oysters were placed in bags of five oysters each 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. The animals were uniformly placed less than a lateral distance of 0.5 m from the sensors at the same tidal height as the instruments. Oysters were housed in exclusion cages to prevent predation. Juvenile oysters remained at each site for a 29-day exposure 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).

Environmental data was treated as follows. Conductivity observations were removed when less than zero, which occurs when the instrument is dry at low tide. Remaining observations were converted to salinity measurements using the *swSCTp* function in the *oce* package in R (Kelley & Richards 2018; R Core Team 2018), with temperature at 25ºC and pressure at 10 dbar. For dissolved oxygen, pH, and salinity datasets, data were removed when collected by probes 1) during low tide or 2) when tidal depth was less than one foot to remove readings where the probes may have been exposed. Values collected during low tide or a depths less than one foot were retained for temperature datasets. Outliers were screened using the Tukey method for temperature, dissolved oxygen, pH, and salinity datasets (Hoaglin et al. 1986). Uniform outplant tidal heights were checked using *prop.test* in R (R Core Team 2018).

179 A non-metric multidimensional scaling analysis (NMDS) was used to evaluate
180 differences in environmental parameters. First, mean and variance were calculated for
181 each day of the outplant. Values were log-transformed, and a separate Gower's
182 distance matrix was calculated for daily mean and daily variances, accounting for
183 missing data. The NMDS was conducted with the Gower's distance matrix to visually
184 compare means or variances between sites and habitats. Significant differences
185 between site and habitat were identified using a One-way Analysis of Similarities
186 (ANOSIM) for each environmental parameter. Pairwise ANOSIM tests for significant
187 one-way ANOSIM results and two-way ANOSIM tests by site and habitat were not
188 conducted due to lack of replicates within each site-habitat combination. R Scripts are
189 available in the supplementary Github repository (Venkataraman et al. 2018).

190 ***Protein Discovery***

191 To identify select protein targets for characterization across locations and
192 environmental conditions, a subset of tissue samples were analyzed with data
193 independent acquisition (DIA) mass spectrometry analysis. Two tissue samples were
194 used from each site to make a peptide library and maximize the amount of protein
195 abundance data collected from each sample.

196 Protein Quantification

197 Tissue samples were homogenized in a solution of 50 mM NH_4HCO_3 with 6M
198 Urea (500µl). Tissues were then sonicated 3 times (Fisher Scientific Sonic
199 Dismembrator Model 100) for 10 seconds each and cooled between sonications in a
200 bath of ethanol and dry ice. Protein quantities were measured with the Pierce BCA

201 Protein Assay Kit microplate assay with a limited quantity of sonicated sample (11 µL).
202 The protein concentration was measured via spectroscopy at 540 nm in a Labsystems
203 (Waltham, MA) Multiskan MCC/340 and accompanying Ascent Software Version 2.6.
204 Protein concentrations were calculated based on a standard curve with BSA (Pierce)
205 per manufacturer's instructions.

206 Protein Digestion

207 Protein digestion followed the protocol outlined in Timmins-Schiffman et al.
208 (2013). To each sample of 30 µg protein,1.5 M Tris pH 8.8 buffer (6.6 µL) and 200 mM
209 TCEP (2.5 µL) were added. After solvent additions, each sample's pH was verified to be
210 basic (pH ≥ 8), and placed on a 37ºC heating block for one hour. Iodoacetamide (200
211 mM, 20 µL) was then added to each sample to maximize digestion enzyme access to
212 protein cleavage sites. Samples were covered with aluminum foil to incubate in the dark
213 for 1 hour at room temperature. Afterwards, diothiolthreitol (200 mM, 20 µL) was added
214 and samples were incubated at room temperature for one hour. Lysyl Endopeptidase
215 (Wako Chemicals) was then added to each sample in a 1 µg enzyme:30 µg oyster
216 protein ratio, followed by one hour of incubation at room temperature. Urea was diluted
217 with NH_4HCO_3 (25 mM, 800 µL) and HPLC grade methanol (200 µL). Trypsin
218 (Promega) was added to each sample in a 1 µg trypsin: 30 µg oyster protein ratio for
219 overnight digestion at room temperature.

220 Peptide Isolation

221 After overnight incubation, samples were evaporated to near dryness at 4ºC with
222 a speedvac (CentriVap ® Refrigerated Centrifugal Concentrator Model 7310021).
223 Samples were then reconstituted in 100 µL of a 5% Acetonitrile and 0.1% Trifluoroacetic
224 Acid (Solvent A) to isolate peptides. If samples were not at pH ≤ 2, 10-20 µL aliquots of
225 10% Formic Acid were added until this pH was achieved.

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

234 To bind peptides to the columns, digested peptides were added to prepared
235 columns, then the columns were spun at 3000 rpm for three minutes. The filtrate was
236 pipetted back onto the column and spun again at 3000 rpm for three minutes. Solvent A
237 (200 µL) was added to each column three separate times, then the column was spun for
238 three minutes at 3000 rpm to wash salts off the column.

239 Peptides were eluted with two additions of 100 µL Solvent B to each column.
240 Columns were spun at 3000 rpm for three minutes and the peptide fraction (filtrate) was

241 reserved. Samples were placed in a speed vacuum at 4ºC until they were nearly dry
242 (approximately two hours) to dry peptides. Peptides were reconstituted with 60 µL of 3%
243 Acetonitrile + 0.1% Formic Acid, and stored at -80ºC.

244 Internal Standard Addition

245 Peptide Retention Time Calibration (PRTC; Pierce) is used as an internal
246 standard to ensure consistency of peptides detected and measured throughout a mass
247 spectrometry run. The stock solution of PRTC was diluted to 0.2 pmol/µL using 3%
248 Acetonitrile with 0.1% Formic Acid. In a clean centrifuge tube, 6 µg of oyster protein and
249 0.376 pmol of PRTC were mixed together as per the PRTC user guide. Sample volume
250 was brought up to 15 µL using a 3% acetonitrile and 0.1% formic acid solution. A quality
251 control solution was also prepared (1 µL PRTC + BSA:3 µL 3% Acetonitrile and 0.1%
252 Formic Acid solution).

253 ***Data Independent Acquisition Mass Spectrometry***

254 Peptides were analyzed on an Orbitrap Fusion Lumos mass spectrometer
255 (Thermo Scientific) using Data Independent Acquisition Mass Spectrometry (DIA). DIA
256 analyses were completed as a comprehensive, non-random analytical method for
257 detecting peptide ions present within a sample to create a peptide library. The peptide
258 library was then leveraged to develop a targeted proteomics assay for quantification
259 (see Selected Reaction Monitoring Assay). A 30 cm analytical column and 3 cm pre-
260 column were packed in-house with 3 µm C18 beads (Dr. Maisch). Samples were run in
261 a randomized order. A blank injection followed each sample, with procedural blanks run
262 at the very end. Every injection was 3 µL, which included 1 µg of oyster protein and

263 0.0752 pmol of PRTC. Peptides were analyzed in MS1 over the *m/z* range of 450-950
264 with 12 *m/z* wide windows with 5 *m/z* overlaps (Egertson et al. 2013). MS1 resolution
265 was 60000 and AGC target was 400000 with a three second cycle time. The MS2 loop
266 count was set to 20 and MS2 data was collected with a resolution of 15000 on charge
267 state of 2 with an AGC target of 50000. No dynamic exclusion was used.

268 ***Peptide-Centric Proteomic Analyses***

269 Unknown peptide spectra from mass spectrometry samples were matched with
270 known peptides using Peptide-Centric Analysis in the PECAN software (Ting et al.
271 2015). Raw mass spectrometry files were converted to mzML files, then demultiplexed
272 using MSConvert (Chambers et al. 2012). The *C. gigas* proteome was digested with in
273 silico tryptic digest using Protein Digestion Simulator (Riviere et al. 2015). All known
274 peptides from the mzML files were identified in comparison to the digested *C. gigas*
275 proteome (Riviere et al. 2015).

276 The PECAN-generated spectral library (.blib) file was used to detect peptides of
277 interest in raw DIA files in Skyline (MacLean et al. 2010). Skyline identified peptides
278 using chromatogram peak picking, where ions that elute at the same time and mass are
279 detected as a peptide (file available at Panorama Public). All PRTC peptides and
280 approximately 100 different oyster proteins and their peptide transitions were manually
281 checked for retention time and peak area ratio consistency to determine a Skyline auto
282 peak picker error rate (24.3% ± 25%, range: 0% to 100%).

283 Proteins had to satisfy four criteria to be considered appropriate targets for the
284 study. 1) After an extensive literature search, functions related to oxidative stress,
285 hypoxia, heat shock, immune resistance, shell formation, growth, and cellular

286 maintenance were determined useful for evaluating environmental response. Proteins
287 with annotations matching these functions were considered potential targets. 2) Protein
288 data was then evaluated in Skyline to ensure there was no missing data for any peptide
289 or sample. 3) Peaks with significant interference from other peptides were not
290 considered. 4) Proteins needed at least two peptides with three transitions per peptide
291 to quality as a potential target for downstream assays. The fifteen proteins (41 peptides
292 and 123 transitions) that matched all of these criteria were selected as targets (Table 2).

293 ***Selected Reaction Monitoring Assay***

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

300 Proteins of interest identified from the DIA analysis were used as targets in a
301 SRM assay following the workflow and informatic pipeline of (Timmins-Schiffman et al.
302 2016). Target peptide transitions were monitored using SRM on a Thermo TSQ
303 Vantage. SRM data were collected during a gradient of 2-60% acetonitrile over 40
304 minutes. All samples were run in technical duplicates in a randomized order with a 1 µg
305 oyster peptide and 0.0752 pmol PRTC injection. A quality control injection and blank
306 injection were run after every five sample injections, and PRTC peptides were
307 monitored throughout the experiment.

308 Target Peptide Specificity

309 To ensure SRM assay specificity to oyster peptides of interest, oyster peptides
310 were diluted in a background matrix of similar complexity (Pacific geoduck — *Panopea*
311 *generosa* — peptides), then analyzed using the oyster SRM assay. An oyster-specific
312 SRM target would decrease in abundance with a decreasing abundance of oyster
313 peptides in a mixture. Non-specific peptides — more likely to be found in background
314 matrix of similar complexity — or peptides susceptible to interference would not
315 correlate with oyster peptide abundance, and therefore, would be uninformative. Five *C.*
316 *gigas* samples used for SRM were randomly selected and pooled in equal quantities. A
317 ten-sample oyster:geoduck dilution series was prepared and run using the same
318 methods as other SRM samples.

319 Target Analysis

320 Raw SRM files, a background *C. gigas* proteome, and the PECAN spectral
321 library file from DIA were used to create a Skyline document (file available at Panorama
322 Public). Correct transition peaks were selected based on predicted retention times from
323 DIA results by comparing the relative retention times between identical PRTC peptides
324 in the DIA and SRM datasets (R^2 = 0.99431). Based on peptide specificity analyses,
325 heat shock protein 70 B2 and one constituent peptide of
326 glucose-6-phosphate 1-dehydrogenase were removed from analyses (S11).
327 Further filters were applied to the data to maintain only high quality peptides and
328 transitions in the analysis. Coefficients of variation were calculated between technical
329 replicates for each peptide transition. Peptides were removed from the dataset if CV >
330 20%. To maintain high sample quality, any sample missing data for more than 50% of

331 peptide transitions was deemed poor quality for downstream analyses and excluded.
332 Abundance data was normalized using total ion current (TIC) values from the mass
333 spectrometer. Consistency between technical replicates was verified in remaining
334 samples using a NMDS with TIC-normalized data and a euclidean dissimilarity matrix.
335 Technical replicates were consistent if replicates lay closer together than to other
336 samples in the NMDS. These replicates were then averaged for multivariate analytical
337 methods.

338 Averaged technical replicate data was used to determine if peptides were
339 differentially abundant between outplant sites and habitats. Before proceeding with
340 analysis, peptide abundances were subjected to a Hellinger transformation to give low
341 weights to any peptides with low counts. A NMDS was used to visually compare relative
342 peptide abundance. One-way ANOSIM tests by site, region (Puget Sound vs. Willapa
343 Bay), and habitat, as well as a two-way ANOSIM test by site and habitat, were used to
344 determine significant differences. Pairwise ANOSIM tests and post-hoc similarity
345 percentage (SIMPER) analyses were conducted for each one- or two-way ANOSIM
346 result significant at the 0.05 level. The first ten SIMPER entries were deemed influential
347 peptides for each significant comparison.

348 The importance of environmental variables for explaining peptide abundance was
349 evaluated with a redundancy analysis (RDA). For each site and habitat combination,
350 mean and variance were calculated for pH, dissolved oxygen, salinity, and temperature
351 over the course of the entire outplant. Environmental variables were then used as
352 predictors to constrain peptide abundance. Predictors with missing values were not
353 included. A triplot was used to visually assess differences in peptide abundance by site

354 and habitat and the influence of individual peptides and environmental parameters.
355 Analysis of Variance (ANOVA) was used to calculate significance of the RDA and
356 environmental variables, with predictors deemed significant at $p < 0.05$. Since estuarine
357 sites are highly variable, a second RDA was conducted constraining peptide abundance
358 by environmental conditions on the day of collection to evaluate robustness of
359 proteomic methods. R Scripts are available in the supplementary Github repository
360 (Venkataraman et al. 2018).

361 **Results**

362 ***Conditions at Outplant Locations***

363 Outplanted oysters experienced environmental variables representative of
364 standard summer conditions in Puget Sound (PS) and WB. Wild or cultured oysters
365 were present at the same tidal elevation as sensors, so outplant conditions represent
366 experiences of non-experimental organisms. NMDS plots revealed mean environmental
367 conditions were more similar among sites than variances. Both NMDS had stress
368 values less than 0.20 and were considered appropriate multivariate representations of
369 environmental data (Mean NMDS: stress = 0.0170, $p = 0.0100$; Variance NMDS: stress
370 = 0.0340, $p = 0.0100$). Mean dissolved oxygen and temperature were significantly
371 different between sites (Table 4; ANOSIM; dissolved oxygen: $R = 0.4063$, $p = 0.0530$;
372 temperature: $R = 1.0000$, $p = 0.0020$), but not between habitats. Variances of all
373 environmental parameters were significantly different between sites (Table 5; ANOSIM;
374 pH: $R = 0.5313$, $p = 0.0180$; dissolved oxygen: $R = 0.6800$, $p = 0.0030$; salinity: $R =$
375 0.8400, $p = 0.0130$; temperature: $R = 0.9400$, $p = 0.0010$). WB had the warmest, but

376 least variable, temperature of 18.0ºC ± 1.3ºC compared to averages ranging from
377 14.8ºC ± 1.8ºC to 16.1ºC ± 1.7ºC in PS (Table 3, Figure 2). All variance values, mean
378 pH, and mean salinity were not significantly different between habitats (Table 4, Table
379 5).

380 Although oyster position was standardized by tidal height, outplanted oysters
381 experienced different amounts of exposure at low tide at each site due to differences in
382 tidal amplitude ($\chi_4^2 = 25.29$, $p < 0.0001$). Oysters at FB spent the highest percent of
383 time emersed at tides less than 1 foot MLLW (13.99%; 188 hours), followed by PG
384 (10.93%; 146 hours), SK (10.29%; 138 hours), CI (9.35%; 125 hours), and WB (8.48%;
385 113 hours). CI and FB outplants ($p = 0.0021$), FB and SK outplants ($p = 0.0324$), and
386 FB and WB outplants ($p < 0.0001$) spent significantly different amounts of time emersed
387 during low tide conditions.

388 ***Data Independent Acquisition Mass Spectrometry***

389 Out of 39816 predicted proteins in the *C. gigas* FASTA proteome, 9047 proteins
390 were detected in *C. gigas* across five sites and two habitats using DIA (Skyline auto
391 peak picker error rate 24.3% ± 25%, range: 0% to 100%). Proteins detected included,
392 but were not limited to, those annotated from processes such as responses to hypoxia
393 and oxidative stress, removal of superoxide radicals, protein folding, muscle organ
394 development, and negative regulation of apoptosis (Raw data: PeptideAtlas accession
395 no. PASS01305).

396 ***Selected Reaction Monitoring Assay***

397 Differential abundance of protein targets was evaluated at the peptide level after
398 combining technical replicates. Proteins were considered differentially abundant if at
399 least one monitored peptide was differentially abundant. There was no significant
400 difference in SRM peptide abundance between unvegetated and eelgrass habitats
401 across sites (One-way ANOSIM; R = 0.0440, p = 0.1220). Abundance data from both
402 habitats were pooled for downstream analyses comparing protein abundances among
403 sites (Raw data: PeptideAtlas accession no. PASS01304).

404 Rank distances between peptide abundances in multivariate space revealed
405 differences in peptide abundance between WB versus the other four sites (NMDS:
406 stress = 0.0750, p = 0.0099). One-way ANOSIM by site demonstrated no significant
407 differences in peptide abundance (R = 0.0640, p = 0.065), but a one-way ANOSIM by
408 region (PS vs. WB) revealed a trend towards peptide abundance differences (R =
409 0.2260, p = 0.0530). Peptide abundance was significantly different between FB and
410 Willapa Bay (Table 6; Pairwise ANOSIM; R = 0.2568, p = 0.0350). Environmental
411 variables explained 29% of variance in peptide abundance, but the proportion of
412 variation explained was not significant (ANOVA for RDA; $F_{6,19}$ = 1.3064; p = 0.1950).
413 Temperature mean and variance were the two most influential environmental predictors
414 of peptide abundance, but were not significant at the 0.05 level (ANOVA on RDA; Table
415 7; temperature mean: $F_{1,19}$ = 2.2375, p = 0.0650; temperature variance: $F_{1,19}$ = 2.1627,
416 p = 0.0670). Peptides differentially abundant between FB and WB are primarily
417 positively loaded onto temperature mean, with two negatively loaded on temperature
418 variance. Robustness of analysis was evaluated by performing a second RDA to predict

419 peptide abundance based on mean and variance of temperature and pH at time of
420 collection. Due to missing values, dissolved oxygen and salinity could not be included in
421 analysis. Temperature variance was the most influential predictor of peptide abundance
422 at time of collection, but was not significant at the 0.05 level (ANOVA on RDA; $F_{1,13}$ =
423 2.2312, p = 0.0800).

424 Peroxiredoxin-5 (PRX), catalase (EC 1.11.1.6) (CAT), NAD(P) transhydrogenase
425 (NADPt), glucose-6-phosphate 1-dehydrogenase (G6PD), carbonic anhydrase (CA),
426 protein disulfide-isomerase 1 and 2 (PDI) had peptides that were identified as
427 differentially abundant between Willapa Bay and Fidalgo Bay by SIMPER analysis
428 (Figure 3). Fidalgo Bay peptide composition was differentiated by CA abundance, while
429 all other significantly abundant proteins differentiated Willapa Bay. These proteins are
430 involved in general cellular stress responses like reactive oxygen species neutralization
431 or protein folding (Table 8). All differentially abundant proteins were detected at higher
432 levels in the WB oysters than in oysters from the other four PS locations (CI, FB, PG,
433 and SK), regardless of protein function (Figure 4). No differences in protein abundance
434 were detected among the PS sites (Table 6).

435 **Discussion**

436 Among sibling Pacific oysters placed at five sites in Washington State, we found
437 higher abundances of seven proteins — antioxidant enzymes and molecular
438 chaperones — in oysters outplanted at WB. Significant differences in protein
439 abundances were detected between WB and either FB or SK; no differences were
440 observed among PS sites. Increased antioxidant and molecular chaperone protein

441 abundance are conserved stress responses consistently documented as responses to
442 extreme temperatures, hyposalinity, low pH and air exposure (Tomanek 2014; Zhang et
443 al. 2015). Warmer and less variable temperature conditions at WB may have driven
444 differential protein abundances (Figure 3). Validation of RDA results by correlating
445 peptide abundance to environmental conditions at the time of collection demonstrates
446 that proteomic methods are suitable for evaluating the influence of month-long
447 environmental variation on organismal physiology.

448 To our knowledge, this is the first time combined use of DIA and SRM analytical
449 methods have been applied in an ecological context. Previously, both gel-based (Parker
450 et al. 2011; Zhang et al. 2015; Campos et al. 2016) and gel-free (Dineshram et al. 2016;
451 Timmins-Schiffman et al. 2017; Müller et al. 2018) proteomic methods have been used
452 in lab settings to evaluate the effects of environmental conditions on organismal
453 physiology, with gel-based methods having a limited dynamic range. Similar gel-free
454 methods have also been used to examine physiology of other non-model marine
455 species (Plumel et al. 2013; Kültz et al. 2015). Our methods allowed for increased
456 acquisition of proteomic data for a non-model organism. We detected over 9000
457 proteins with DIA — far more than previous studies have detected. The application of a
458 novel, two step, gel-free proteomic approach broadened the scope of proteins detected
459 in the DIA phase, thus revealing more possibilities for SRM target design and also
460 completing the first step for future proteomic studies in this species. The SRM assay
461 allowed investigation of specific biomarkers with a high-throughput analysis.

462 ***Antioxidant enzymes and Acid-Base Regulation***

463 Higher antioxidant enzyme abundances can be a direct response to an increase
464 in reactive oxygen species (ROS) (Limón-Pacheco & Gonsebatt 2009; Zhang et al.
465 2015). During electron transport, oysters can produce ROS that induce oxidative stress
466 if not neutralized (Abele et al. 2007; Limón-Pacheco & Gonsebatt 2009). Peroxiredoxin-
467 5 (PRX), and catalase (CAT) scavenge these ROS and degrade them before they
468 cause cellular harm, while NAD(P) transhydrogenase (NADPt) maintains the cellular
469 redox state (Limón-Pacheco & Gonsebatt 2009; Sussarellu et al. 2012; Flores-Nunes et
470 al. 2015). Higher abundances of antioxidant enzymes in WB oysters suggests the need
471 for ROS scavenging to acclimatize to local conditions.

472 Elevated antioxidant enzyme abundance at WB may be a response to higher
473 levels of ROS brought on by heat stress. Mollusc species, like *C. gigas*, have been
474 known to increase ROS production at higher temperatures (Abele et al. 2007; Tomanek
475 2014). Warmer and more variable temperature conditions at WB could be driving ROS
476 production and in turn, higher abundances of PRX, CAT, and NADPt to ameliorate
477 ROS-associated stress (Zhang et al. 2015). The shallow bathymetry of WB may have
478 also contributed to elevated ROS presence and the need for antioxidant enzymes. At
479 low tide, shallow waters would warm faster, leading to the higher temperatures
480 observed at WB (Table 1). Warmer waters at low tide may prompt oysters to spend
481 more time with their shells closed, inducing hypoxia and hypercapnia. Low oxygen
482 concentrations within the shell could then augment ROS levels (Guzy & Schumaker
483 2006). Oysters could respond through increased abundance of antioxidant enzymes
484 (Sussarellu et al. 2012).

485 The need for WB outplants to regulate internal acid-base conditions is
486 demonstrated by higher abundance of carbonic anhydrase (CA) in these oysters. *C.*
487 *gigas* can regulate hemolymph pH by increasing HCO_3^- concentration via the
488 conversion of CO2 to HCO_3^-, catalyzed by CA (Michaelidis et al. 2005; Wei et al. 2015).
489 If warmer water conditions at WB prompted shell closure, *C. gigas* would need to switch
490 to anaerobic metabolism, inducing a significant reduction in hemolymph pH (Michaelidis
491 et al. 2005). Buffering of hemolymph pH could be accomplished by elevated CA
492 abundance. Although oysters at FB spent more time in low tide conditions that would
493 also prompt shell closure and similar molecular responses, upregulated protein
494 abundance at WB implies conditions at this site required a greater proteomic response
495 in these specific biomarkers for acclimatization.
496 Higher abundance of glucose-6-phosphate 1-dehydrogenase (G6PD) in WB
497 oysters indicates these oysters maintained metabolic activity in warmer temperature
498 conditions. G6PD catalyzes the oxidative portion of the pentose phosphate pathway,
499 and products from this pathway are often the precursors for nucleic and aromatic amino
500 acids (Livingstone 1981). Additionally, G6PD activity generates NADPH and can
501 indirectly regulate the redox environment and ameliorate effects of ROS (Livingstone
502 1981). For example, exposure to ROS-inducing pollutants led to increased G6PD
503 activity in *C. gigas* ctenidia (Flores-Nunes et al. 2015). Increased abundance of G6PD
504 in WB not only could have maintained transcription and translation processes, but also
505 levels of cellular and metabolic activity by indirectly dealing with ROS.
506 ROS are produced in response to many environmental changes, thus biomarkers
507 of ROS scavenging are difficult to link to a single environmental difference in a variable

508 and complex setting. For example, reduction of ROS was found to be a common
509 response to both increased temperatures and aerial exposure in *C. gigas* (Zhang et al.
510 2015). Since ROS mediation is a conserved response to several stressors, it is possible
511 that environmental parameters we did not measure (e.g., contaminants, microbiota
512 abundance, trace metals), or a combination of environmental parameters, could explain
513 the observed variation in antioxidant enzyme abundance. Future work at these locations
514 should take these variables into account.

515 ***Molecular Chaperones Involved in Protein Folding***

516 Much like cellular response to ROS, increased levels of protein disulfide
517 isomerase 1 and 2 (PDI) demonstrate a generalist response to conditions at WB. PDI is
518 a general chaperone protein that forms disulfide bonds and assists with protein folding
519 (Vargas-Albores et al. 2009). Higher PDI abundance would reflect a need to refold
520 misshapen proteins. In this regard, PDI would function similarly to molecular
521 chaperones like heat shock proteins. Several invertebrate taxa demonstrate higher PDI
522 abundance when faced with an immune challenge or metal contamination. When faced
523 with an immune challenge, Pacific white shrimp (*Litopenaeus vannamei*) hemocytes
524 synthesized higher abundances of immune response proteins, followed by elevated
525 abundance of PDI to correct disulfide bonds in these proteins (Vargas-Albores et al.
526 2009). An immune challenge could also lead to more misfolded proteins that PDI would
527 need to refold to avoid cellular damage (Zhang et al. 2014). In flat oysters (*Ostrea*
528 *edulis*), PDI expression increased in response to disseminated neoplasia (Silvestre et
529 al. 2006; Martín-Gómez et al. 2013). Metal contamination at WB could also elevate PDI
530 abundance: Chinese mitten crabs (*Eriocheir sinensis*) chronically exposed to cadmium

over-expressed PDI (Silvestre et al. 2006; Martín-Gómez et al. 2013). Since neither disease burden or environmental contamination was measured in this study, it is impossible to know if either triggered the PDI response. Examination of these factors in future studies may elucidate the specifics of elevated PDI abundance in WB.

Proteomic Responses in Puget Sound and Between Habitats

Due to the observed differences across environmental parameters between Willapa Bay and Puget Sound locations, similar abundances for proteins involved in various environmental responses may be evidence of physiological plasticity. One particular protein that we expected to be differentially abundant was heat shock 70 kDA protein 12A (HSP12A), since Willapa Bay had higher average temperatures (Table 3). However, this trend was not observed. Higher abundances of heat shock proteins (HSPs) are generally induced when organisms are exposed to thermal stress (Hamdoun et al. 2003; Zhang et al. 2015). In our experiment, average temperatures *C. gigas* experienced were lower than the 30ºC threshold to induce elevated HSP expression in a controlled setting (Figure 2; Table 3), which would explain why we did not see elevated HSP12A expression in WB (Hamdoun et al. 2003).

The lack of differential abundance for protein targets — both among Puget Sound sites and between unvegetated and eelgrass habitats — was unexpected. These similar proteomic profiles could be due to four factors. First, it is possible that a different suite of environmental response proteins in the SRM assay could have yielded a different view of acclimatization to the various outplant sites; however, the targets we chose have proven to yield insight into a range of environmental responses in previous studies (ex. Table 8). Second, outplant duration could have been too short to capture

554 varied physiological response within Puget Sound, or oysters could have also
555 acclimatized to conditions in their outplant locations. Third, post-translational
556 modifications may have influenced how we detected proteins. Abundance between sites
557 or habitats may have been similar, but addition of post-translational modifications may
558 have varied. Finally, it is possible that the proteomic response was not different because
559 the environmental conditions that would elicit up- or down-regulation of monitored
560 proteins were similar across these five locations. For example, we found no differences
561 in environmental conditions between eelgrass and unvegetated areas, nor any
562 differences in protein responses between these habitats (Table 4; Table 5). Our results
563 suggest that the potential vegetation-based systems of remediation could be limited
564 under current conditions in the field. A recent comparison of stable isotopes and fatty
565 acid composition in *C. gigas* at eelgrass and unvegetated habitats found no differences
566 in $\delta^{13}C$, $\delta^{15}N$, total fatty acids, or proportional fatty composition in WB outplants,
567 providing another line of evidence that suggests habitat may not affect *C. gigas*
568 physiological performance (Lowe et al. 2018).

569 **Conclusion**

570 Differential abundance of target proteins observed between sibling oysters
571 placed for 29 days in WB or PS indicates that environmental factors at WB tended to
572 increase antioxidant enzyme and molecular chaperone protein abundance in Pacific
573 oysters. This study is one the first to link regional environmental conditions to
574 physiological responses in Puget Sound and Willapa Bay, as well as compare
575 responses between Puget Sound and Willapa Bay. Understanding the difference

between these two estuaries is important for the persistence of oyster reefs and aquaculture in the face of climate change. Of the environmental parameters measured, higher mean temperature, as well as less variable temperature conditions, at Willapa Bay may have influenced protein abundance. The lack of protein abundance differences between PS sites may imply that 2016 conditions were well-within the tolerances of outplanted *C. gigas*, so patterns of stress response witnessed under laboratory conditions were not apparent in the field. Together, the results generate a suite of new hypotheses for lab and field experiments comparing the effects of environmental conditions on physiological responses of marine invertebrates.

As global climate change continues to rapidly influence estuarine dynamics, this study illustrates the importance of investigating the effect of multiple modes of change on organismal physiology *in situ*. Our finding that oysters used generalist proteins to ameliorate stressors implies that assays for elucidating responses to multiple drivers *in situ* should include these conserved responses in addition to specific, stressor-related proteins identified in laboratory experiments. Pacific oysters are grown commercially throughout the Pacific Northwest region of the United States, so it is possible the population used for this study has a broad environmental tolerance. Proteomic assays allow for quantification of that tolerance, which is crucial for aquaculture industry and natural resource management. Future *in situ* explorations of environmental drivers on physiology should include a longer outplant duration, additional environmental variables, and multiple sampling points to provide a clearer connection between ecosystem dynamics and physiological performance.

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810

811 **Tables**

812 **Table 1.** Latitude and longitude of *C. gigas* outplants, as well as time of day and tidal
813 height at collection. Oysters were placed at five locations sites: Case Inlet (CI), Fidalgo
814 Bay (FB), Port Gamble Bay (PG), Skokomish River Delta (SK), and Willapa Bay (WB).

Location	Latitude	Longitude	Time at Collection	Tidal Height (MLLW, feet) at Collection
CI	47.3579367	-122.7957627	12:15	-1.8
FB	48.481691	-122.58353	12:12	-1.7
PG	47.842676	-122.583832	11:11	-1.6
SK	47.35523	-123.1572	11:51	-1.8
WB	46.4944789	-124.0261356	9:28	-1.7

815

816 **Table 2.** Proteins used as targets for a Selected Reaction Monitoring Assay (SRM).
817 Targets were identified based on differential abundance and stress-related annotations.
818 At least two peptides and six transitions were included in the assy for each protein. The
819 protein Catalase had two isoforms under separate proteome IDs —
820 CHOYP_CATA.1.3|m.11120 and CHOYP_CATA.3.3|m.21642 — and target peptides
821 were chosen from both IDs. A total of four peptides and twelve associated transitions
822 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 1	CHOYP_PDIA1.1.1|m.5297	3	9
Protein disulfide-isomerase 2	CHOYP_PDIA3.1.1|m.60223	3	9
Puromysin-sensitive amirase	CHOYP_PSA.1.1|m.27259	3	9

823

824 **Table 3.** Environmental conditions (mean ± standard deviation) at outplant locations
825 (Case Inlet (CI), Fidalgo Bay (FB), Port Gamble Bay (PG), Skokomish River Delta (SK),
826 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

827

828 **Table 4.** Analysis of Similarities (ANOSIM) R statistic and p-value for mean values of
829 environmental variables by site and habitat. Values significant at the 0.05 level are
830 marked by **, and values significant at the 0.10 level are marked by *.

Environmental Variable	Contrast	R statistic	p-value

pH	Site	0.3281	0.1350
	Habitat	0.2125	0.1510
Dissolved oxygen	**Site**	**0.4063 ***	**0.0530 ***
	Habitat	-0.14375	0.9220
Salinity	Site	-0.0133	0.4820
	Habitat	-0.1667	0.9710
Temperature	**Site**	**1.0000 ** **	**0.0020 ** **
	Habitat	-0.1920	0.905

Table 5. Analysis of Similarities (ANOSIM) R statistic and p-value for environmental variable variances by site and habitat. Values significant at the 0.05 level are marked by **, and values significant at the 0.10 level are marked by *.

Environmental Variable	Contrast	R statistic	p-value
pH	**Site**	**0.5313 ** **	**0.0180 ** **
	Habitat	-0.1813	0.9340
Dissolved oxygen	**Site**	**0.6800 ** **	**0.0030 ** **
	Habitat	-0.1240	0.8480

Salinity	**Site**	**0.8400 ****	**0.0130 ****
	Habitat	-0.1250	0.7080
Temperature	**Site**	**0.9400 ****	**0.0010 ****
	Habitat	-0.2200	0.9870

Table 6. Results of pairwise ANOSIM tests between sites for protein abundance data. R values are listed above the diagonal, and p values are listed below the diagonal. Values significant at the 0.05 level are marked by **, and values significant at the 0.10 level are marked by *.

	CI	**FB**	**PG**	**SK**	**FB**
CI	-	0.0197	-0.0707	-0.0583	0.0926
FB	0.3310	-	-0.0313	0.0949	0.2568
PG	0.7800	0.5992	-	-0.0061	0.0727
SK	0.7330	0.0860	0.3820	-	0.1547
WB	0.1600	**0.0350 ****	0.192	**0.0790 ***	-

Table 7. Significance of each predictor in the RDA. Salinity mean and variance were not included due to missing values. Predictors significant at the 0.10 level are marked by *.

Environmental Variable	Df	Variance	F	Pr(>F)
Temperature Mean	1	0.0014	**2.3275 ***	**0.0650 ***
Temperature Variance	1	0.0013	**2.1627 ***	**0.0670 ***
pH Mean	1	0.0003	0.5264	0.7810
pH Variance	1	0.0007	1.2171	0.3010
Dissolved Oxygen Mean	1	0.0006	1.0354	0.3490
Dissolved Oxygen Variance	1	0.0003	0.5692	0.7250
Residual	19			

843

844 **Table 8.** Functions of differentially abundant proteins

Protein	Function	Citations
Peroxiredoxin-5 (PRX)	Scavenges ROS located in mitochondria using	(Tomanek 2014; Sussarellu et al. 2012)

	cysteine residuals to reduce substrates	
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)
Carbonic anhydrase (CA)	Maintenance of acid-base balance by catalyzing CO_2 conversion to HCO_3^-	(David et al. 2005; L. M. Parker, Ross, and O'Connor 2011; Wei et al. 2015)
Glucose-6-phosphate 1-dehydrogenase (G6PD)	Catalyzes first step in the pentose phosphate pathway and controls rate of pathway's oxidative portion	(L., n.d.)
Protein disulfide-isomerase 1 (PDI1) and Protein disulfide-isomerase 2 (PDI2)	General chaperone protein that aids in protein disulfide bond formation and isomerization	(Silvestre et al. 2006; Vargas-Albores et al. 2009; P. Zhang et al. 2014)

845

Figures

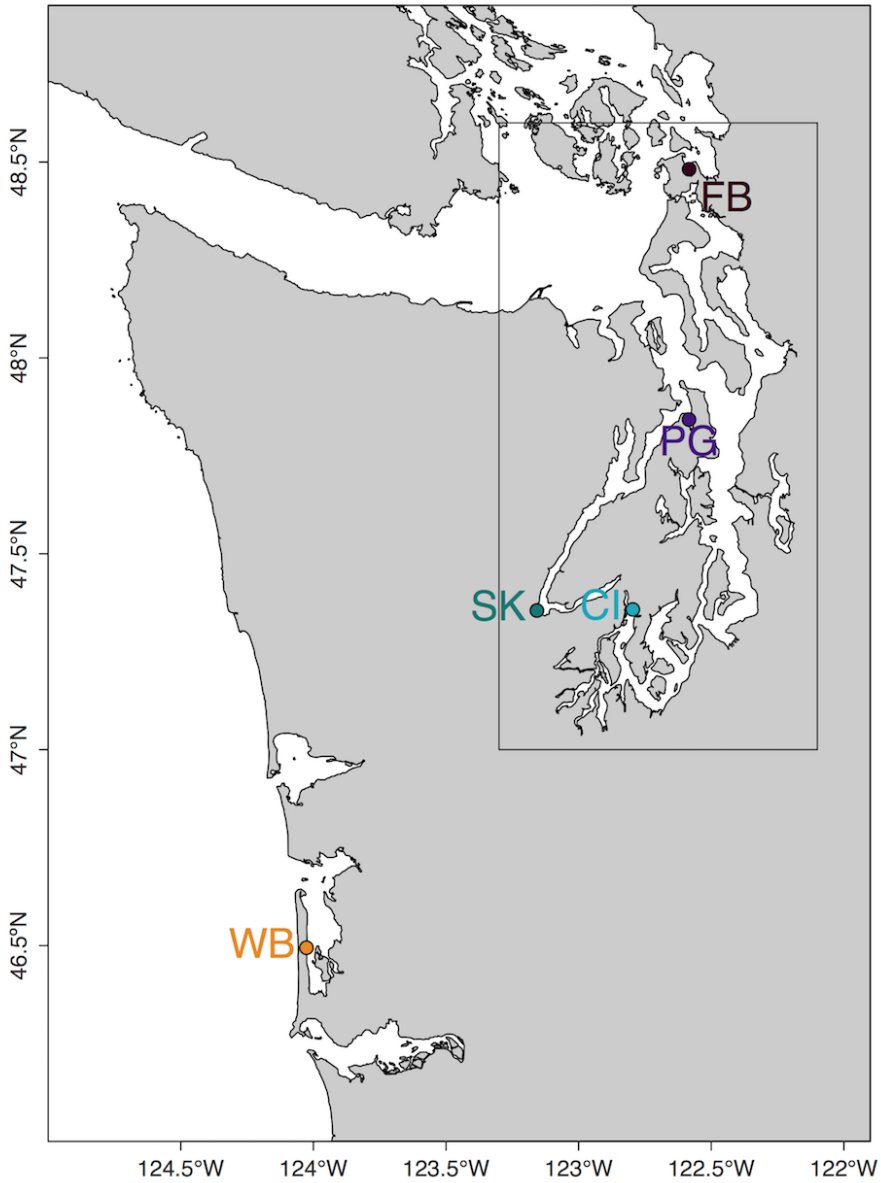


Figure 1. Map of outplant locations. Oysters were placed at five sites: Case Inlet (CI), Fidalgo Bay (FB), Port Gamble Bay (PG), Skokomish River Delta (SK), and Willapa Bay (WB). Sites in Puget Sound are outlined in a grey box.

Figure 2. Environmental variables for each site (Case Inlet, Fidalgo Bay, Port Gamble Bay, Skokomish River Delta, and Willapa Bay) and habitat (unvegetated and eelgrass) over the course of the 29 day outplant.

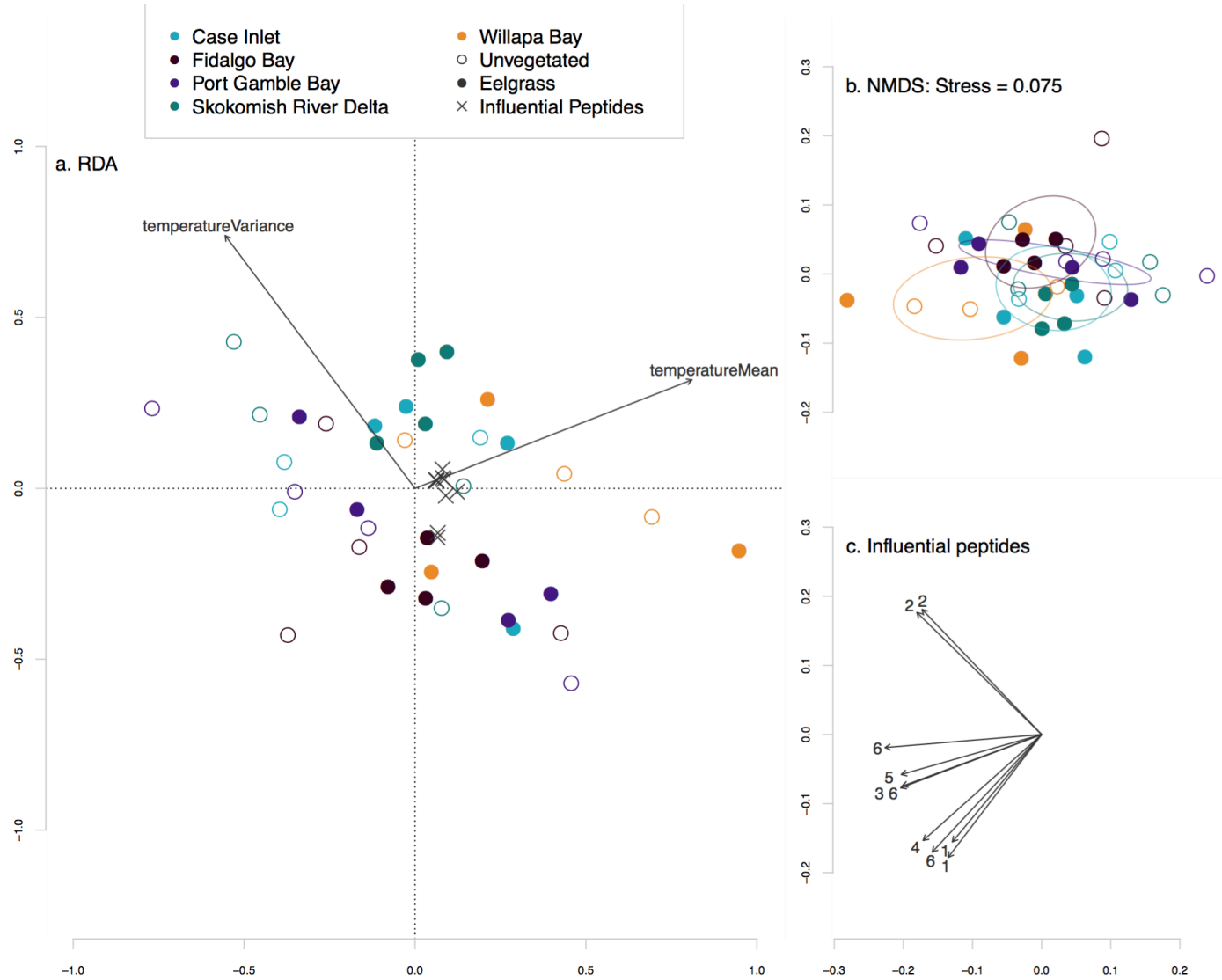
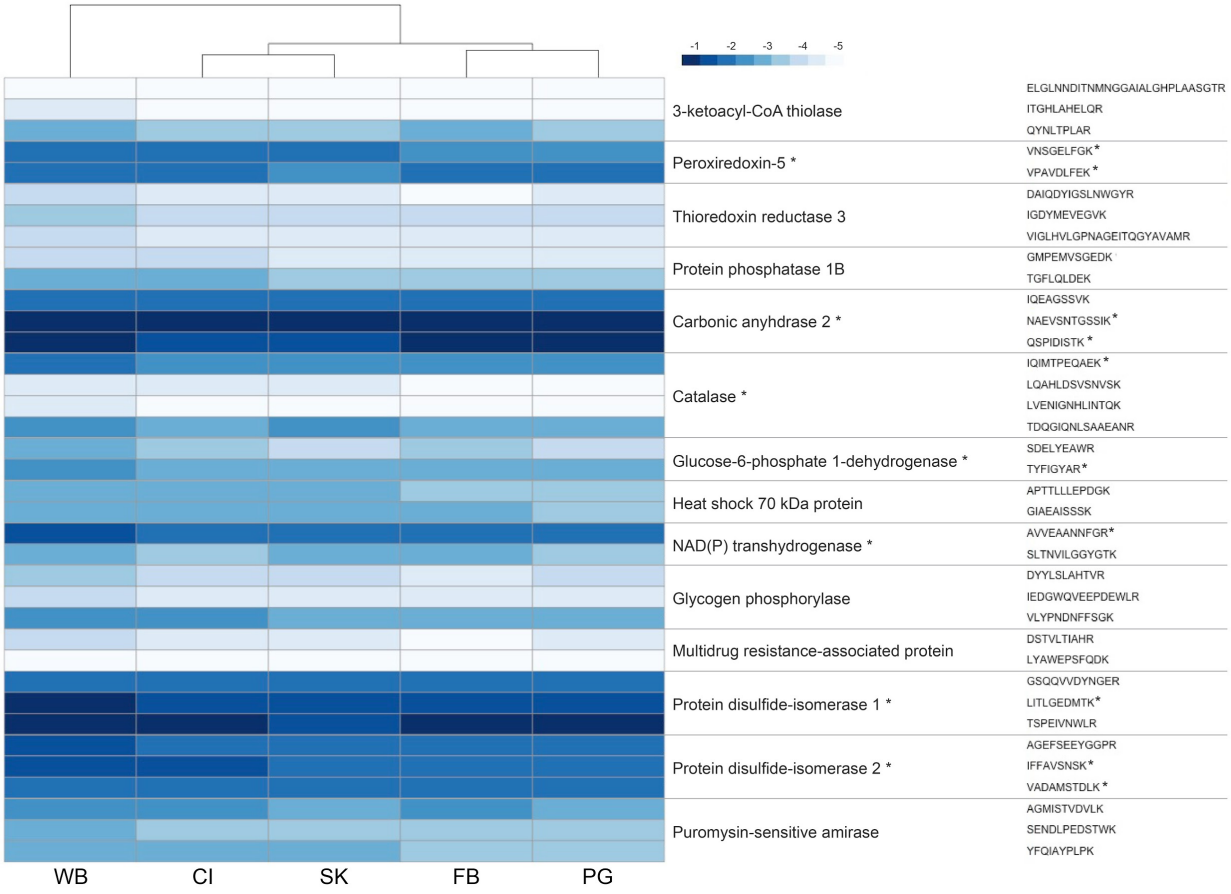


Figure 3. Ordination results for peptide abundance and environmental data. a) Environmental variables (pH, dissolved oxygen, salinity, and temperature) explained 30% of variance in peptide abundance data; however, redundancy analysis (RDA) for peptide abundance constrained by environmental variable was not significant (ANOVA; $F_{6,19} = 1.306$, $p = 0.195$). Temperature mean and variance were the most influential, yet nonsignificant, environmental predictors (ANOVA; temperature mean: $F_{1,25} = 2.3275$, $p = 0.065$; temperature variance: $F_{1,25} = 2.1527$, $p = 0.067$). Peptides differentially

abundant between Fidalgo Bay (FB) and Willapa Bay (WB) are primarily positively loaded onto temperature mean, with two negatively loaded on temperature variance. b) Non-metric multidimensional scaling plot depicting peptide abundance by site and habitat, with 95% confidence ellipses around each site (stress = 0.075, p = 0.0099). c) Peptides that contributed to significant peptide abundance differences between FB and WB, as determined by post-hoc similarity percentage analysis (SIMPER). Peptides denoted 1 correspond to peroxiredoxin-5 (PRX), 2 for carbonic anhydrase (CA), 3 for catalase (CAT), 4 for glucose-6-phosphate 1-dehydrogenase (G6PD), 5 for NADP transhydrogenase (NADPt), and 6 for protein disulfide isomerases 1 and 2 (PDI). FB peptide composition was differentiated by CA abundance, while all other proteins influenced peptide abundances at WB.



889

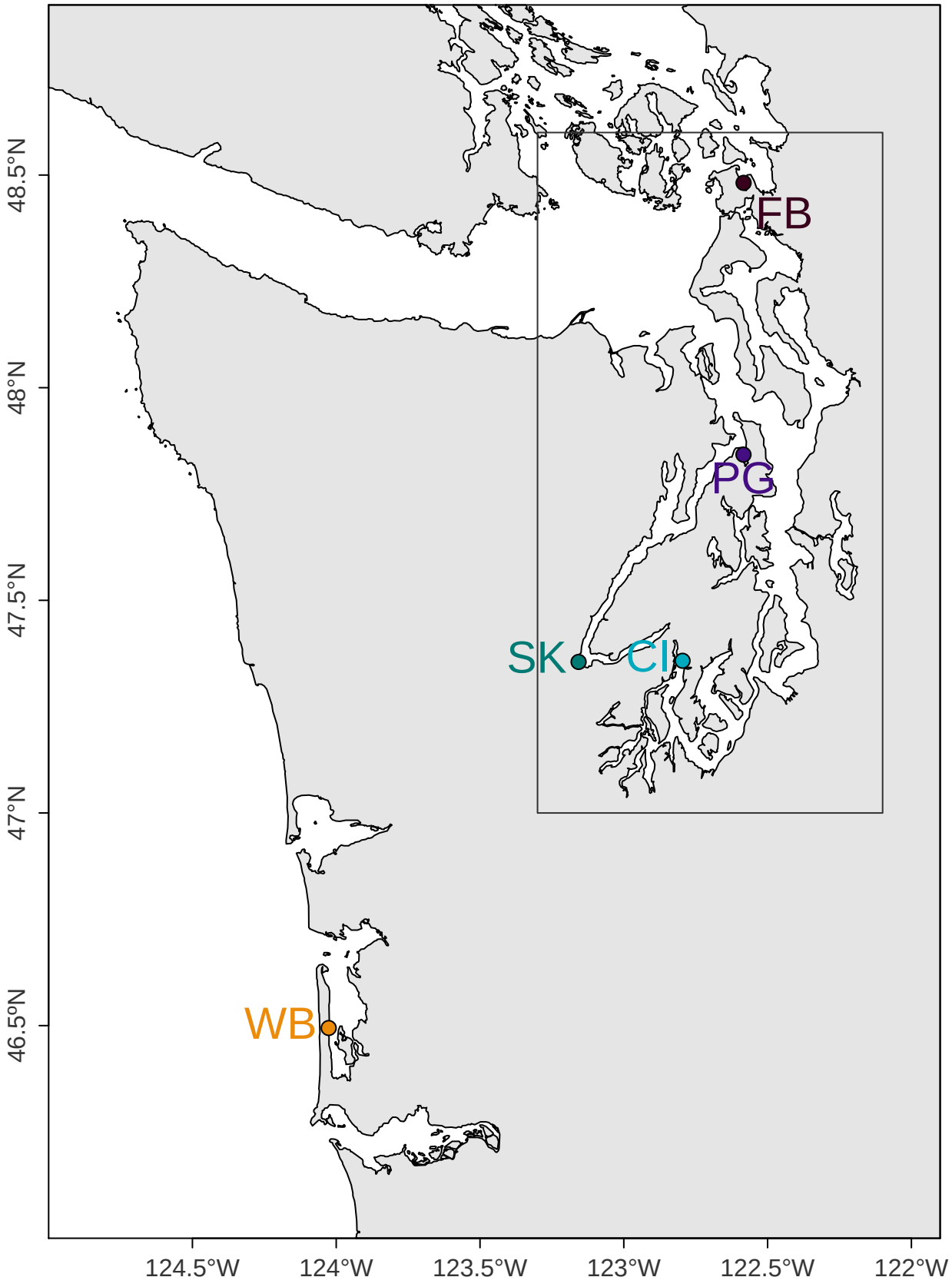
890 **Figure 4.** Average protein abundance by constituent peptides across experimental sites
891 from SRM. Peptide abundance data at each site was averaged, then log transformed.
892 Proteins were considered differentially abundant if at least one constituent peptide was
893 significantly different (indicated by an asterisk). There were no significant differences in
894 protein abundance among the Puget Sound locations, or between unvegetated and
895 eelgrass habitats. Proteins were only significantly different between Fidalgo Bay and
896 Willapa Bay or Skokomish River Delta and Willapa Bay.

897

898 **Supplementary Material**

899

900 Raw data can be accessed in the PeptideAtlas under accession PASS01304 and PASS01305.
901 Skyline documents can be found on Panorama Public.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PG
SK
Cl
WB
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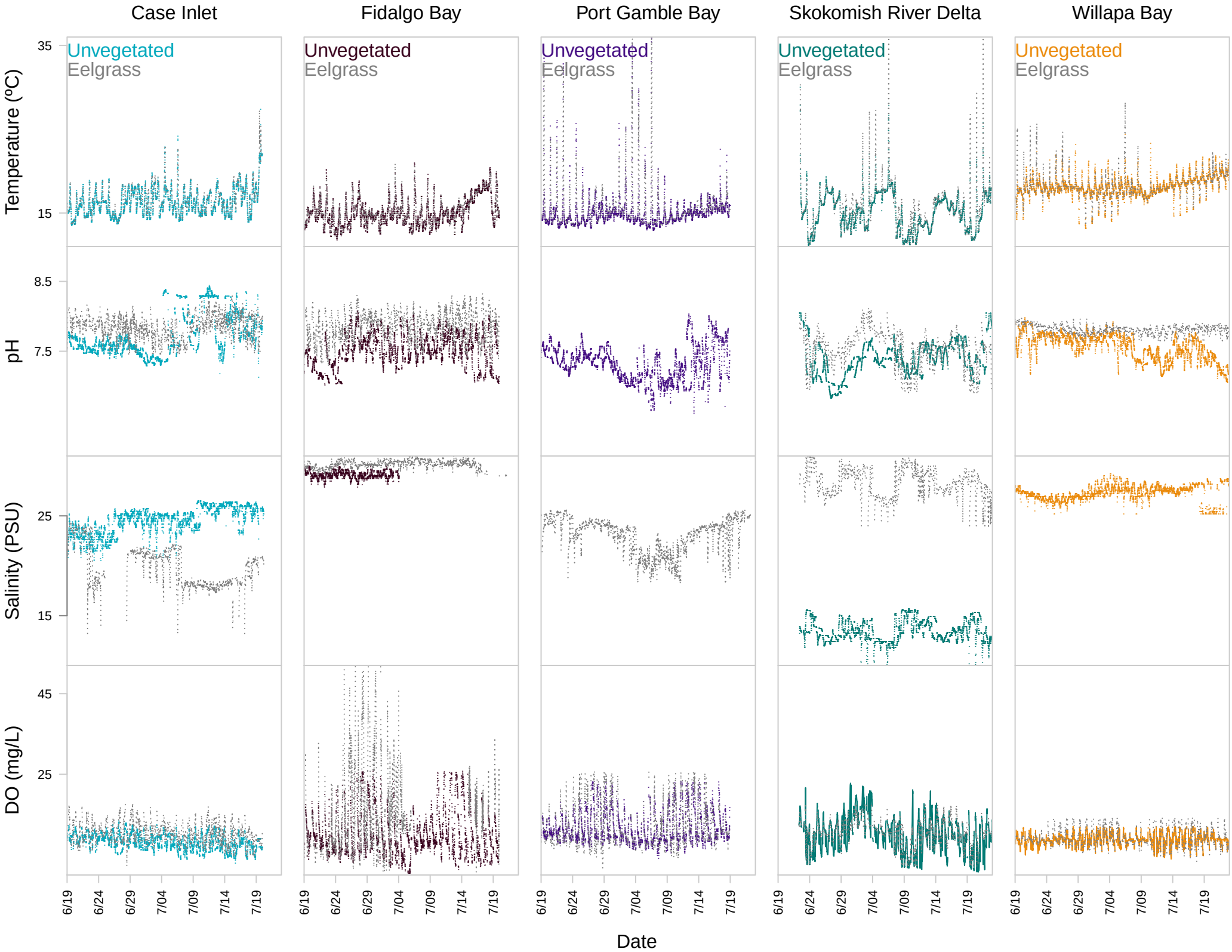
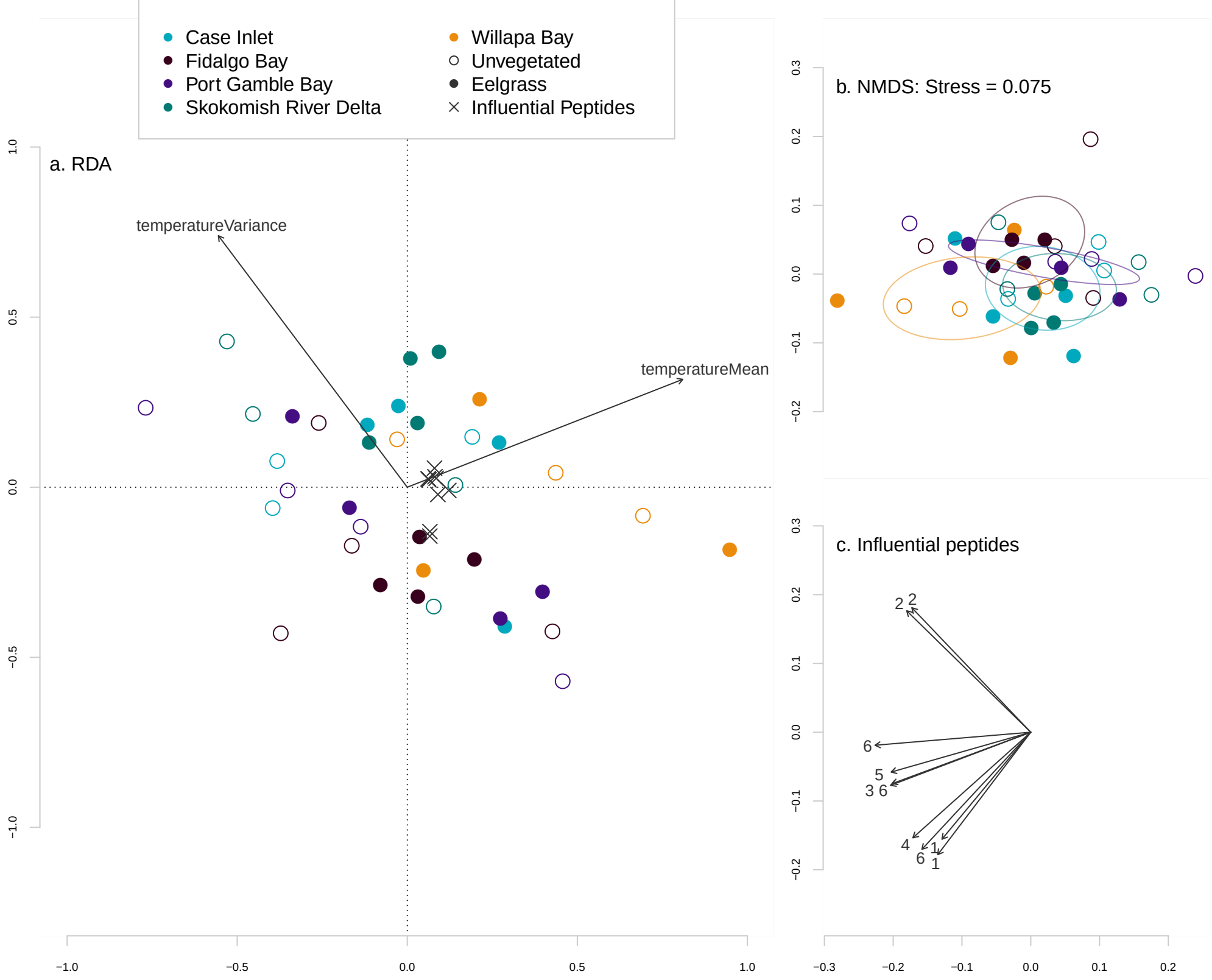
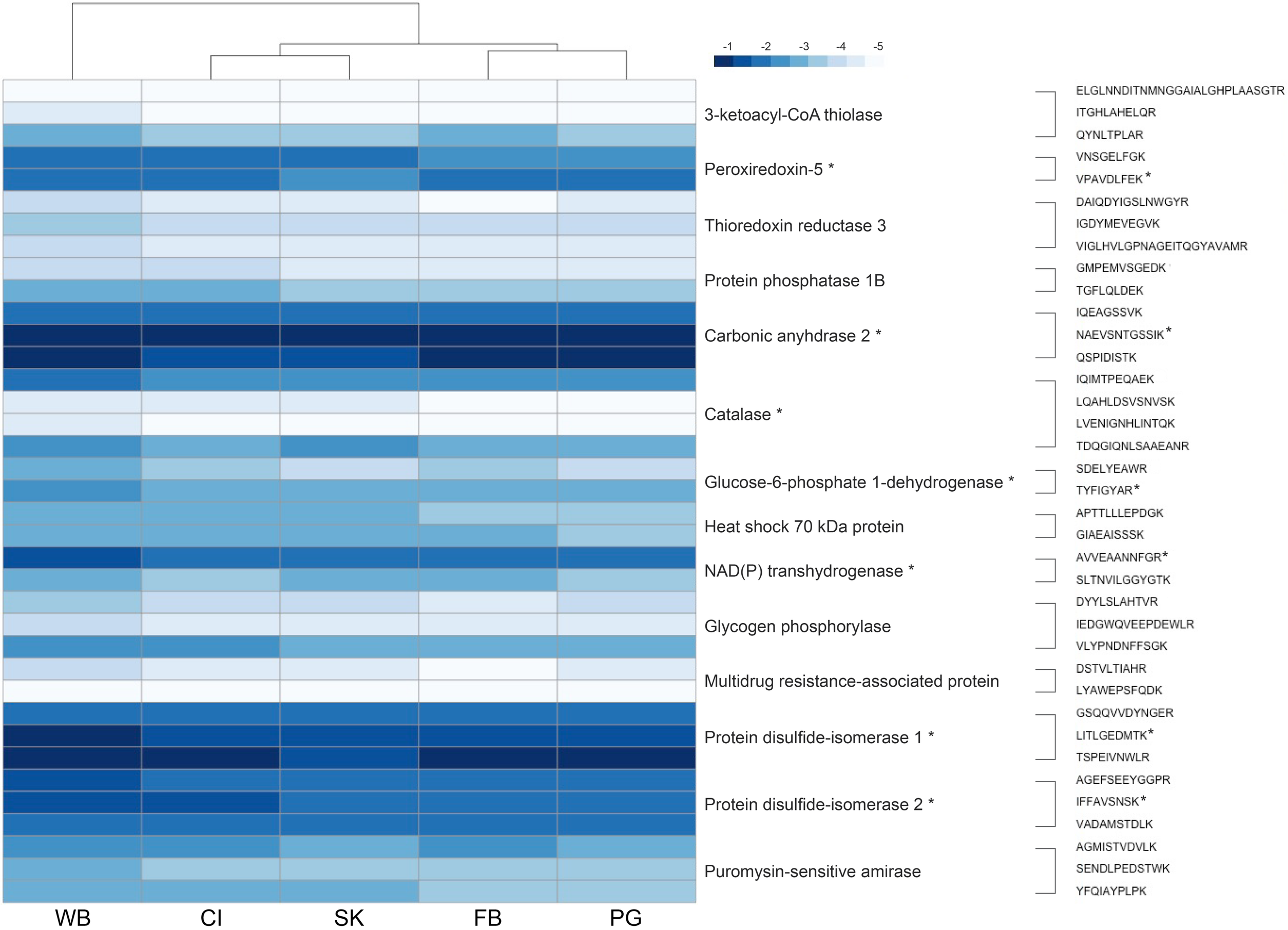
123.5°W
123°W
122.5°W
122°W


Case Inlet
Fidalgo Bay
Port Gamble Bay
Skokomish River Delta
Willapa Bay
Unvegetated
Eelgrass
Temperature (ºC)
35
15
pH
8.5
7.5
Salinity (PSU)
25
15
DO (mg/L)
45
25
6/19
6/24
6/29
7/04
7/09
7/14
7/19
Date


Case Inlet
Fidalgo Bay
Port Gamble Bay
Skokomish River Delta
Willapa Bay
Unvegetated
Eelgrass
Influential Peptides
a. RDA
temperatureVariance
temperatureMean
b. NMDS: Stress = 0.075
c. Influential peptides
2 2
6
5
3 6
4
6 1
1


-1
-2
-3
-4
-5
3-ketoacyl-CoA thiolase
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QYNLTPLAR
Peroxiredoxin-5 *
VNSGELFGK
VPAVDLFEK*
Thioredoxin reductase 3
DAIQDYIGSLNWGYR
IGDYMEVEGVK
VIGLHVLGPNAGEITQGYAVAMR
Protein phosphatase 1B
GMPEMVSGEDK
TGFLQLDEK
Carbonic anyhdrase 2 *
IQEAGSSVK
NAEVSNTGSSIK*
QSPIDISTK
Catalase *
IQIMTPEQAEK
LQAHLDSVSNVSK
LVENIGNHLINTQK
TDQGIQNLSAAEANR
Glucose-6-phosphate 1-dehydrogenase *
SDELYEAWR
TYFIGYAR*
Heat shock 70 kDa protein
APTTLLLEPDGK
GIAEAISSSK
NAD(P) transhydrogenase *
AVVEAANNFGR*
SLTNVILGGYGTK
Glycogen phosphorylase
DYYLSLAHTVR
IEDGWQVEEPDEWLR
VLYPNDNFFSGK
Multidrug resistance-associated protein
DSTVLTIAHR
LYAWEPSFQDK
Protein disulfide-isomerase 1 *
GSQQVVDYNGER
LITLGEDMTK*
TSPEIVNWLR
Protein disulfide-isomerase 2 *
AGEFSEEYGGPR
IFFAVSNSK*
VADAMSTDLK
Puromysin-sensitive amirase
AGMISTVDVLK
SENDLPEDSTWK
YFQIAYPLPK
WB
CI
SK
FB
PG