LAY SUMMARY

The oyster gill proteome (expressed proteins) was sequenced using shotgun proteomics. This effort represents the first time that a global, non-gel based approach has been used to characterize proteins from oyster gill. The data provide insight into the dynamic functions of this tissue and demonstrate the viability of this approach.

Shotgun proteomics as a viable approach for biological discovery in the Pacific oyster

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ABSTRACT

Shotgun proteomics offers an efficient means to characterize proteins in a complex mixture, particularly when sufficient genomic resources are available. In order to assess the practical application of shotgun proteomics in the Pacific oyster (*Crassostrea gigas*), liquid chromatography coupled with tandem mass spectrometry was used to characterize the gill proteome. Using information from the recently published Pacific oyster genome, 1,043 proteins were identified. Biological samples (n=4) and corresponding technical replicates (3) were similar in both specific proteins identified and expression, as determined by normalized spectral abundance factor. A majority of the proteins identified (703) were present in all biological samples. Functional analysis of the protein repertoire illustrates these proteins represent a wide range of biological processes, supporting the dynamic function of the gill. These insights are important for understanding environmental influences on the oyster since the gill tissue acts as the interface between the oyster and its environment. *In silico* analysis indicated this sequencing effort identified a large proportion of the complete gill proteome. Together these data demonstrate that shotgun sequencing is a viable approach for biological discovery and will play an important role in future studies of oyster physiology.

INTRODUCTION

Fluctuation in gene and protein expression can be sensitive and specific indicators of biological processes. At the transcript level, several methodologies can be used to characterize expression from the gene-centric to systems level, including qPCR (e.g. Griffitt et al. 2006; Stumpp et al. 2011), microarrays (e.g. Todgham and Hofmann 2009; Lockwood et al. 2010), and high-throughput sequencing (e.g. Polato et al. 2011; Philipp et al. 2012). The use of high-throughput sequencing technology has exponentially increased available genome and transcript information for taxa of ecological interest in recent years. While these results provide an accurate portrayal of changes at the molecular level, it is common that proteins have a more direct role in regulating physiological processes and responding to environmental change.

Historically there have been several technical and analytical challenges in characterizing global protein expression. One challenge is the need to have sufficient genomic resources available to describe proteins of interest. Specifically, protein sequencing generally produces short amino acid fragments that require a known corresponding gene for identification and annotation purposes. However, the lack of genomic resources has not completely hampered proteomic studies. For example, researchers characterized the physiological response of *Gillichthys mirabilis* gill tissue exposed to osmotic and temperature stress using two dimensional gel electrophoresis without sequencing proteins (Kültz and Somero 1996). In another study, researchers used Surface Enhanced Laser Desorption/Ionisation and identified 11 differentially expressed proteins in the gill tissue of *Oncorhynchus mykiss* exposed to zinc stress (Hogstrand et al. 2002). Four proteins were identified based on a combination of their physical properties (i.e.mass and binding) coupled with sequence similarity comparisons with limited teleost protein sequences in the SwissProt database (Hogstrand et al. 2002).

Using predicted protein sequences in closely related species can assist in annotation, but species specific information will provide more accurate results. This is evident in a study on protein expression in pea (*Pisum sativum*) chloroplasts where concurrent cDNA sequencing facilitated a greater number of protein identifications compared to identifications through homology searches with closely related model species (Bräutigam et al. 2008). The reason that species specific information provides such an advantage is due to how modern day protein sequence identification is executed. The vast majority of high-throughput mass spectrometry proteomics is accomplished by matching observed peptide fragmentation patterns (tandem mass spectra) to theoretical spectra. This is possible because peptides fragment in a predictable manner allowing for theoretical tandem mass spectra to be created *in silico* from a given protein sequence, stressing the importance of the database used. These correlation-based algorithms require the peptide mass (precursor mass) and peptide fragmentation (tandem mass spectrum). Even when employing databases of closely related species a large number of peptides’ viable tandem mass spectra might not accurately be assigned to a protein as a single amino acid mutation could significantly alter the peptide mass and resulting fragmentation pattern.

As technological advances have continued to increase accessibility of whole transcriptomes and genomes to researchers, there is increasing interest in leveraging these data to carry out proteomic studies for both biological discovery and for better characterizing physiological responses to environmental change. Recently, the Pacific oyster genome was sequenced (Zhang et al. 2012). Given the availability of this resource, our objective was to quantify the level of information (and respective variability) attainable in proteomic studies in oysters. There have been a several prior studies examining protein expression in oysters using liquid chromatography coupled with tandem mass spectrometry (MS) with samples separated by two-dimensional gel electrophoresis (2-DE) beforehand. In larval oysters, these proteomic techniques have identified specific proteins that are responsible for early developmental changes in *C. gigas* (Huan et al. 2012) and larval *C. gigas* response to elevated *p*CO2 (ocean acidification) (Dineshram et al. 2012). These methods have also been used to identify and sequence proteins that are differentially regulated in a range of physiological situations in adult oyster species. Discoveries include up-regulation of antioxidant proteins in response to ocean acidification (Tomanek et al. 2011); expression profiles denoting high quality oocytes (Corporeau et al. 2012); differing proteomic profiles between disease resistant and susceptible oysters (Simonian et al. 2009); and specific responses to metal exposure (Liu and Wang 2012; Thompson et al. 2011; Thompson et al. 2012a; Thompson et al. 2012b) and acid sulfate runoff (Amaral et al. 2012). These seminal studies in marine invertebrate proteomics demonstrate that analysis of global protein expression is a powerful tool towards understanding the molecular physiological response to environmental stressors.

An alternative to 2-DE approaches is to perform shotgun proteomics. Shotgun proteomics is the sequencing of a complex mixture of peptides using liquid chromatography and tandem MS without prior separation (*i.e.* 2-DE). One of the main advantages of using 2-DE methods is that information on the proteins’ physical properties (mass, isoelectric point) can be used in the protein identification, whereas these empirical data are lost in the strictly tandem MS approaches. However, tandem MS has significantly greater data efficiency than gel-based approaches. The use of shotgun proteomics allows for a greater number of proteins to be rapidly identified from a single sample providing a more complete metabolic picture of cellular function and physiology. This method has been demonstrated by Muralidharan et al. (2012), who used shotgun proteomics to uncover *Saccostrea glomerata* hemocyte proteomic responses to metal contamination, and by Dheilly et al. (2012 and 2013), who explored the proteomic response of coelomocytes to immune challenge in two urchin species.

In this study, we used shotgun proteomics to sequence the gill proteome of the Pacific oyster, *Crassostrea gigas*. The gill is the interface between bivalves and their environment, necessitating that the tissue performs a variety of physiological functions in response to the environment (e.g. David et al. 2007; Wang et al. 2010). The identification of proteins that are expressed in gill tissue supports the development of tools that can help to guide future research on the molecular physiology of molluscs faced with stresses such as climate change and disease. The goal of this study was to determine the effectiveness of using a shotgun proteomics approach and to functionally characterize proteins expressed in gill tissue.

MATERIALS AND METHODS

*Oysters*

Pacific oysters (*Crassostrea gigas*, 18 months old) were collected in Shelton, Washington, United States. Oysters were transferred to Friday Harbor Laboratories (Friday Harbor, WA, USA) into a flow-through system at 13°C for six weeks. Eight 4-L vessels containing six oysters each were kept in a water bath with seawater flowing through at 57.5 mL/min. Vessels were cleaned every other day with fresh water and salt water rinses. Oysters were fed Shellfish Diet 1800 (Reed Mariculture, Campbell, CA, USA). At the end of six weeks, gill tissue was removed from four oysters and immediately flash frozen in liquid nitrogen for proteomic analysis.

*Protein Digestion and Desalting*

Gill tissue samples (50-100 mg) were homogenized in 50 mM NH4HCO3 (100 ul) using RNAse-free plastic pestles. Each homogenized gill sample was sonicated four times with a probe sonicator and stored on dry ice between sonications. After sonication, protein concentrations were measured using the Bradford assay, following the manufacturer’s protocol (Pierce, Thermo Fisher Scientific, Rockford, IL, USA). Urea (36mg) was added to each sample (for a total concentration of 6M) to stabilize peptides. Next, 1.5 M Tris (pH 8.8) (6.6 µl) was added followed by 200 mM TCEP (2.5 µl). Samples were incubated for 1 hour at 37°C on a shaker. To alkylate the proteins, 200 mM iodoacetamide (IAA) (20 µl) was added. Samples were then vortexed, and incubated for 1 hour at room temperature in the dark. To absorb excess IAA, 200 mM dithiolthreitol (20 µl) was added, followed by vortexing and incubation at room temperature for 1 hour. A volume equal to approximately 100 µg was removed and the remainder was discarded. NH4HCO3 (200 µl of 25 mM) was added to dilute the urea and then HPLC grade MeOH (50 µl) was added to each tube. Trypsin was solubilized in a trypsin dilution buffer (20 µl) to a concentration of 1 µg/µl (Promega, Madison, WI, USA) and 3 µl of this solution was added to each sample to enzymatically digest the proteins. The samples were incubated overnight at 37°C. The next day, dilute formic acid was added and the samples were evaporated on the speed vac to near dryness. Samples were reconstituted in 200 µl of 5% ACN and 0.1% trifluoroacetic acid.

Samples were desalted by passage through a pre-prepared MacroSpin column following the manufacturer’s specifications (The Nest Group, Southborough, MA, USA). After desalting, the remaining solvent was evaporated using a speed vac.

*Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS)*

Mass spectrometry was performed at the University of Washington Proteomics Resource (Seattle, WA, USA). Samples were resuspended in 2% acetonitrile and 0.1% formic acid in water (100 µl). Samples were then vortexed to mix and spun down at 15,000 rpm for 10 minutes. The supernatant was aliquoted to autosampler vials. Nano LC separation was performed with a nanoACUITY system (Waters, Milford, MA, USA) interfaced to a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA, USA). Peptides were trapped on a 100 µm i.d. x 20 mm long pre-column packed with 200 angstrom (5 µm) Magic C18 particles (C18AQ; Michrom, Auburn, CA, USA). For separation, a 75 µm i.d. x 250 mm long analytical column with a laser pulled emitter tip packed with 100 angstrom (5 µm) Magic C18 particles (C18Q; Michrom) was used and analyzed in positive ion mode. For each LC-MS/MS analysis, an estimated amount of 0.5 µg of peptides was loaded onto the pre-column at 2 µL/min in water/acetonitrile (98%/2%) with 0.1% (v/v) formic acid. Peptides were eluted using an acetonitrile gradient flowing at 240 nL/min using mobile phase consisting of the following: Solvent C (water, 0.1% formic acid) and Solvent D (acetonitrile, 0.1% formic acid). The gradient program was as follows: 0 - 1 minute: Solvent C (98%) Solvent D (2%); 1 minute: Solvent C (90%) Solvent D (10%); 90 minutes: Solvent C (65%) Solvent D (35%); 91 - 101 minutes: Solvent C (20%) Solvent D (80%); 102 - 120 minutes, Solvent C (98%) Solvent D (2%). Peptide spectra were acquired by scans in the Orbitrap followed by the ion trap.

*Data acquisition*

High resolution full precursor ion scans were acquired at 60,000 resolution in the Orbitrap over 400-2000 m/z while six consecutive tandem mass spectra (MS/MS) were acquired by collision induced dissociation (CID) in the Linear ion trap (LTQ). Data dependent ion threshold was set at 5,000 counts for MS/MS, and the maximum allowed ion accumulation times were 400 ms for full scans and 100 ms for MS/MS measurements. The number of ions accumulated was set to 1 million for Orbitrap scans and 10,000 for linear ion trap MS/MS scans. An angiotensin and neurotensin standard was run after every eight injections. Each sample was injected in triplicate in a novel randomized order.

*Protein Identification and Data Analysis*

Peptide sequence and corresponding protein identification for all mass spectra were carried out using SEQUEST (Eng et al. 1994) and the *Crassostrea gigas* proteome version 9 (Zhang et al. 2012, <http://dx.doi.org/10.5524/100030>). A DECOY database was created by reversing the *C. gigas* proteome and adding it to the forward database. This was completed in order to determine false positive matches of peptide spectra matching and yielded a false discovery rate of ~0.6%. Search parameters included trypsin as the assigned enzyme and a precursor mass accuracy of ± 3 Da. SEQUEST results were analyzed using PeptideProphet and ProteinProphet in order to statistically evaluate peptide matches and assign protein probabilities (Nesvizhskii et al. 2003). Only proteins with a probability of ≥0.9 (estimated false discovery rate of 0.6%), a minimum of two unique peptide hits within a single replicate, and a minimum of four total tandem mass spectral assignments in the combined technical and biological replicates were used in further characterizations described below.

In order to annotate corresponding proteins, the *C. gigas* proteome (version 9) was compared to the UniProtKB/Swiss-Prot database (www.uniprot.org) using Blastp with an e-value limit of 1E-10. Associated Gene Ontology terms were used to classify sequences based on biological process as well as categorize genes into parent categories (GO Slim). Enrichment analysis was used to identify overrepresented biological processes in the gill proteome compared to the entire proteome (Database for Annotation, Visualization and Integrated Discovery (DAVID, v. 6.7; Huang et al. 2009a and 2009b, <http://david.abcc.ncifcrf.gov/>)). The results of the enrichment analysis were visualized in REViGO (Reduce and Visualize Gene Ontology; Supek et al. 2011, <http://revigo.irb.hr/>). Normalized spectral abundance factor (NSAF, Florens et al. 2006) was used to calculate expression for each protein in each oyster. Technical replicates were pooled by taking the sum of total independent tandem mass spectra for each protein (SpC). For each protein SpC was divided by protein length (L). NSAF is calculated from SpC/L divided by the sum of all SpC/L for the proteins for a particular oyster. Comparisons of proteins identified across biological samples were visualized using Venny (Oliveros 2007).

The minimum number of peptides needed to be sequenced to optimize unique protein identifications was determined using an *in silico* approach. A list was constructed of all sequenced peptides and their matching protein identification. Redundancies were maintained in this list, so that if a certain peptide was sequenced multiple times it was included multiple times in the list. Randomized subsets of this list were generated using the sample function in R (R core Development Core Team 2009). The number of hypothetically sequenced peptides in these lists ranged from 500-70,000. A plot was generated to visualize the relationship between each sample size of randomly chosen peptides and the number of unique proteins identified.

RESULTS

*LC-MS/MS*

A combined total of 175,818 tandem MS spectra were generated across all four biological and three technical replicates using the Orbitrap mass spectrometer. Expression values were comparable between biological replicates with R2 ranging from 0.800 to 0.889 (Supplementary Data 1). A total of 54,521 unique peptides contributed to the identification of 2,850 proteins with probability score threshold of 0.9 (Supplementary Data 2). 1,043 of these proteins had at least two unique peptide hits and four tandem mass spectra in the combined replicates. The mean amino acid coverage across all proteins was 13.3%. Protein identifications for each injection including protein probability scores, number of total and unique spectra, and peptide sequences are provided in Supplementary Data 3. NSAF values for each protein are provided in Supplementary Data 4.

For all biological samples, the number of proteins identified in each technical replicate was consistent with minimal standard deviation (1.2-3.5%). In each biological replicate the proteins were identified from between 43,275-44,720 sequenced peptides (standard deviation as percentage of mean ranged from 4.8-7.4%). For each oyster, 54-55% of the identified proteins were present in all three technical replicates. Using spectral counts as a proxy for relative expression, protein expression levels were consistent across technical replicates (Figure 1).

The number of proteins identified in each oyster (after pooling technical replicates, see Methods) was 923, 959, 883, and 875 (Table 1). Most proteins (n=703) were identified across all biological samples (Figure 2).

In order to evaluate general protein expression and assess sample variability, the ten most highly expressed proteins in all oysters were identified. These twelve proteins represent core cell structure and function, such as nucleosome assembly, cytoskeleton structure, muscle components, turnover of intracellular proteins, and protection against oxidative stress. Eight out of twelve of these proteins (arginine kinase, actin, histone H2A, histone H2B.3, histone H4, peptidyl-prolyl cis-trans isomerase, extracellular superoxide dismutase, and cytosol aminopeptidase) were identified in the top ten most expressed proteins in all four oysters.

Of the 1,043 proteins expressed across all samples, 1,033 were annotated using the UnitProt-KB/SwissProt database. Of the annotated proteins, 888 were associated with Gene Ontology classifications. A majority of proteins were associated with the biological process of protein metabolism (n=273), followed by cell organization and biogenesis (n=201), and transport (n=165).

Enrichment analysis was carried out to determine which biological processes were overrepresented in gill tissue as compared to the entire proteome. Several of the functional groups identified were associated with the abundant proteins involved in metabolism and transport as well as structural processes (i.e. actin-filament, microtubule) and oxidation-reduction. The most significantly enriched biological process was generation of precursor metabolites and energy. Protein IDs (CGI numbers) corresponding to the proteins that contributed to GO term enrichment are listed in Supplementary Data 5.

The number of unique proteins identified with different numbers of sequenced peptides created an exponential curve (Figure 4). The plateau began around 30,000-40,000 sequenced peptides with a total of 2,400-2,516 unique peptides identified. New unique peptides were still identified in larger sample sizes of peptides, but the return per sequenced peptide diminished.

DISCUSSION

Technical and analytical challenges have resulted in limited focus on quantitative proteomics approaches in environmental physiology. Given the recent technological advances in the proteomics field (Yates et al. 2009) and release of the Pacific oyster genome (Zhang et al. 2012), we set out to assess the practical use of quantitative proteomics in this model species. For all biological samples a majority of the proteins identified (54-55%) were present in all respective technical replicates. Relative expression across technical and biological replicates was also consistent (Figure 1, Supplementary Data 1). However, there were some proteins not identified in all technical replicates. Thus, proteins with limited expression might not be detected and/or expression levels might not be accurately reflected. It should be noted that the inclusion of proteins in our analysis is highly dependent on threshold selection. In the current study a protein was included only if it had two unique spectral hits within a replicate and had four total spectra across the combined technical replicate data. If the threshold were adjusted to be more conservative (i.e. a greater total spectral count threshold) variability would be reduced. With a total spectral count threshold of five, 983 proteins are identified and 56-57% of the proteins are in all three technical replicates; with a threshold of ten, 845 proteins are identified and 61-63% of the proteins are in all technical replicates (data not shown).

The number of proteins identified and subsequently annotated can vary tremendously based on experimental design, target tissue, match thresholds, and genomic resources available. In the present study, the majority of the proteins (703) were identified in all biological samples. Based on *in silico* analysis (Figure 4) we have sequenced a relatively complete proteome for oyster gill tissue. In a study of European whitefish, *Coregonus lavaretus*, proteomics on fish larvae yielded sequencing of peptides corresponding to 1,500 proteins (Papakostas et al. 2012). The similar number of protein identifications in whitefish compared to our study (1,043) is likely associated with the tissue complexity. In the whitefish study whole body tissue was examined. In a metaproteomics study of marine microbes, 2,273 distinct proteins were identified across ten samples (Morris et al. 2010). The large number of proteins identified by Morris et al. (2010) is evidence of the large number of organisms and ecological niches that were sampled in their study. Previous proteomics studies on Sydney rock oyster hemolymph have found relatively few proteins compared to the current study in gill tissue, with number of identified proteins ranging from 49 to 514 (Simonian et al. 2009; Thompson et al. 2011; Muralidharan et al. 2012; Thompson et al. 2012a; Thompson et al. 2012b). The identification of fewer proteins in hemolymph is likely due to fewer cell types present in hemolymph compared to the gill.

In addition to assessing the feasibility of shotgun proteomics in the Pacific oyster we also were able to provide a functional characterization of the gill proteome. Gene ontology (GO) characterization identified a majority of proteins associated with protein metabolism, cell organization and biogenesis, and transport. These biological functions would be expected since gill tissue is the primary interface between the oyster and the environment (water) where the tissue’s major functions include ion regulation, respiration, and sorting of food particles. The high number of proteins involved in these GO categories is not necessarily unique to gill tissue but does likely reflect the multifunctional nature of a tissue that responds to variable environments.

Enrichment analysis was performed to identify which functional groups of proteins expressed in gill tissue were overrepresented as compared to the complete protein repertoire. Several of the functional groups identified were associated with the abundant proteins involved in metabolism and transport as well as cellular structure. These enrichment analysis findings were consistent with a previous transcriptomic comparison between *C. gigas* gill tissue and other tissues with genes predominantly expressed in the gill involved in epithelia morphogenesis, cilia movement, and detoxification and defense (Dheilly et al. 2011). Some of the cytoskeletal proteins identified in gill were tektin-3, microtubule-associated protein futsch, and actin. Tektin is part of cilia and flagellar microtubules and has been found to change expression in response to elevated *p*CO2 (Dineshram et al. 2013) and has also been identified in Sydney rock oyster hemolymph (Thompson et al. 2012b). Transport proteins included ATP synthases and v-type proton ATP synthase. ATP synthase is a good marker of environmental stress in *C. gigas*, as its transcript expression is altered in response to hypoxia (David et al. 2005) and pesticide exposure (Tanguy et al. 2005). The most significantly enriched biological process was generation of precursor metabolites and energy. Many of the proteins that contributed to the overrepresentation of this GO category in the gill tissue are involved in metabolic processes, such as 2-oxoglutarate dehydrogenase, dihydroplipoyllisin-residue acetyltransferase, glycogen phosphorylase, triose phosphate isomerase, and hexokinase. These enzymes are all involved in the breakdown of carbohydrates and other food inputs and thus underline the important metabolic processes that occur in the gill.

Proteins involved in oxygen and reactive oxygen species (ROS) metabolic process were also enriched in gill tissue, providing further support for the importance of gill tissue in response to environmental change. Previous transcriptomic based studies of oysters support that oxidative stress response is an important role of the gill tissue (e.g. David et al. 2007; Fleury and Huvet 2012). Genes and proteins responding to reactive oxygen species production increase in oysters in many instances of environmental stress, such as exposure to contaminants (e.g. David et al. 2007; Muralidharan et al. 2012) as well as exposure to ocean acidification (Tomanek et al. 2011) and temperature stress (Meistertzheim et al. 2007). Specific proteins that contributed to ROS response are enzymes instrumental in the physiological response to oxidative stress such as the anti-oxidants superoxide dismutase, peroxiredoxin, and catalase.

The success of the shotgun sequencing effort was due in part to the recent publication of the *C. gigas* genome, emphasizing that the dissemination of genomic resources provides invaluable opportunities for advancement for the scientific community. The sharing of these large datasets, such as the genome and the gill proteome, will support further research into the effects of environmental changes on the oyster in terms of both acclimatization and adaptation. The characterization of the scope of acclimatization and adaptation are instrumental in understanding how the Pacific oyster, an ecologically and economically important species, can respond to climate change at the physiological and population levels. These research results demonstrate shotgun sequencing as a viable approach for biological discovery and that it will likely play an important role in future studies on oyster physiology.

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SUPPLEMENTARY DATA

**Supplementary Data 1**: Correlations of log(NSAF) data between all oyster pairs. The 1:1 line is plotted in pink on each graph and the R2 value is provided in the upper lefthand corner of the graph. Also included is a histogram showing the frequency of proteins for each log(NSAF) value, which follows a normal distribution.

**Supplementary Data 2**: Proteins identified based on tandem mass spectra compared to the *Crassostrea gigas* proteome. The protein accession number is provided for each protein identified (n=2,850). When proteins could be annotated, SwissProt Accession Number (SPID), e-value for the BLASTp search, and gene description are provided. For all proteins, the total number of tandem mass spectra is provided.

**Supplementary Data 3**: Spectra, peptide, and corresponding protein information for all 12 injections (4 biological samples x 3 technical replicates). Sheets are labeled as oyster ID and technical replicate (i.e. B\_03 is the third technical replicate for oyster B). Each protein search result includes an entry number, one or more protein accession numbers, the probability that the protein assignment is correct, the percent coverage of the protein by the sequenced peptides, the number of unique peptides (non-redundant) used to identify the protein, the total number of peptides used to identify the protein, and the peptide sequences. Some of the entries have multiple protein accession numbers because numbers correspond to proteins with the same or highly similar sequences.

**Supplementary Data 4**: Calculated normalized spectral abundance factors (NSAF) for each oyster. NSAF was calculated by dividing spectral counts for each oyster (SpC, summed across three technical replicates) by protein length (SpC/L) and then dividing SpC/L by the sum of all SpC/L for that particular oyster (Florens et al. 2006). Proteins included in this file satisfy the thresholds of at least two unique peptide hits in a biological replicate and at least four spectral counts across all biological replicates.

**Supplementary Data 5:** List of proteins associated with enriched Gene Ontology terms in the gill tissue compared to the entire oyster proteome. Protein accession numbers are associated with UniProt accession numbers as well as the enriched GO term(s).

FIGURE CAPTIONS

Figure 1. Total independent spectral counts for three technical replicates for oyster “A” plotted for each protein (n=1,500). Similar patterns were observed for the other three oysters (data not shown).

Figure 2. Venn diagram of proteins identified among biological samples. Proteins identified in oyster A are in the blue ellipse, B are in yellow, C are in green, and oyster D proteins are in red.

Figure 3. Representation of biological processes corresponding to the proteins identified from oyster gill tissue.

Figure 4. Predicted number of unique proteins that would be identified based on a sequential increase in peptides sequences.

TABLES

Table 1. Summary of the number of peptides sequenced proteins identified for each oyster (labeled A-D).

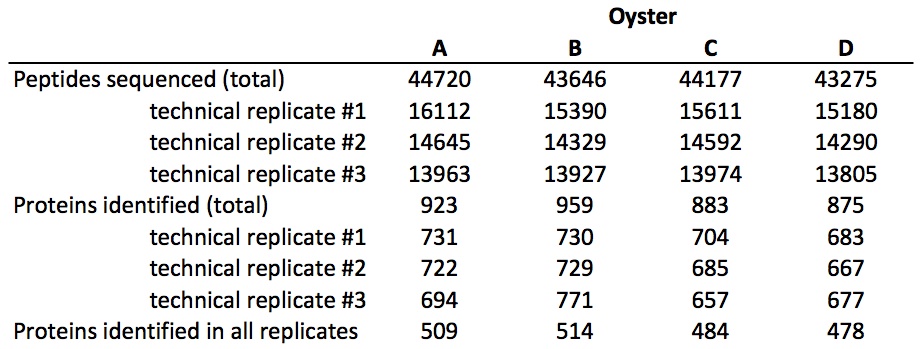
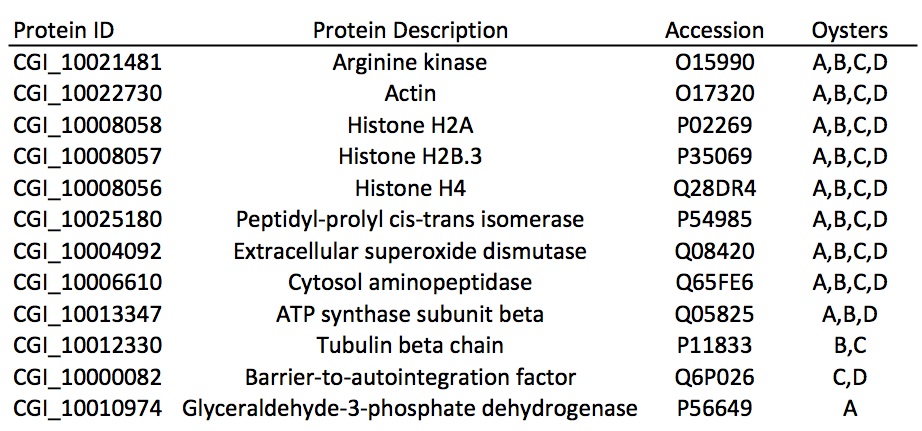


Table 2. Twelve most abundant proteins in gill proteome as determined by identifying the ten most abundant proteins in each oyster. Protein ID is given, as well as protein description from UniProt-KB/SwissProt, SwissProt Accession Number, and the oysters in which the protein was detected.



FIGURES

Figure 1

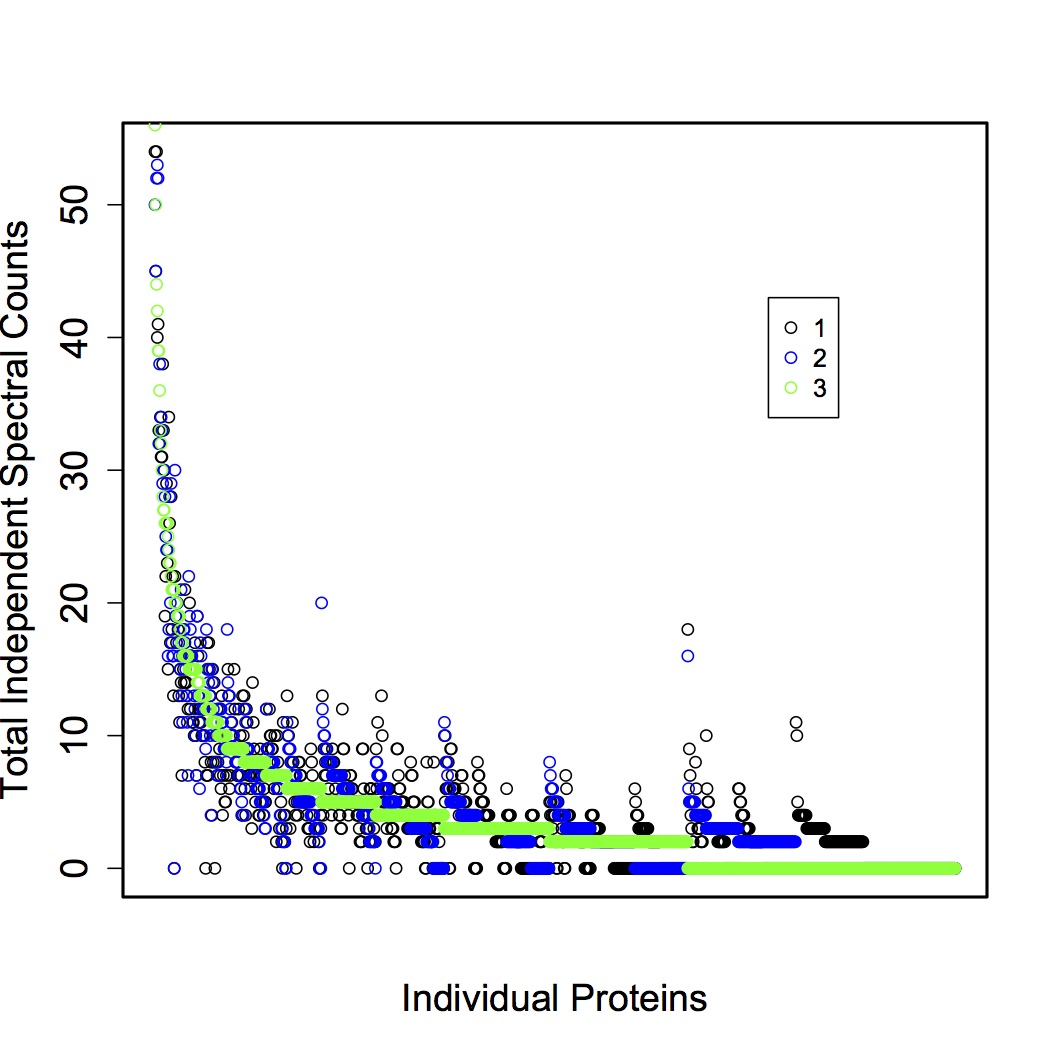


Figure 2

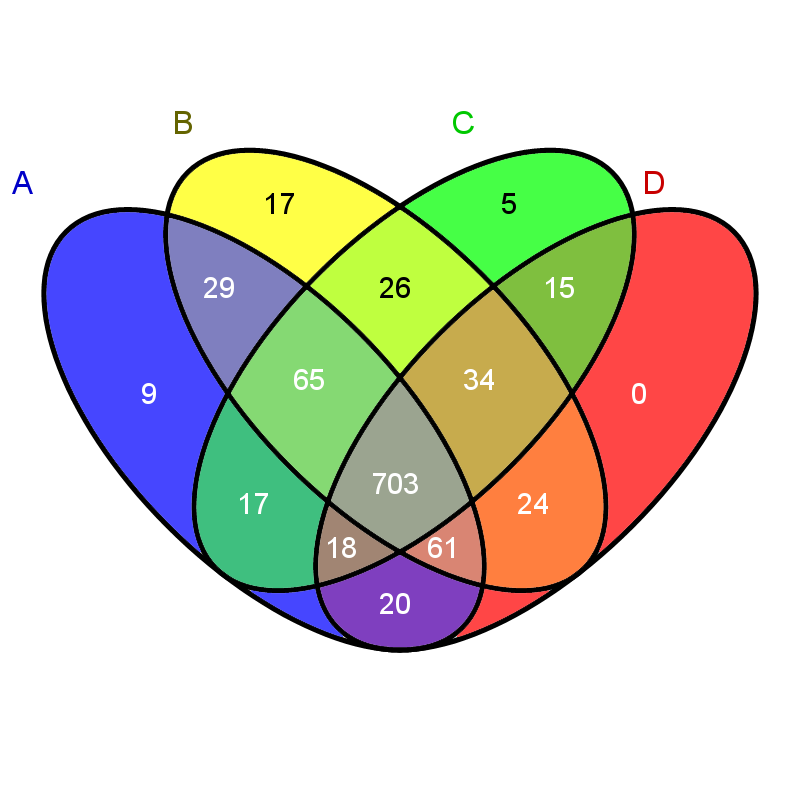


Figure 3

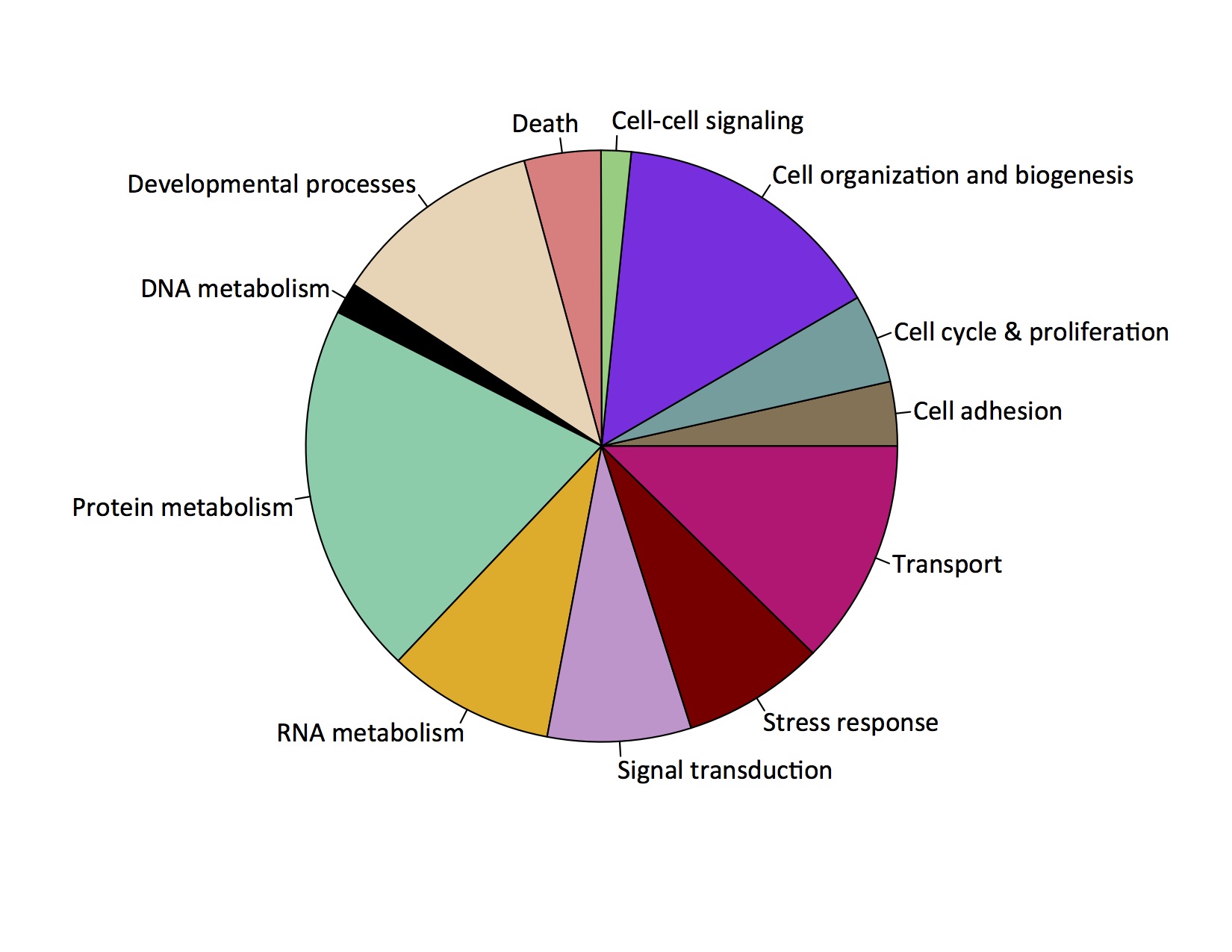
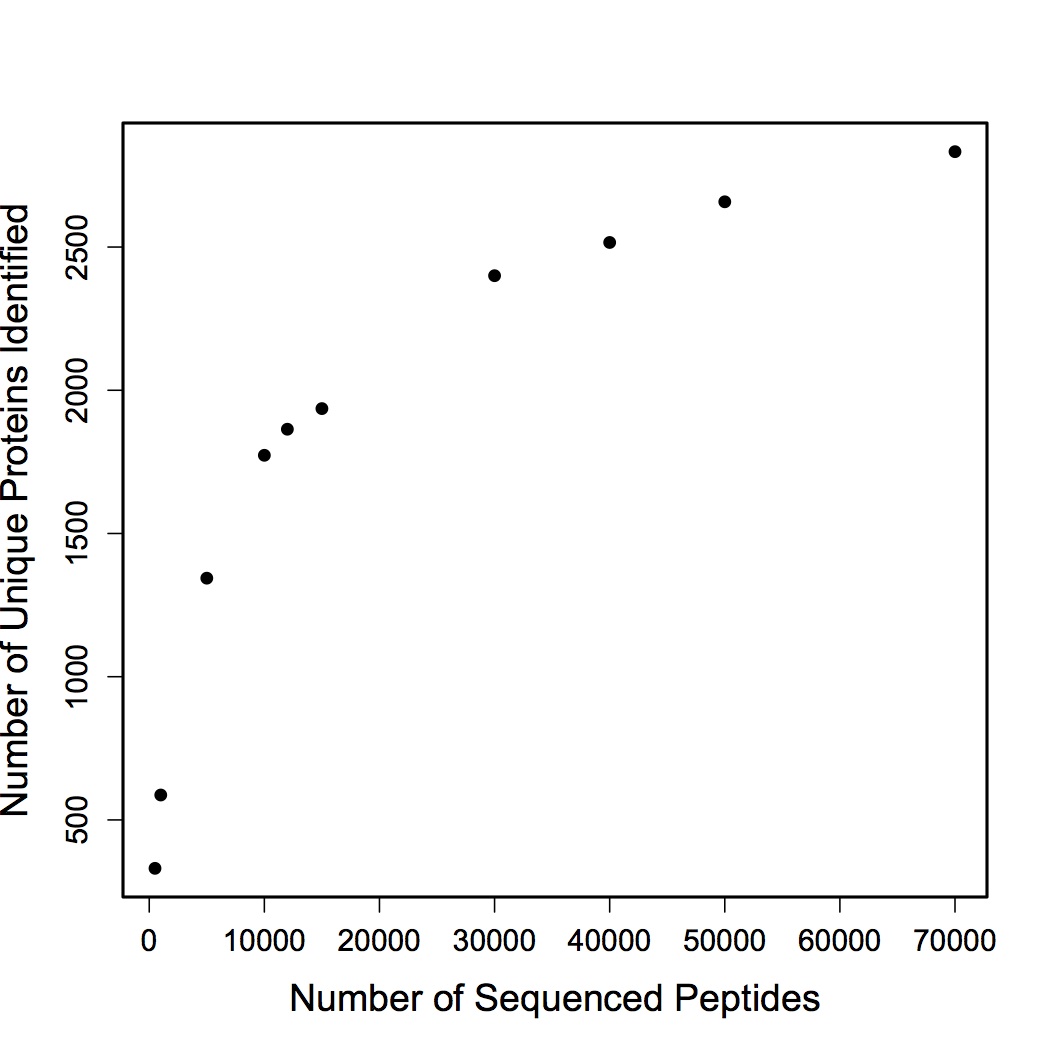


Figure 4



1. ETS performed the experiment and lab work, performed the data analysis, and was primary author of the manuscript. [↑](#footnote-ref-1)
2. BLN assisted with the lab work and helped to author and revise the manuscript. [↑](#footnote-ref-2)
3. DRG provided support to do the lab work and assisted in authoring and revising the manuscript. [↑](#footnote-ref-3)
4. SBR helped with the experimental design, data analysis, and writing and revising of the manuscript. [↑](#footnote-ref-4)