Proteomic response of early juvenile Pacific oysters (*Crassostrea gigas*) to temperature

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Pacific oysters (Crassostrea gigas) are a valuable aquaculture product that provides important ecosystem benefits. Among other threats, climate-driven changes in ocean temperature can impact oyster metabolism, survivorship, and immune function. We investigated how elevated temperature impacts larval oysters during settlement (19-33 days post-fertilization), using shotgun proteomics with data-independent acquisition to identify proteins present in the oysters after two weeks of exposure to 23°C or 29°C. Oysters maintained at elevated temperatures were larger and had a higher settlement rate, with 86% surviving to the end of the experiment; these oysters also had higher abundance trends of proteins related to metabolism and growth. Oysters held at 23°C were smaller, had a decreased settlement rate, displayed 100% mortality, and had elevated abundance trends of proteins related to immune response. This novel use of proteomics was able to capture characteristic shifts in protein abundance that hint at important differences in the phenotypic response of Pacific oysters to temperature regimes. Additionally, this work has produced a robust proteomic product that will be the basis for future research on bivalve developmental processes.

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16 ## Abstract

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18 provides important ecosystem benefits. Among other threats, climate-driven changes in
19 ocean temperature can impact oyster metabolism, survivorship, and immune function.
20 We investigated how elevated temperature impacts larval oysters during settlement (19-
21 33 days post-fertilization), using shotgun proteomics with data-independent acquisition
22 to identify proteins present in the oysters after two weeks of exposure to 23℃ or 29℃.
23 Oysters maintained at elevated temperatures were larger and had a higher settlement
24 rate, with 86% surviving to the end of the experiment; these oysters also had higher
25 abundance trends of proteins related to metabolism and growth. Oysters held at 23℃
26 were smaller, had a decreased settlement rate, displayed 100% mortality, and had
27 elevated abundance trends of proteins related to immune response. This novel use of
28 proteomics was able to capture characteristic shifts in protein abundance that hint at
29 important differences in the phenotypic response of Pacific oysters to temperature
30 regimes. Additionally, this work has produced a robust proteomic product that will be the
31 basis for future research on bivalve developmental processes.

32

33 **Keywords:** *Crassostrea gigas*, Pacific oysters, proteomics, data-independent
34 acquisition, temperature, ciliates

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Introduction

Oysters, such as the Pacific oyster (*Crassostrea gigas*), are a valuable aquaculture product and keystone species that provide essential ecosystem services within marine environments. Oysters are sessile organisms that form reefs that provide habitat for fish, invertebrates, and marine flora, and as filter feeders, oysters can have a positive influence on water quality (Coen et al., 2007; reviewed in Newell 2004). Pacific oysters are broadcast spawners, releasing eggs and sperm into the water column where fertilization occurs. Larval settlement generally occurs two to three weeks after fertilization and requires the metamorphosis of free-swimming pelagic larvae to sessile juvenile oysters. Metamorphosis is an energy-intensive process during which larvae undergo complex behavioral and morphological changes.

As with many coastal marine organisms, changes in temperature can impact oyster physiology. In Ireland, mass mortality events of hatchery-produced adult Pacific oysters out-planted at two sites have been linked to high-temperature stress during the summer months with companion laboratory studies demonstrating decreased immune function at 21°C compared to 12°C by way of decreased phagocytosis in the hemolymph (Malham et al., 2009). Often, temperature changes are accompanied by changes in other environmental factors that can also have negative impacts. For example, (Ko et al., 2014) found that low pH and low salinity combined with high temperature delays Pacific oyster larval growth rate before settlement and metamorphosis. These impacts of temperature can affect both cultured and wild oysters and are likely to be realized on a more frequent basis with climate-change-induced ocean warming (Helmuth & Hofmann, 2001).

While natural and uncontrollable temperature increases can be harmful, commercial hatcheries often rear oyster larvae in elevated temperatures under controlled conditions to achieve better outcomes. For many bivalves, increased temperature is often used to initiate spawning in the hatchery by mimicking spring conditions, when spawning naturally occurs (FAO, 2004). Increased temperature is also used to improve metabolism and growth in young oysters in hatchery settings. A comparison of larval physiology and early juvenile development of the Pacific oyster at five different temperatures (17, 22, 25, 27, and 32°C) concluded that optimal growth rates and greatest settlement occurred at 27°C (Rico-Villa, Pouvreau & Robert, 2009). Higher temperatures accelerate biological processes, including respiration and metabolism, allowing for faster development given that the larvae's energetic needs are met, perhaps allowing young bivalves to "cruise" through the stressful metamorphosis period. However, there is likely a limit to any realized benefit with respect to temperature and duration of exposure.

Insight into molecular physiology at the protein level could help to explain the differences in larval success between different temperatures. The presence of certain proteins will show which genes are being translated and which proteins can be available for cell physiological function. An unbiased global proteomics survey can identify many of the proteins present in organisms at the time of sampling. This information can be

84 leveraged to decipher the biological processes at play in various temperature
85 treatments. Although it is still considered a novel approach, proteomics has been used
86 in several studies of Pacific oyster response to a range of relevant environmental
87 drivers and life stages (Venkataraman et al., 2019; Huan et al., 2012; Dineshram et al.,
88 2012; Corporeau et al., 2012).
89
90   In this study, we used shotgun proteomics to understand how temperature
91 impacts the physiology of recently settled oysters in a hatchery setting. We employed
92 data-independent acquisition (DIA), which is a method of tandem mass spectrometry
93 that achieves a more in-depth proteome coverage by acquiring all MS2 spectra for a
94 given MS1 scan, unlike data dependent acquisition which only acquires the top *n* MS2
95 for each MS1 scan (Venable et al., 2004; Gillet et al., 2012). The goal of this experiment
96 was to compare the proteomic responses of the oysters in the two temperature regimes,
97 a typical rearing temperature of 23˚C and an elevated temperature thought to promote
98 faster larval growth and better survival of 29˚C, and in the process develop a robust
99 proteome that could build upon current *Crassostrea gigas* proteomic resources. These
100 results contribute to our understanding of how temperature conditions impact oysters in
101 hatchery settings, and suggest that oysters in the wild may be impacted by increasing
102 temperature during the post-settlement stage.

103 ## Methods

104 Oyster Rearing and Treatment Conditions

105   Adult oysters (n=20) were strip spawned and eggs and sperm were combined for
106 fertilization. Adult oysters (approximately 7cm in size) were from Hood Canal,
107 Washington. Oyster larvae were reared for 19 days post fertilization (dpf) before
108 competent larvae were split between two silos, each a different temperature regime —
109 conventional commercial conditions (23˚C) and elevated temperature (29˚C) — with 1.1
110 million *Crassostrea gigas* larvae reared in one fiberglass silo (46 cm diameter) per
111 treatment. All seawater was pumped from Dabob Bay, WA, filtered through sequentially
112 decreasing filter bags of 25 μm, 10 μm, and 5 μm, and treated with sodium carbonate
113 (Na_2CO_3) to reach a pH set point of 8.4 (NBS scale). All oysters received the same
114 mixed high-density microalgae diet (flagellates: *Isochrysis* spp., *Pavlova* spp.,
115 *Nannochloropsis* spp., *Rhodomonas* spp., and *Tetraselmis* spp.). Effluent algal
116 densities were targeted at 100K cells ml^{-1}. Crushed oyster shell (microcultch) graded
117 from 180-315 μm was used as substrate for settlement and added to the oyster silos at
118 20 dpf. At 24 dpf (5 days after initiation of temperature treatment), oysters were
119 screened to determine size and settlement rate, and then were returned to their
120 respective silos. Settled oyster seed from each temperature regime was sampled for
121 proteomic analysis on 24 and 27 dpf, after 5 and 8 days of temperature exposure. Dry
122 oyster seed (approximately 500 μl) was flash frozen in liquid nitrogen prior to storage at
123 -80˚C. The remaining seed was reared until 33 dpf, then screened to determine size
124 and mortality (**Figure 1**).

Proteomic Sample Preparation

125

126   For proteomic analyses, four samples of pooled dry larvae (~250ul equivalent)
127   were prepared and analyzed representing two samples each from 23°C and 29°C, one
128   from 24 dpf and one from 27 dpf. For each of the 4 samples, 50mM ammonium
129   bicarbonate (NH_4HCO_3) + 6M urea (500 µl) was added and larvae were homogenized
130   using a pestle. Samples were centrifuged at 2000 x g for 5 minutes. Supernatant (150
131   µl) was pipetted from each sample and placed into new tubes. The supernatant was
132   sonicated three times each for 5 seconds at room temperature, cooling samples in
133   between sonication rounds using an ethanol and dry ice bath for 5 seconds. Protein
134   concentration was determined using a Pierce™ BCA Protein Assay Kit according to the
135   manufacturer's protocol, at 100 µg diluted in 50mM NH_4HCO_3 (ThermoFisher Scientific,
136   Waltham, MA).
137
138   Each sample (100 µg) was digested using trypsin and desalted for mass
139   spectrometry as previously described (Timmins-Schiffman et al., 2017). Dried peptides
140   were reconstituted in 100 µl 3% acetonitrile + 0.1% formic acid and stored at -80°C.
141   Data Independent Acquisition (DIA) was performed to assess protein abundance
142   patterns via liquid chromatography tandem mass spectrometry (LC-MS/MS) (gradient of
143   2-35% acetonitrile over 60 minutes) with a Q-Exactive mass spectrometer (Thermo
144   Fisher). Full MS (MS1) settings included 60,000 resolution with 100 ms maximum
145   injection time, and an AGC target of 3e6. DIA settings included 30,000 resolution, 1e6
146   AGC target, maximum injection time of 50 ms, loop count of 20, isolation window of 4
147   *m/z*, and an offset window of 0.0 *m/z* (Timmins-Schiffman et al., 2017).

148   Proteomic Data Analysis

149   Raw mass spectrometry files were converted to .mzML format using MSConvert
150   from the ProteoWizard Toolkit version 3.0 (Chambers et al., 2012). Resulting files and
151   the *Crassostrea gigas* deduced proteome (Supplemental File 1) were used to create a
152   chromatogram library using EncyclopeDIA with Walnut version 0.6.14 (Searle et al.,
153   2018). Specific protocol details are provided in supplementary material (Supplemental
154   File 2). The chromatogram library, *Crassostrea gigas* proteome, and .mzML files were
155   subsequently imported into Skyline Daily version 4.1.9.18271 (MacLean et al., 2010),
156   which provides a means of setting filters, viewing spectral data for quality inspection,
157   and exporting the data for downstream analyses (Supplemental File 3).
158
159   Spectral data and proteins detected were exported for use in MS Stats (version
160   3.12.3, (Choi et al., 2014)). Within MS Stats, the two sampling dates (5 and 8 days of
161   temperature treatment) were combined and treated as replicates to compare protein
162   abundances between temperatures (Supplemental File 4). Pooling the sampling dates
163   provided a more robust analysis of the dominant trends in temperature response to
164   compensate for the small number of samples. From the list of proteins, proteins that
165   achieved the threshold of >2.00 and <-2.00 log-2 fold change (log2FC) were
166   considered of interest for potential physiological importance, even though they were not
167   significantly differentially abundant proteins (Supplemental File 5). Specific protocol
168   details are provided (Supplemental File 6). DAVID, version 6.8 (Huang, Sherman &

169 Lempicki, 2009a,b), was used to identify the enriched Gene Ontology (GO) terms from
170 the list of proteins of physiological interest in relation to all detected proteins.
171 Additionally, enriched GO terms from the detected proteins were characterized in
172 relation to the *Crassostrea gigas* proteome to capture the abundant biological
173 processes present at this developmental stage, irrespective of temperature treatment,
174 and enriched GO terms were identified using a < 0.05 FDR cutoff.

Results

Phenotype

177 At 24 dpf (5 days into temperature treatment), oysters reared at 29˚C had a 22.6%
178 settlement rate with a weighted average screen size of 560 μm. Approximately 25% of
179 seed grown at 29˚C were 710 μm and larger. Oysters grown at 23˚C had a 9.2%
180 settlement rate with a size of 363 μm at 24 dpf, with no seed exceeding 710 μm. At 29
181 dpf (10 days into temperature experiment), ciliates were visible at 23˚C. By 33 dpf, no
182 oysters were alive at 23˚C, while survival of oysters grown at 29˚C was 86%.

Proteomics

184 There were 2,808 detected proteins (Supplemental File 7), ~6.9% of the predicted
185 proteins described in the published *Crassostrea gigas* proteome (Supplemental File 8).
186 Of the 2,808 detected proteins, 1,256 were associated with GO slim terms, the majority
187 of which were related to metabolism, growth, and development (**Figure 2**). These
188 detected proteins were enriched for 108 biological process GO terms compared to the
189 full, predicted *Crassostrea gigas* proteome.
190

191 Of the 2,808 detected proteins, 69 followed changing abundance trends that suggested
192 physiological importance between the 23˚C and 29˚C treatments when using thresholds
193 of >2.00 and <-2.00 log-2 fold change. Thirty-six proteins were higher in the 29˚C
194 treatment, while 33 were higher in 23˚C treatment (**Table 1**). These proteins of interest
195 contributed to 18 enriched GO biological processes (**Figure 3**). Further analysis of this
196 set of proteins identified enriched biological processes in the samples when compared
197 with the full predicted proteome. Proteins higher in oysters grown in 29˚C were
198 associated with biological processes primarily related to growth (**Table 2**), while those
199 higher in oysters grown in 23˚C may contribute to the immune response (**Table 3**).

Discussion

201   Using novel proteomics techniques, this study identified proteins of physiological
202 interest when comparing larval Pacific oysters exposed to two different temperatures.
203 While this study focuses primarily on hatchery applications, oysters can face
204 temperatures of 23˚C and 29˚C in natural settings as well. As summarized in Lim et al.
205 (2016), Pacific oysters can live in temperatures ranging 5 - 35 ˚C, and can handle acute

206 exposure to 43 °C (Shamseldin et al. 1997; Lim et al., 2016). Temperature can have
207 different effects on different stages of development; for example, the optimal range of
208 temperature for gametogenesis is thought to be between 20-25°C (Mann et al. 1991;
209 Lim et al. 2016). With tools like proteomics, we can get a deeper understanding of the
210 mechanisms for different life stages, such as the development of oocytes (Corporeau et
211 al., 2012), and with more research, investigate how different environmental conditions
212 such as temperature impact the proteomic response of oysters. This has applications
213 not only for developing optimal hatchery conditions for growth and production, but also
214 for understanding how wild populations may fare in the face of climate change and other
215 stressors.
216
217   Across the two temperature treatments, the detected proteins primarily
218 represented those involved in metabolism, growth, and development. These findings
219 are in agreement with the life history stage sampled, where growth rates are elevated
220 and significant physiological changes are occurring related to somatic organization. In
221 another study, researchers used *in silico* approaches to identify genes associated with
222 larval settlement in *Crassostrea gigas* (Foulon et al., 2019). Approximately 27% of
223 genes described by Foulon et al (2019) had protein complements expressed in the
224 current study. This not only validates the relevance of the developmental role of these
225 proteins but also provides valuable resources for future work focused on
226 metamorphosis and larval adhesion. Additionally, this comparison highlights the robust
227 nature of the proteome developed as part of this study, along with the value of the Data
228 Independent Acquisition proteomic approach.
229
230   Based on protein abundance comparison, we found that the GO Biological
231 Processes detected in oysters in the 29°C treatment were related to metabolism and
232 growth, while the Biological Processes detected in oysters exposed to the 23°C
233 treatment were involved in immune system response. The presence of and interaction
234 with ciliates in bivalve larval cultures have previously elicited a similar proteomic
235 response in geoduck clam larvae (Timmins-Schiffman et al., 2020). Among the
236 processes that were elevated at 23°C in the oysters were Mitogen-activated protein
237 kinase 4 (MAPK-4), which plays a role in the initiation of innate immune response
238 (Krzyzowska et al., 2010), and allograft inflammatory factor 1 (AIF1), which has been
239 shown to be a component in invertebrate immune response against pathogens and
240 injury (Vizioli, Verri & Pagliara, 2020).
241
242   The growth and development of Pacific oyster larvae were positively impacted by
243 exposure to 29°C. The higher temperature likely promoted elevated metabolic rates,
244 which in turn supported elevated growth and development. Higher temperatures (28-
245 30°C) have been shown to promote higher rates of metabolism and growth in another
246 oyster species, *Crassostrea corteziensis,* in the juvenile spat life stage (Cáceres-Puig et
247 al., 2007). Another possibility for the observed proteomic trend is that in the absence of
248 other stressors, such as the ciliates observed at the lower temperature, there was an
249 increased relative allocation of energy towards growth and development at the higher
250 temperature. This is further supported by the phenotype data, which revealed that the
251 oysters in the 29°C treatment had higher settlement rates, greater size, no ciliates, and

252 86% survival, as compared to the 23℃ treatment group which had lower settlement
253 rate, smaller size, and 100% mortality by the end of the experiment.
254
255   The oyster larvae samples from the 23℃ treatment had evidence of a greater
256 physiological role of proteins associated with immune response when compared to the
257 larvae at elevated temperature. At 29 dpf, ciliates were observed in the silo at 23℃ and
258 by 33 dpf, all the oysters in the 23℃ treatment were dead. The predominant proteomic
259 response was an immune response to parasites, supporting the idea that the oysters
260 were initiating immune responses. Ciliate presence likely negatively impacted survival,
261 either directly through parasitism or indirectly through increased energy allocation
262 towards immune responses and away from critical maintenance processes. Ciliates
263 have been a problem in hatcheries for decades and are associated with significant
264 mortality events in early development bivalves (Elston et al., 1999). Ciliates may prefer
265 colder temperatures or may not be able to survive at higher temperatures, protecting
266 larval oysters at 29°C against potential infections and the associated cost of launching
267 an immune response. Alternatively, larvae may be physiologically compromised at lower
268 temperatures, making them more susceptible to ciliates. In natural, non-hatchery
269 settings, oyster susceptibility to ciliates increases with increasing salinity (Gauthier,
270 Soniat & Rogers, 1990), and increases in summer and fall seasons when temperatures
271 are roughly between 23°C and 25°C (McGurk, Ford & Bushek, 2016). This seasonal
272 change observed could be related to laboratory findings where oysters were more
273 susceptible to infection from OsHV-1 (oyster herpesvirus) in warmer temperatures,
274 specifically 21°C and 26°C, though at an elevated temperature of 29°C, the susceptibility
275 of the oysters to OsHV-1 declined, and oysters at 29°C had high survival rates (Delisle
276 et al., 2018, 2020). Future research is needed to attempt to disentangle these
277 phenomena and continue to elucidate factors contributing to improved survival in
278 oysters.
279
280   Our findings can help hatchery workers, managers, and conservationists predict
281 how temperature is and will impact oysters at the settlement stage in hatchery settings.
282 The findings support an improved practice of increasing the temperature during the
283 early developmental stage in the days preceding settlement to improve growth and
284 survival. However further studies should investigate the optimal length of time and
285 during which phase of development the larvae should be reared at elevated
286 temperature. In addition, the annotated proteome developed as part of this work will be
287 a valuable tool for future studies on bivalve development including providing specific
288 targets for protein regulation studies in oysters as well as a reference for gene discovery
289 in less studied bivalves.

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Supplemental Information

294

295   Supplemental files, scripts, and data can be found at the associated repository:
296 DOI: https://doi.org/10.5281/zenodo.3906831. Proteomic data can be accessed via
297 PRIDE, at http://www.ebi.ac.uk/pride/archive/projects/PXD015434, Accession:
298 PXD015434.

References

300 Cáceres-Puig JI, Abasolo-Pacheco F, Mazón-Suastegui JM, Maeda-Martínez AN,
301   Saucedo PE. 2007. Effect of temperature on growth and survival of Crassostrea
302   corteziensis spat during late-nursery culturing at the hatchery. *Aquaculture*
303   272:417–422. DOI: 10.1016/j.aquaculture.2007.06.030.
304 Chambers MC, Maclean B, Burke R, Amodei D, Ruderman DL, Neumann S, Gatto L,
305   Fischer B, Pratt B, Egertson J, Hoff K, Kessner D, Tasman N, Shulman N, Frewen
306   B, Baker TA, Brusniak M-Y, Paulse C, Creasy D, Flashner L, Kani K, Moulding C,
307   Seymour SL, Nuwaysir LM, Lefebvre B, Kuhlmann F, Roark J, Rainer P, Detlev S,
308   Hemenway T, Huhmer A, Langridge J, Connolly B, Chadick T, Holly K, Eckels J,
309   Deutsch EW, Moritz RL, Katz JE, Agus DB, MacCoss M, Tabb DL, Mallick P. 2012.
310   A cross-platform toolkit for mass spectrometry and proteomics. *Nature*
311   *biotechnology* 30:918–920.
312 Choi M, Chang C-Y, Clough T, Broudy D, Killeen T, MacLean B, Vitek O. 2014.
313   MSstats: an R package for statistical analysis of quantitative mass spectrometry-
314   based proteomic experiments. *Bioinformatics* 30:2524–2526. DOI:
315   10.1093/bioinformatics/btu305.
316 Coen LD, Brumbaugh RD, Bushek D, Grizzle R, Luckenbach MW, Posey MH, Powers
317   SP, Tolley SG. 2007. Ecosystem services related to oyster restoration. *Marine*
318   *Ecology Progress Series* 341:303–307. DOI: 10.3354/meps341303.
319 Corporeau C, Vanderplancke G, Boulais M, Suquet M, Quéré C, Boudry P, Huvet A,
320   Madec S. 2012. Proteomic identification of quality factors for oocytes in the Pacific
321   oyster Crassostrea gigas. *Journal of proteomics* 75:5554–5563.
322 Delisle L, Pauletto M, Vidal-Dupiol J, Petton B, Bargelloni L, Montagnani C, Pernet F,
323   Corporeau C, Fleury E. 2020. High temperature induces transcriptomic changes in
324   that hinder progress of ostreid herpesvirus (OsHV-1) and promote survival. *The*
325   *Journal of experimental biology* 223. DOI: 10.1242/jeb.226233.
326 Delisle L, Petton B, Burguin JF, Morga B, Corporeau C, Pernet F. 2018. Temperature
327   modulate disease susceptibility of the Pacific oyster Crassostrea gigas and
328   virulence of the Ostreid herpesvirus type 1. *Fish & shellfish immunology* 80:71–79.
329 Dineshram R, Wong KKW, Xiao S, Yu Z, Qian PY, Thiyagarajan V. 2012. Analysis of
330   Pacific oyster larval proteome and its response to high-CO2. *Marine pollution*
331   *bulletin* 64:2160–2167.
332 Elston RA, Cheney D, Frelier P, Lynn D. 1999. Invasive orchitophryid ciliate infections in

333   juvenile Pacific and Kumomoto oysters, Crassostrea gigas and Crassostrea

334   sikamea. *Aquaculture* 174:1–14. DOI: 10.1016/s0044-8486(98)00512-2.

335   FAO. 2004. *Hatchery culture of bivalves*.

336   Foulon V, Boudry P, Artigaud S, Guérard F, Hellio C. 2019. In Silico Analysis of Pacific

337   Oyster (Crassostrea gigas) Transcriptome over Developmental Stages Reveals

338   Candidate Genes for Larval Settlement. *International journal of molecular sciences*

339   20. DOI: 10.3390/ijms20010197.

340   Gauthier JD, Soniat TM, Rogers JS. 1990. A Parasitological Survey of Oysters Along

341   Salinity Gradients in Coastal Louisiana. *Journal of the World Aquaculture Society*

342   21:105–115. DOI: 10.1111/j.1749-7345.1990.tb00530.x.

343   Gillet LC, Navarro P, Tate S, Röst H, Selevsek N, Reiter L, Bonner R, Aebersold R.

344   2012. Targeted data extraction of the MS/MS spectra generated by data-

345   independent acquisition: a new concept for consistent and accurate proteome

346   analysis. *Molecular & cellular proteomics: MCP* 11:O111.016717.

347   Helmuth BS, Hofmann GE. 2001. Microhabitats, thermal heterogeneity, and patterns of

348   physiological stress in the rocky intertidal zone. *The Biological bulletin* 201:374–

349   384.

350   Huang DW, Sherman BT, Lempicki RA. 2009a. Systematic and integrative analysis of

351   large gene lists using DAVID bioinformatics resources. *Nature protocols* 4:44–57.

352   Huang DW, Sherman BT, Lempicki RA. 2009b. Bioinformatics enrichment tools: paths

353   toward the comprehensive functional analysis of large gene lists. *Nucleic acids*

354   *research* 37:1–13.

355   Huan P, Wang H, Dong B, Liu B. 2012. Identification of differentially expressed proteins

356   involved in the early larval development of the Pacific oyster Crassostrea gigas.

357   *Journal of Proteomics* 75:3855–3865. DOI: 10.1016/j.jprot.2012.04.051.

358   Ko GWK, Dineshram R, Campanati C, Chan VBS, Havenhand J, Thiyagarajan V. 2014.

359   Interactive Effects of Ocean Acidification, Elevated Temperature, and Reduced

360   Salinity on Early-Life Stages of the Pacific Oyster. *Environmental Science &*

361   *Technology* 48:10079–10088. DOI: 10.1021/es501611u.

362   Krzyzowska M, Swiatek W, Fijalkowska B, Niemialtowski M, Schollenberger A. 2010.

363   The role of MAP kinases in immune response. *Medical Journal of Cell Biology*

364   2:125–138.

365   Lim H-J, Kim B-M, Hwang IJ, Lee J-S, Choi I-Y, Kim Y-J, Rhee J-S. 2016. Thermal

366   stress induces a distinct transcriptome profile in the Pacific oyster Crassostrea

367   gigas. *Comparative biochemistry and physiology. Part D, Genomics & proteomics*

368   19:62–70.

369   MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R,

370   Tabb DL, Liebler DC, MacCoss MJ. 2010. Skyline: an open source document editor

371   for creating and analyzing targeted proteomics experiments. *Bioinformatics*

372   26:966–968.

373 Malham SK, Cotter E, O'Keeffe S, Lynch S, Culloty SC, King JW, Latchford JW,
374   Beaumont AR. 2009. Summer mortality of the Pacific oyster, Crassostrea gigas, in
375   the Irish Sea: The influence of temperature and nutrients on health and survival.
376   *Aquaculture* 287:128–138.
377 Mann R, Burreson EM, Baker PK. 1991. The decline of the Virginia oyster fishery in
378   Chesapeake Bay: considerations for introduction of a non-endemic species,
379   Crassostrea gigas (THUNBERG, 1793). *Journal of Shellfish Research* 10,
380   379–388.
381 McGurk ES, Ford S, Bushek D. 2016. Unusually abundant and large ciliate xenomas in
382   oysters, Crassostrea virginica, from Great Bay, New Hampshire, USA. *Journal of*
383   *invertebrate pathology* 137:23–32.
384 Newell RIE. 2004. Ecosystem influences of natural and cultivated populations of
385   suspension-feeding bivalve molluscs: a review. *Journal of Shellfish Research* 23
386   (1).
387 Rico-Villa B, Pouvreau S, Robert R. 2009. Influence of food density and temperature on
388   ingestion, growth and settlement of Pacific oyster larvae, Crassostrea gigas.
389   *Aquaculture* 287:395–401. DOI: 10.1016/j.aquaculture.2008.10.054.
390 Searle BC, Pino LK, Egertson JD, Ting YS, Lawrence RT, MacLean BX, Villén J,
391   MacCoss MJ. 2018. Chromatogram libraries improve peptide detection and
392   quantification by data independent acquisition mass spectrometry. *Nature*
393   *Communications* 9. DOI: 10.1038/s41467-018-07454-w.
394 Shamseldin AA, Clegg JS, Friedman CS, Cherr GN, Pillai MC. 1997. Induced
395   thermotolerance in the Pacific oyster Crassostrea gigas. *Journal of Shellfish*
396   *Research* 16, 487–491.
397 Timmins-Schiffman EB, Crandall GA, Vadopalas B, Riffle ME, Nunn BL, Roberts SB.
398   2017. Integrating Discovery-driven Proteomics and Selected Reaction Monitoring
399   To Develop a Noninvasive Assay for Geoduck Reproductive Maturation. *Journal of*
400   *proteome research* 16:3298–3309.
401 Timmins-Schiffman E, Guzmán JM, Elliott Thompson R, Vadopalas B, Eudeline B,
402   Roberts SB. 2020. Larval Geoduck (Panopea generosa) Proteomic Response to
403   Ciliates. *Scientific reports* 10:6042.
404 Venable JD, Dong M-Q, Wohlschlegel J, Dillin A, Yates JR. 2004. Automated approach
405   for quantitative analysis of complex peptide mixtures from tandem mass spectra.
406   *Nature methods* 1:39–45.
407 Venkataraman YR, Timmins-Schiffman E, Horwith MJ, Lowe AT, Nunn B, Vadopalas B,
408   Spencer LH, Roberts SB. Characterization of Pacific oyster (Crassostrea gigas)
409   proteomic response to natural environmental differences. DOI: 10.1101/460204.
410 Vizioli J, Verri T, Pagliara P. 2020. Allograft Inflammatory Factor-1 in Metazoans: Focus
411   on Invertebrates. *Biology* 9. DOI: 10.3390/biology9110355.

Figure 1

Experimental timeline.

At 19 days post-fertilization (dpf), *Crassostrea gigas* larvae were exposed to either 23°C or 29°C for 14 days. Proteomic samples were taken at 24 and 27 dpf, or days 5 and 8 of temperature exposure respectively. Settlement, growth, and mortality was assessed at 24 dpf and 33 dpf, representing 5 and 14 days of temperature exposure, respectively.

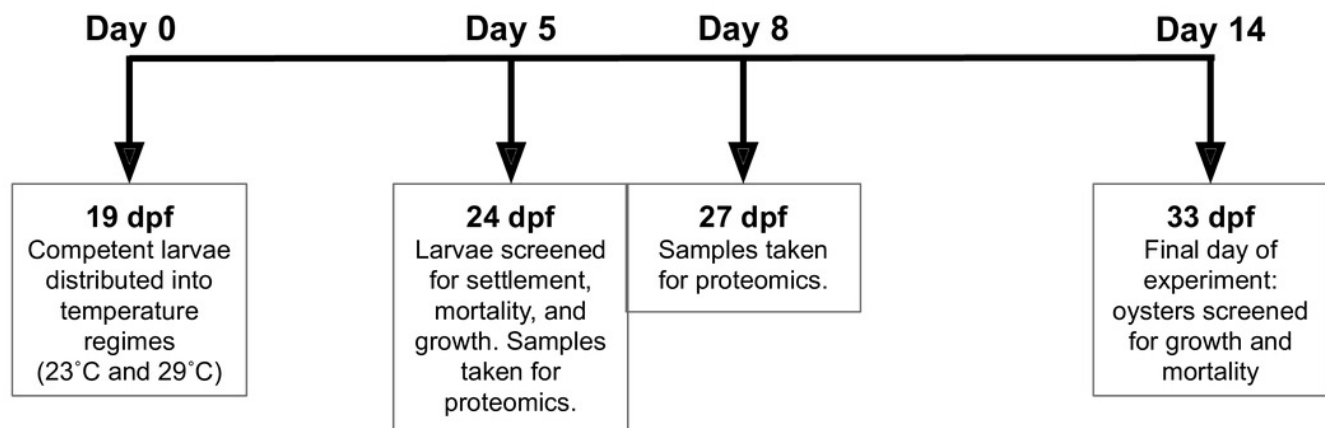



Figure 2

Proportions of GOslim terms of all detected proteins

Pie graph of the proportions of 1,256 detected proteins that fall within the Gene Ontology Slim terms listed in the legend: other biological processes; other metabolic processes; cell organization and biogenesis; developmental processes; transport; protein metabolism; RNA metabolism; signal transduction; stress response; cell cycle and proliferation; death; cell-cell signaling; cell adhesion; DNA metabolism.

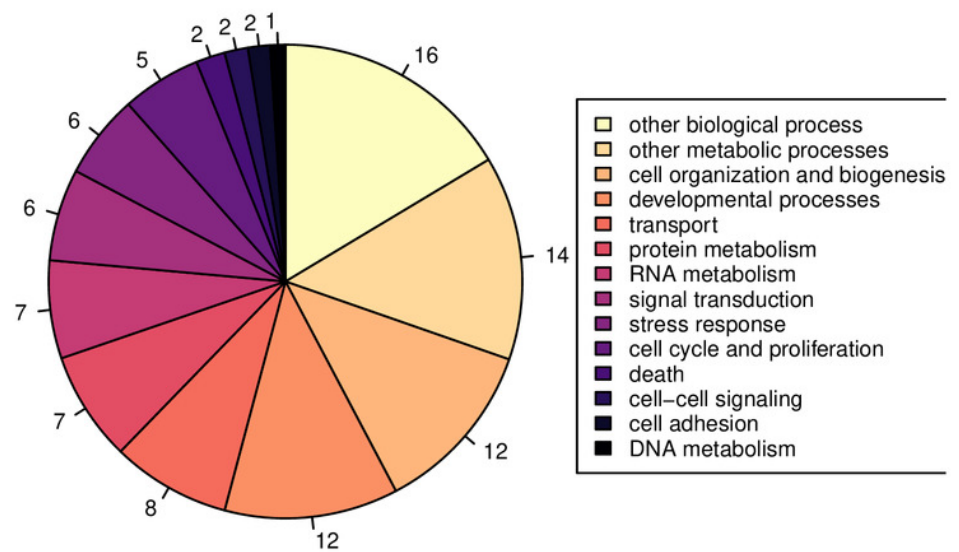
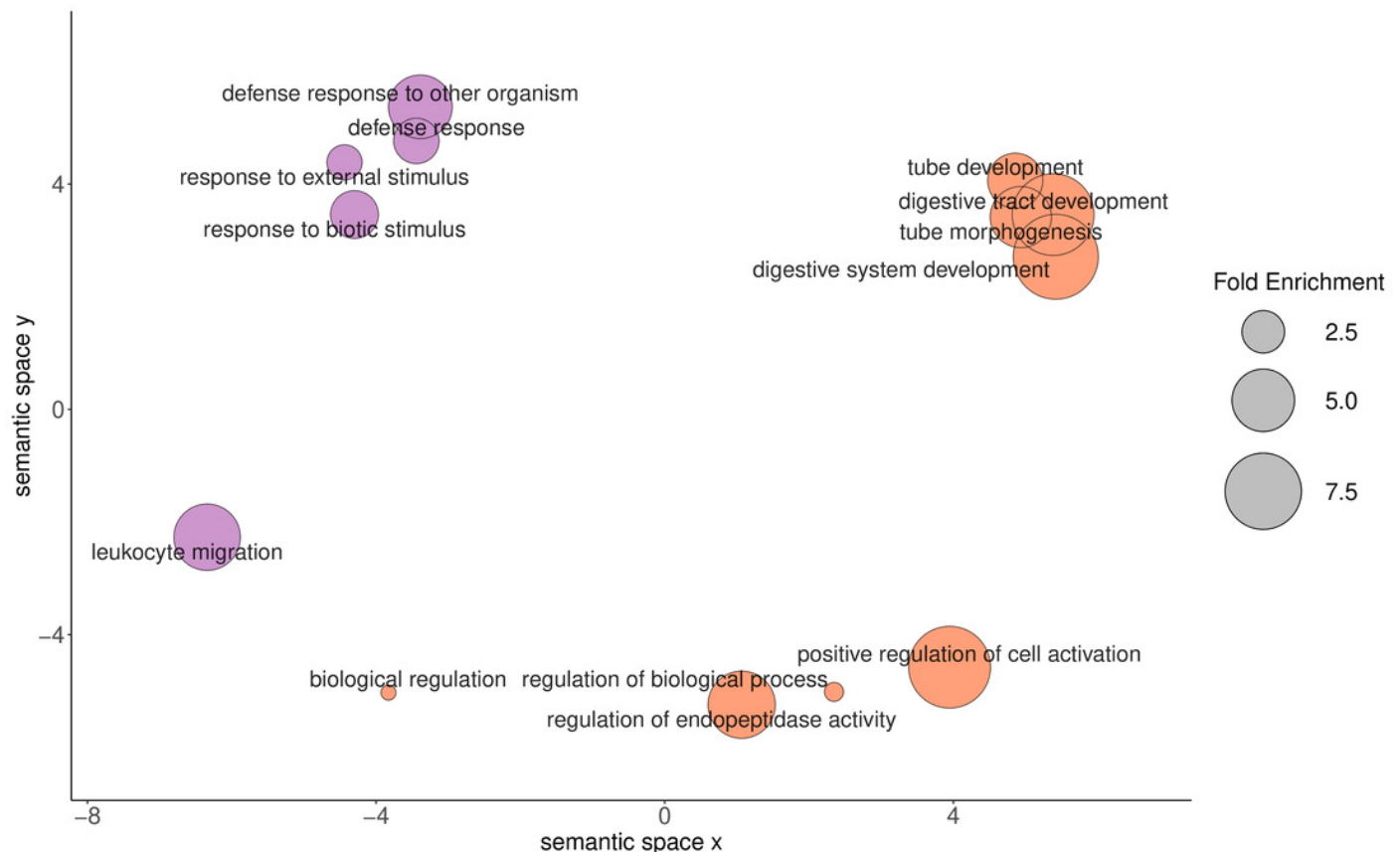



Figure 3

Relationship between the enriched Gene Ontology biological process terms from the proteins of physiological interest found between larval oysters held at two different temperatures.

The size of the circle represents the fold enrichment, which demonstrates how enriched the process is in relation to the detected proteins. The color represents the different temperature treatments in which the gene ontology terms were more abundantly present. Lavender circles represent enriched processes that were higher in the 23 ˚C treatment, with larger circles being more enriched relative to the C. gigas proteome. Orange circles represent enriched processes that were higher in the 29 ˚C treatment, with larger circles being more enriched relative to the C. gigas proteome.

Proteins of Physiological Interest

Proteins identified using a <-2.00 log2FC and >2.00 log2FC cut-off. Proteins with log2FC values <-2.00 log2FC were more abundant in the oysters kept at 23˚C, while the proteins with log2FC values >2.00 log2FC were more abundant in the oysters kept at 29˚C.

Protein	Log2FC	E-value	Annotation
CHOYP_CCDC146.1.1|m.11347	-6.8061724	0	Coiled-coil domain-containing protein 146
CHOYP_AAEL_AAEL005639.1.1|m.10386	-4.8210246	0	ER degradation-enhancing alpha-mannosidase-like protein 2
CHOYP_PCCB.2.2|m.62988	-4.4373158	0	Propionyl-CoA carboxylase beta chain, mitochondrial
CHOYP_BRAFLDRAFT_120058.1.1|m.9895	-4.3588618	8.57E-20	DPY30 domain-containing protein 1
CHOYP_LRRF2.2.2|m.40846	-4.0072733	3.63E-81	Leucine-rich repeat flightless-interacting protein 2
CHOYP_BRAFLDRAFT_126379.1.3|m.5102	-3.7331439	1.09E-17	Tctex1 domain-containing protein 1-B (Fragment)
CHOYP_BRAFLDRAFT_265162.1.3|m.58969	-3.4663681	3.11E-62	tRNA 2'-phosphotransferase 1
CHOYP_LOC100377780.8.11|m.53259	-3.3450726	9.13E-41	Interferon-induced protein 44-like
CHOYP_LOC100890099.1.1|m.11505	-3.2295286	2.99E-166	Actin, cytoplasmic 3
CHOYP_LOC100748625.1.1|m.24479	-3.219579	1.35E-37	Nuclear pore complex protein Nup50
CHOYP_LOC101174856.1.1|m.47585	-3.1898223	0	Mitogen-activated protein kinase kinase kinase 4
CHOYP_LOC100372915.3.7|m.25794	-3.0750837	2.02E-102	Patched domain-containing protein 3
CHOYP_ANR31.1.1|m.61075	-3.0736726	3.11E-13	Putative ankyrin repeat domain-containing protein 31
CHOYP_ATS6.3.3|m.56109	-3.0371165	1.07E-43	A disintegrin and metalloproteinase with thrombospondin motifs 16
CHOYP_LOC101160066.1.1|m.65775	-3.030505	3.18E-123	ADP-dependent glucokinase
CHOYP_FASN.3.4|m.60278	-2.9470953	0	Fatty acid synthase
CHOYP_ISCW_ISCW021350.1.1|m.10332	-2.9209024	7.71E-92	Protein SET
CHOYP_IFIH1.10.14|m.603	-2.9117919	1.89E-54	Interferon-induced helicase C

93			domain-containing protein 1
CHOYP_FUCO2.1.1|m.641 39	-2.5948448	7.90E-178	Plasma alpha-L-fucosidase
CHOYP_BRAFLDRAFT_81 509.1.1|m.31565	-2.5764331	1.81E-42	Allograft inflammatory factor 1
CHOYP_BRAFLDRAFT_87 662.1.1|m.5474	-2.5756398	2.57E-151	N-acylglucosamine 2-epimerase
CHOYP_ACTC.5.6|m.58242	-2.5403778	0	Actin, cytoplasmic
CHOYP_TEKT5.1.1|m.6003 1	-2.5227287	2.55E-107	Tektin-3
CHOYP_FCER2.8.9|m.2722 7	-2.4483624	3.69E-21	Perlucin
CHOYP_DDX46.1.1|m.5247 3	-2.3581865	0	Probable ATP-dependent RNA helicase DDX46
CHOYP_CRE_01395.2.2|m. 44269	-2.3270263	2.35E-34	Tenascin-X
CHOYP_NRDC.1.1|m.2852 6	-2.2979781	0	Nardilysin
CHOYP_LOC410403.1.1|m. 31535	-2.2622511	8.53E-95	Proteasome activator complex subunit 3
CHOYP_LOC100709493.2. 2|m.59425	-2.1893171	1.40E-68	Calpain-8
CHOYP_BRAFLDRAFT_20 8293.6.20|m.36168	-2.1359859	3.04E-114	Heat shock 70 kDa protein 12B
CHOYP_LYAM3.2.4|m.3333	-2.0863555	6.25E-18	P-selectin
CHOYP_ISCW_ISCW01439 8.1.3|m.15838	-2.0670706	7.57E-84	Serine/arginine-rich splicing factor 1
CHOYP_DNMT1.1.1|m.549 90	-2.0263393	0	DNA (cytosine-5)-methyltransferase 1
CHOYP_LOC100876012.1. 1|m.8280	2.01458152	1.19E-95	Uridine phosphorylase 1
CHOYP_PCKG.2.2|m.5013 9	2.04701648	0	Phosphoenolpyruvate carboxykinase [GTP]
CHOYP_UNC89.1.19|m.784 8	2.05672775	1.02E-38	Rab effector MyRIP
CHOYP_LOC100377195.1.	2.06246173	1.95E-28	Mesenchyme-specific cell surface

2|m.28624			glycoprotein
CHOYP_SCP.9.12|m.62606	2.0903096	2.29E-59	Sarcoplasmic calcium-binding protein
CHOYP_BRAFLDRAFT_90098.2.4|m.5630	2.106258	5.32E-44	Betaine--homocysteine S-methyltransferase 1
CHOYP_BRAFLDRAFT_227287.1.1|m.14669	2.14098269	5.42E-51	Metallo-beta-lactamase domain-containing protein 1
CHOYP_TSP_03740.1.1|m.61194	2.14664447	1.24E-72	Peptidyl-prolyl cis-trans isomerase B
CHOYP_PPA5.1.1|m.36087	2.15478979	2.67E-101	Tartrate-resistant acid phosphatase type 5
CHOYP_LOC100024935.1.1|m.2615	2.1972962	6.34E-43	Thioredoxin, mitochondrial
CHOYP_LOC100371209.1.1|m.49090	2.2051407	2.95E-108	LRP2-binding protein
CHOYP_ARB_02478.1.1|m.29725	2.21514424	1.28E-39	Uncharacterized FAD-linked oxidoreductase ARB
CHOYP_BRAFLDRAFT_76885.1.1|m.20259	2.24122259	6.79E-35	Gigasin-6
CHOYP_NTF-2.1.1|m.38498	2.2417884	4.95E-30	Probable nuclear transport factor 2
CHOYP_JNK.1.1|m.1540	2.24390832	0	Stress-activated protein kinase JNK
CHOYP_BIRC6.1.2|m.51516	2.25147409	0	Baculoviral IAP repeat-containing protein 6
CHOYP_BRAFLDRAFT_93126.2.3|m.15451	2.27030459	0	Mitochondrial Rho GTPase 1
CHOYP_ZN706.1.2|m.11926	2.2779611	2.01E-29	Zinc finger protein 706
CHOYP_TMEM2.4.4|m.28440	2.2865413	1.44E-99	Cell surface hyaluronidase
CHOYP_LOC577383.1.1|m.8364	2.30433808	2.35E-65	RNA polymerase II-associated protein 3
CHOYP_LOC100673983.1.1|m.10072	2.30829285	3.25E-128	Peroxisomal sarcosine oxidase
CHOYP_VRK1.1.1|m.10917	2.35944728	2.61E-139	Serine/threonine-protein kinase VRK1

CHOYP_LOC100374471.1.1|m.55948	2.42727851	1.89E-66	StAR-related lipid transfer protein 7, mitochondrial
CHOYP_LOC100878123.1.1|m.55730	2.43195478	0	Procollagen galactosyltransferase 1
CHOYP_BRAFLDRAFT_283451.1.1|m.41825	2.43609067	0	Integrator complex subunit 4
CHOYP_BRAFLDRAFT_72357.1.1|m.59063	2.62270046	2.62E-101	Renalase
CHOYP_DS.1.1|m.62106	2.64058171	0	Protein dachsous
CHOYP_LOC100378619.2.2|m.36047	2.71093822	6.50E-40	Lamin tail domain-containing protein 1
CHOYP_FAIM1.1.1|m.36651	2.74126935	8.46E-81	Fas apoptotic inhibitory molecule 1
CHOYP_CLK2.1.1|m.3082	2.79615673	9.50E-156	Propionyl-CoA carboxylase alpha chain, mitochondrial
CHOYP_NCBP2.1.1|m.60410	2.91829504	7.65E-86	Nuclear cap-binding protein subunit 2
CHOYP_LOC100208382.1.1|m.12145	3.06964605	2.01E-22	Calmodulin
CHOYP_GLOD5.1.1|m.25831	3.10869924	2.00E-61	Glyoxalase domain-containing protein 5
CHOYP_CBR1.1.1|m.62756	3.45577173	3.73E-67	Carbonyl reductase [NADPH] 3
CHOYP_contig_027593|m.31285	3.49679313	1.45E-146	Collagen alpha-2(I) chain
CHOYP_HS90A.2.3|m.21992	3.57946239	0	Heat shock protein HSP 90-alpha 1

Enriched GO terms of proteins that were higher in the 29˚C-exposed oyster seed than in the 23˚C seed.

Each row contains an enriched GO term ID, fold enrichment of the differentially abundant protein in relation to all detected proteins, and the function of the GO term.

Term	Fold Enrichment	Function
GO:0055123	14.37	digestive system development
GO:0035295	4.57	tube development
GO:0048568	7.66	embryonic organ development
GO:0007389	6.21	pattern specification process
GO:0045428	19.16	regulation of nitric oxide biosynthetic process
GO:0006809	19.16	nitric oxide biosynthetic process
GO:0009791	5.34	post-embryonic development

Enriched GO terms of proteins that were higher in the 23˚C-exposed oyster seed than in the 29˚C-exposed seed.

Each row contains an enriched GO term ID, fold enrichment of the differentially abundant protein in relation to all detected proteins, and the function of the GO term.

Term	Fold Enrichment	Function
GO:0044712	6.68	single-organism catabolic process
GO:0044419	5.60	interspecies interaction between organisms
GO:0044403	5.60	symbiosis, encompassing mutualism through parasitism
GO:1901136	20.50	carbohydrate derivative catabolic process