Proteomic response of early juvenile Pacific oysters (*Crassostrea gigas*) to temperature (#67862)

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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 also provides important ecosystem benefits. Among other threats, climate-driven changes in ocean temperature can negatively 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 and 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 abundances 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 abundances of proteins related to immune response. This novel use of proteomics was able to capture characteristic shifts in protein abundance that indicate 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.

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

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Abstract

Pacific oysters (*Crassostrea gigas*) are a valuable aquaculture product that also provides important ecosystem benefits. Among other threats, climate-driven changes in ocean temperature can negatively 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℃ and 29℃. 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 abundances of proteins related to metabolism and growth. Oysters held at 23℃ were smaller, had a decreased settlement rate, displayed 100% mortality, and had elevated abundances of proteins related to immune response. This novel use of proteomics was able to capture characteristic shifts in protein abundance that indicate 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.

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

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 as adults and 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.

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 they’re 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 since the presence of certain proteins will show which genes are being translated as a physiological response to the given environment. An unbiased global proteomics survey can identify all proteins present in organisms at the time of sampling. This information can be leveraged to

decipher the biological processes at play in various temperature treatments. Although it is still considered a novel approach, proteomics has been used in several studies of Pacific oyster response to a range of relevant environmental drivers and life stages (Venkataraman et al., 2019; Huan et al., 2012; Dineshram et al., 2012).

In this study, we used shotgun proteomics to understand how temperature impacts the physiology of recently settled oysters in a hatchery setting. We employed data-independent acquisition (DIA), which is a method of tandem mass spectrometry that achieves a more in-depth proteome coverage by acquiring all MS2 spectra for a given MS1 scan, unlike data dependent acquisition which only acquires the top *n* MS2 for each MS1 scan (Venable et al., 2004; Gillet et al., 2012). The goal of this experiment was to compare the proteomic responses of the oysters in the two temperature regimes and in the process develop a robust proteome that could build upon current *Crassostrea gigas* proteomic resources. These results contribute to our understanding of how temperature conditions impact oysters in hatchery settings, and suggest that oysters in the wild may be impacted by increasing temperature during the post-settlement stage.

Methods

Oyster Rearing and Treatment Conditions

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

Proteomic Sample Preparation

Prior to sample preparation, four larval samples (24 and 27 dpf, duplicates at each time point) were pooled for each treatment (~250ul equivalent). For each pooled

sample, 50mM ammonium bicarbonate (NH_4HCO_3) + 6M urea (500 µl) was added and larvae were homogenized using a pestle. Samples were centrifuged at 2000 x g for 5 minutes. Supernatant (150 µl) was pipetted from each sample and placed into new tubes. The supernatant was sonicated three times each for 5 seconds, cooling samples in between sonication rounds using an ethanol and dry ice bath for 5 seconds. Protein concentration was determined using a Pierce™ BCA Protein Assay Kit according to the manufacturer's protocol (ThermoFisher Scientific, Waltham, MA).

Samples were digested and desalted for mass spectrometry as previously described (Timmins-Schiffman et al., 2017). Dried peptides were reconstituted in 100 µl 3% acetonitrile + 0.1% formic acid and stored at -80°C. Data Independent Acquisition (DIA) was performed to assess protein abundance patterns via liquid chromatography tandem mass spectrometry (LC-MS/MS) with a Q-Exactive mass spectrometer (Thermo Fisher). Samples were analyzed in MS1 over 400–900 *m*/*z* range with 30k resolution in four separate injections with ranges of 400-525 *m/z*, 535-650 *m/z*, 650-775 *m/z,* and 775-900 *m/z*, and from 450 to 850 *m*/*z* in MS2 with 4*m*/*z* isolation windows with a 60 K resolution.

Proteomic Data Analysis

Raw mass spectrometry files were converted to .mzML format using MSConvert from the ProteoWizard Toolkit version 3.0 (Chambers et al., 2012). Resulting files and the *Crassostrea gigas* deduced proteome (Supplemental File 1) were used to create a chromatogram library using EncyclopeDIA with Walnut version 0.6.14 (Searle et al., 2018). Specific protocol details are provided in supplementary material (Supplemental File 2) The chromatogram library, *Crassostrea gigas* proteome, and .mzML files were subsequently imported into Skyline Daily version 4.1.9.18271 (MacLean et al., 2010), which provides a means of setting filters, viewing spectral data for quality inspection, and exporting the data for downstream analyses (Supplemental File 3).

Spectral data and proteins detected were exported for use in MS Stats (version 3.12.3, Choi et al., 2014). Within MS Stats, the two sampling dates (5 and 8 days of temperature treatment) were combined and treated as replicates to compare protein abundances between temperatures (Supplemental File 4). Pooling the sampling dates provided a more robust analysis of the dominant trends in temperature response to compensate for the small number of samples. From the list of proteins, significantly differentially abundant proteins were identified from proteins detected by MS Stats using a threshold of >2.00 and <-2.00 log-2 fold change in RStudio (version 1.1.453, R Core Team, 2015) (Supplemental File 5). Specific protocol details are provided (Supplemental File 6). DAVID, version 6.8 (Huang, Sherman & Lempicki, 2009a,b), was used to identify the enriched Gene Ontology (GO) terms from the list of differentially abundant proteins in relation to all detected proteins. Additionally, enriched GO terms from the detected proteins were characterized in relation to the *Crassostrea gigas* proteome to capture the abundant biological processes present at this developmental stage, irrespective of temperature treatment, and enriched GO terms were identified using a < 0.05 FDR cutoff.

Results

Phenotype

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

Proteomics

There were 2,808 detected proteins (Supplemental File 7) - ~6.9% of the proteins described in the *Crassostrea gigas* proteome as determined by MS Stats. Of the 2,808 detected proteins, 1,256 were associated with GO slim terms, the majority of which were related to metabolism, growth, and development (**Figure 2**). These detected proteins were enriched for 108 biological process GO terms compared to the full *Crassostrea gigas* proteome (Supplemental File 8).

Of the 2,808 detected proteins, 69 were differentially abundant between the 23˚C and 29℃ treatments. Thirty-six proteins were more abundant in the 29˚C treatment, while 33 were more abundant in 23˚C treatment (Supplemental File 9). The differentially abundant proteins contributed to 18 enriched GO biological processes (**Figure 3**). Further analysis of the differentially abundant proteins identified enriched biological processes in the samples when compared with the proteome. Proteins significantly more abundant in oysters grown in 29˚C were to do with biological processes primarily related to growth (**Table 1**), while those in oysters grown in 23˚C contributed to immune response (**Table 2**).

Discussion

Using novel proteomics techniques, this study identified proteins that occur in different abundances when comparing larval Pacific oysters exposed to two different temperatures. Based on differential protein abundance, we found that the biological processes detected in oysters in the high-temperature treatment were related to metabolism and growth, while the biological processes detected in oysters exposed to the low-temperature treatment were involved in immune system response. Given the observance of ciliates at lower temperatures, the latter wasn't unexpected.

Across the two temperature treatments, the detected proteins primarily consisted of proteins related to metabolism, growth, and development. These findings are in agreement with the life history stage sampled, where growth rates are elevated and significant physiological changes are occurring related to somatic organization. In another study, researchers used *in silico* approaches to identify genes associated with

larval settlement *Crassostrea gigas* (Foulon et al., 2019). Approximately 27% of genes described by Foulon et al (2019) had protein complements expressed in the current study. This not only validates the relevance of the developmental role of these proteins but also provides valuable resources for future work focused on metamorphosis and larval adhesion. Additionally, this comparison highlights the robust nature of the proteome developed as part of this study, along with the value of the Data Independent Acquisition proteomic approach.

The growth and development of Pacific oyster larvae were positively impacted by exposure to 29˚C. The higher temperature likely promoted elevated metabolic rates, which in turn supported elevated growth and development. Higher temperatures between 28-30˚C have been shown to promote higher rates of metabolism and growth in another oyster species, *Crassostrea corteziensis,* in the juvenile spat life stage (Cáceres-Puig et al, 2007). Another possibility for the observed proteomic trend is that in the absence of other stressors, such as the ciliates observed at the lower temperature, there was an increased relative allocation of energy towards growth and development. This is further supported by the phenotype data, where the oysters in the 29℃ treatment had higher settlement rates, greater size, no ciliates, and 86% survival, as compared to the 23℃ treatment group which had lower settlement rate, smaller size, and 100% mortality by the end of the experiment.

The oyster larvae samples from the 23℃ treatment had higher abundances of proteins associated with immune response when compared to the larvae at elevated temperature. At 29 dpf, ciliates were observed in the silo at 23℃ and by 33 dpf, all the oysters in the 23℃ treatment were dead. The predominant proteomic response was an immune response to parasites, supporting the idea that the oysters were initiating immune responses. Ciliate presence could have negatively impacted survival, either directly through parasitism or indirectly through increased energy allocation towards immune responses and away from critical maintenance processes. Ciliates have been a problem in hatcheries for decades, and are associated with significant mortality events in early development bivalves (Elston et al., 1999). Ciliates may prefer colder temperatures or may not be able to survive at higher temperatures, protecting larval oysters at 29˚C against potential infections and the associated cost of launching an immune response. Alternatively, larvae may be physiologically compromised at lower temperatures, making them more susceptible to ciliates. In natural, non-hatchery settings, oyster susceptibility to ciliates increases with increasing salinity (Gauthier, Soniat & Rogers, 1990), and increases in summer and fall seasons when temperatures are roughly between 23˚C and 25˚C (McGurk, Ford & Bushek, 2016). This seasonal change observed could be related to laboratory findings where oysters were more susceptible to infection from OsHV-1 in warmer temperatures, specifically 21˚C and 26˚C, though at 29˚C, the susceptibility of the oysters to OsHV-1 declined, and oysters at this temperature had high survival rates (Delisle et al., 2018). Future research is certainly needed to attempt to disentangle these phenomena and continue to elucidate factors contributing to improved survival in oysters.

Our findings can help hatchery workers, managers, and conservationists predict how temperature is and will impact oysters at this developmental stage in hatchery settings. The findings support an improved practice of increasing the temperature during the early developmental stage after settlement to improve growth and survival. However further studies should investigate the optimal length of time and during which phase of development the larvae should be reared at elevated temperature. In addition, the annotated proteome developed as part of this work will be a valuable tool for future studies on bivalve development including providing specific targets for protein regulation studies in oysters as well as a reference for gene discovery in less studied bivalves.

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Data Availability

All supplemental files, scripts, data, and analyses can be found at DOI: 10.5281/zenodo.5706425.

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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.

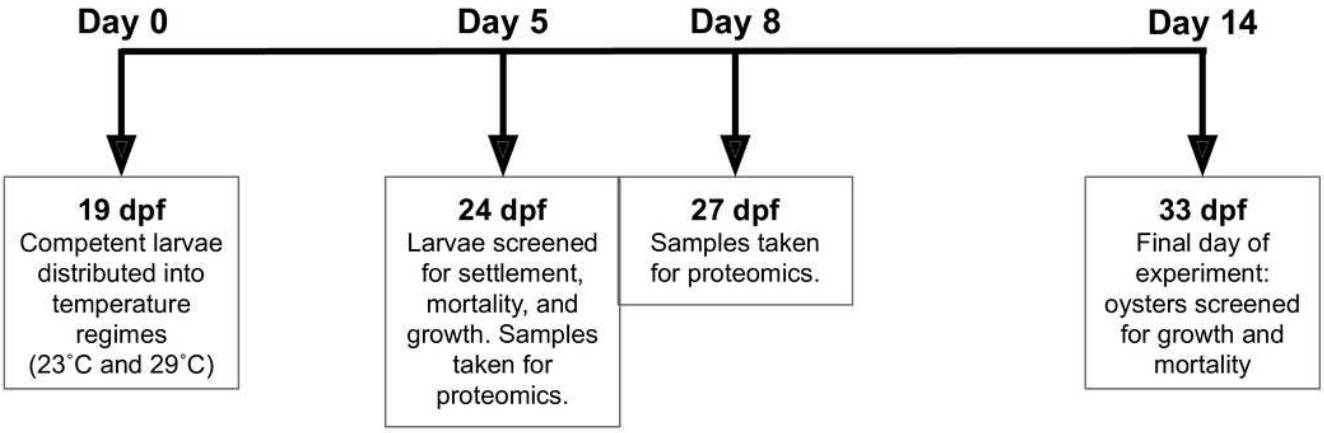
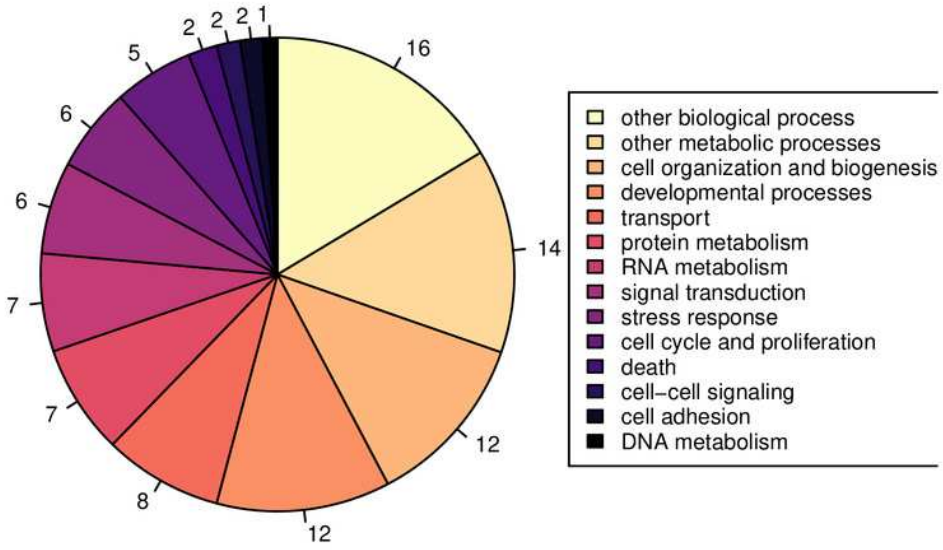


Figure 2

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.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death
cell–cell signaling
cell adhesion
DNA metabolism

Figure 3

Relationship between the enriched Gene Ontology biological process terms from the differentially abundant proteins 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. Pink/purple circles represent enriched processes that were more abundant in the 23 ˚C treatment, with larger circles being more enriched relative to the C. gigas proteome. Orange circles represent enriched processes that were more abundant in the 29 ˚C treatment, with larger circles being more enriched relative to the C. gigas proteome.

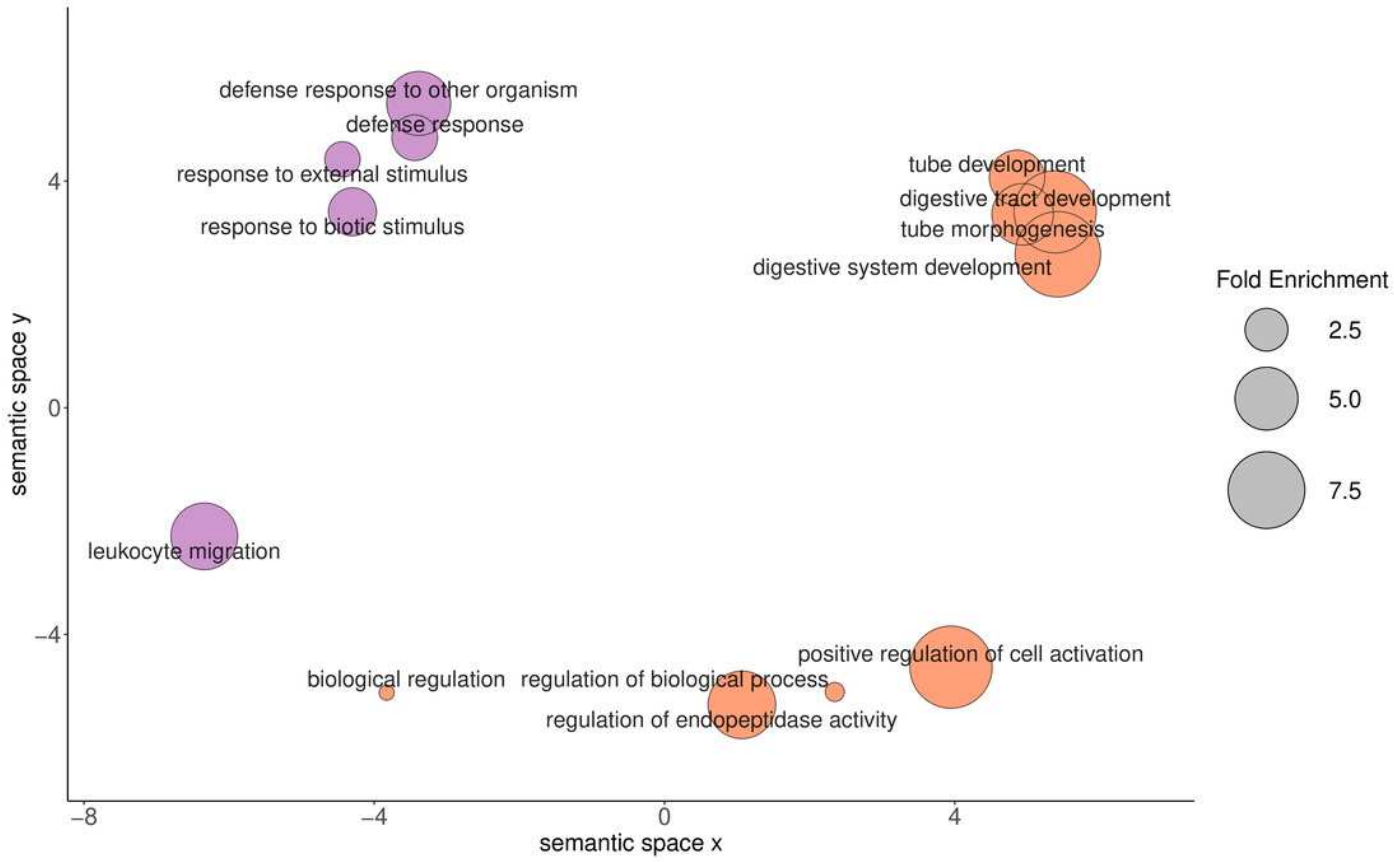

Table 1(on next page)

Enriched GO terms of differentially abundant proteins that were more abundant in the 29C treatment than in the 23C treatment.

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

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Table 2 (on next page)

Enriched GO terms of differentially abundant proteins that were more abundant in the 23C treatment than in the 29C treatment.

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

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