



Probing SWATH-MS as a tool for proteome level quantification in a nonmodel fish

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Abstract

Quantitative proteomics via mass spectrometry can provide valuable insight into molecular and phenotypic characteristics of a living system. Recent mass spectrometry developments include data-independent acquisition (SWATH/DIA-MS), an accurate, sensitive and reproducible method for analysing the whole proteome. The main requirement for this method is the creation of a comprehensive spectral library. New technologies have emerged producing larger and more accurate species-specific libraries leading to a progressive collection of proteome references for multiple molecular model species. Here, for the first time, we set out to compare different spectral library constructions using multiple tissues from a coral reef fish to demonstrate its value and feasibility for nonmodel organisms. We created a large spectral library composed of 12,553 protein groups from liver and brain tissues. Via identification of differentially expressed proteins under fish exposure to elevated pCO₂ and temperature, we validated the application and usefulness of these different spectral libraries. Successful identification of significant differentially expressed proteins from different environmental exposures occurred using the library with a combination of data-independent and data-dependent acquisition methods as well as both tissue types. Further analysis revealed expected patterns of significantly up-regulated heat shock proteins in a dual condition of ocean warming and acidification indicating the biological accuracy and relevance of the method. This study provides the first reference spectral library for a nonmodel organism. It represents a useful guide for future building of accurate spectral library references in nonmodel organisms allowing the discovery of ecologically relevant changes in the proteome.

KEYWORDS

climate change, data-independent acquisition, fish, quantitative proteomics, spectral libraries, SWATH-MS

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1 | INTRODUCTION

Proteomics provides insight into complex biological mechanisms and cellular phenotypes by measuring the presence and abundance of proteins controlling the execution of molecular processes (Aebersold & Mann, 2016; Liu, Beyer, & Aebersold, 2016). The possible post-translational modifications inferred from proteomics can have a stronger correlation to phenotypic observations than transcriptomics, although the technology to quantify the proteome lags behind that of the transcriptome (Liu, Xin, et al., 2016; Tang et al., 2015). In the past decade, proteomic techniques have been developing rapidly, especially those dealing with quantitative proteomics. Previously, quantitative methods were defined in two categories: shotgun and targeted, each with different strengths and weaknesses. The shotgun method identifies more peptides, but has reduced quantitative accuracy and reduced reproducibility due in part to the necessity for prefractionation prior to liquid chromatography–mass spectrometry (LC-MS) analysis (Michalski, Cox, & Mann, 2011). These methods also require costly chemical labelling that increase data processing times. Targeted proteomics (S/MRM), on the other hand, is better for reproducibility if the proteins in question are known. However, this method is limited in the number of measurements and therefore peptides found (Gillet et al., 2012).

Sequential window acquisition of all theoretical spectra (SWATH/DIA-MS) is a newer method that combines the strengths of shotgun and targeted proteomics (Gillet et al., 2012). SWATH/DIA-MS is a label-free and relatively cheap mass spectrometry method using data-independent acquisition (DIA) combined with proteome-wide spectral libraries created from data-dependent acquisition (DDA) methods against which the fragment ion maps of the DIA method are aligned (Gao et al., 2017; Gillet et al., 2012; Huang et al., 2015). Using the sequential isolation window, acquisition has achieved high fragment ion specificity and the ability to identify and quantify thousands of proteins in one measurement (Gillet et al., 2012; Tang et al., 2015). Recent studies have also shown high reproducibility of results when using the same cell cultures across different laboratories as well as different computational software (Collins et al., 2017; Navarro et al., 2016). Most SWATH/DIA-MS studies to date have used model organisms such as mice, humans, zebrafish or yeast with high-quality genomic resources and little biological variation to great success (Blattmann et al., 2019; Braccia, Espinal, Pini, De Pietri Tonelli, & Armirotti, 2018; Bruderer et al., 2015; Collins et al., 2017; Krasny et al., 2018; Rosenberger et al., 2014; Zhang et al., 2019). A recent study has looked into the heat stress response of broiler chickens (*Gallus gallus domesticus*) and found significant changes across the proteome in ecologically relevant proteins with ties to heat stress responses (Tang et al., 2015). However, over generations this organism was kept in controlled conditions and lacks the natural variation of wild organisms. The above qualities of SWATH-MS suggest that it is an ideal method to quantify peptides at the proteome level across large numbers of biological samples with high-quality reproducible results and also an impactful way to understand complex

mechanistic and phenotypic differences in particular in nonmodel organisms which usually lack genomic and proteomic resources. Currently, however, it is still unknown how effective it will be at identifying protein expression differences in wild populations with intrinsic individual variation.

The key to the SWATH/DIA-MS method is the creation of a high-quality spectral library against which to measure peptide quantities in the DIA data of the samples of interest. This reference can provide a standardized set of protein identifications making it easier to compare proteomes across experiments and laboratories (Blattmann et al., 2019; Rosenberger et al., 2017). Although study-specific libraries are easier to create, in the long run a large spectral library is more beneficial to the future studies of an organism by reducing the cost of sample preparation and measurement time. However, it has been understudied whether any loss of information comes from using a study-specific versus a general spectral library. Larger libraries require more stringent cut-offs to control the false discovery rate (FDR) that could lead to a loss of ecologically relevant information, while study-specific libraries could restrict identification at the proteome level due to the smaller reference (Blattmann et al., 2019; Rosenberger et al., 2017).

The SWATH/DIA-MS method provides the technological advances needed to study the whole proteome of nonmodel organisms. Due to the high biological variability of individuals from nonmodel or even wild organisms, a greater number of biological replicates are required to establish statistical significance when studying changes in expression (Todd, Black, & Gemmell, 2016). This method's reductions in cost and sample preparation time allow for the quantification of that required high number of individual samples. Also, the creation of a reference library erases the need for all compared samples to be injected in the mass spectrometer as one, creating the ability to compare a larger number of individuals and/or treatments. Due to this increased sample size and the proteome-wide exploration, we may be able to determine ecologically relevant differences in protein expression both at the individual and population levels for any organism. In order to examine the usefulness of different SWATH libraries, we turned to a commonly used nonmodel fish *Acanthochromis polyacanthus*. Most research in the past decade has focused on its behavioural and physiological changes under different environmental conditions, with a more recent focus on transcriptomic and epigenetic modifications (Jarrold & Munday, 2018; Schunter et al., 2016; Welch, Watson, Welsh, McCormick, & Munday, 2014). To date, the majority of the molecular studies on coral reef fish have focused on the liver and brain tissue due to the physiological effects on metabolism and aerobic scope under heat stress, and the behavioural effects under ocean acidification, respectively (Bernal et al., 2018; Schunter et al., 2016; Veilleux et al., 2015). Only one previous study has measured the changes in protein expression: the method used was iTRAQ labelled shotgun proteomics of pooled samples hence not allowing for the comparison between many individuals or treatments (Schunter et al., 2016). The creation of a SWATH library for this fish will increase ecologically relevant proteomic studies by cutting the cost, reducing overall preparation and quantification times

and allowing for accurate and reproducible measurements of the proteome across high numbers of individuals.

Here, we created several spectral libraries to evaluate the performance of a study-specific versus a large species-specific library for the nonmodel fish *A. polyacanthus*. We focused on liver and brain tissues due to previous studies demonstrating the effects on these tissues by climate change stressors. Recent approaches have created spectral libraries composed of the combined DDA runs, previously used to create the libraries, with the experiment-specific targeted DIA runs (Gandhi et al., 2017). Our aim was to investigate the utility of this new combined library method in the identification of peptides via targeted DIA-MS, using a complex experimental design of fish exposed to multiple climate change stressors. This manuscript provides a first reference library in a nonmodel fish species, as well as a guide on the efficiency, cost-effectiveness and utility of this method in creating future proteomics references in nonmodel organisms aiming to evaluate genome-wide and ecologically relevant differential protein expression.

2 | METHODS

2.1 | Fish rearing and tissue dissection

Acanthochromis polyacanthus offspring were reared in several different conditions (Figure S1). These include control conditions (29°C, 400 μ atm), elevated pCO₂ (750 μ atm or 1,000 μ atm), elevated temperature (31°C) and combined elevated temperature and elevated pCO₂ (31°C, 1,000 μ atm, Jarrold & Munday, 2018; Welch et al., 2014). All experiments were undertaken at James Cook University following the university's animal ethics guidelines (Ethics committee permits: A1828, A2210). Fish were euthanized between 3–5 months of age. For quantitative analysis, a total of 49 fish were collected equating to ~12 biological replicates from each of the four conditions. Brain and liver tissues from each of the fish were dissected out and snap-frozen in liquid nitrogen and then kept at -80°C for further processing.

2.2 | Protein extraction and digestion

Total protein was extracted using the Qiagen All prep mini kit (Qiagen) after elution through the RNA spin column. The flow-through liquid was transferred to a new Eppendorf tube with 3.5 μ l of Halt protease inhibitor cocktail (Thermo Fisher Scientific) and kept on ice. The liquid was then split in half between two Eppendorf tubes (~250 μ l in each), and 1,000 μ l of cold acetone was added to each tube. After 30 min on ice, all tubes were centrifuged at 4°C for 10 min. The samples were then moved to the fume hood, and all the liquid was removed via pipetting. Samples were left to dry for 10 min, and the resulting protein pellet was stored at -80°C for further processing. Protein pellets were resuspended in 8 M urea buffer combined with protease inhibitor (Promega) and purified

using the chloroform-methanol precipitation method (Wessel & Flügge, 1984). The resulting pellet was then resuspended in 8 M UA buffer (8 M urea in 0.1 M Tris/HCl pH 8.5) and sonicated. From this, the protein quantity was measured using the Micro BCA protein assay kit (Thermo Fisher Scientific) and a SpectraMax microplate reader.

For the spectral library preparation, 800 μ g of total protein was combined from across all experimental conditions. This was done separately for liver and brain samples. During individual sample quantification, 20 μ g of protein extract from each biological replicate was used for both liver ($n = 47$) and brain ($n = 49$) samples. Protein alkylation, reduction and digestion were done using the filter-aided sample preparation (FASP) protocol (Wisniewski, Zougman, Nagaraj, Mann, & Wi, 2009). Following digestion with trypsin, samples were desalted using C18 filter pipette tips (Agilent) or a reversed-phase C18 Sep-Pak cartridge (Cat. WAT023590; Waters Corp.) containing an oligo R3 reversed-phase resin (Cat. 1133903; Applied Biosystems) depending on protein quantity. Elution from both the C18 cartridge and pipette tip was done using 75% acetonitrile (ACN) in 0.1% trifluoroacetic acid, and all samples were subsequently dried in a SpeedVac (Thermo Fisher Scientific).

For individual sample quantification with DIA-MS, all samples were resuspended in 15 μ l of the buffer 3% ACN in 0.1% formic acid (FA). Samples were then quantified using a NanoDrop (Thermo Fisher Scientific), and the amount of buffer was adjusted to normalize the concentration amount of each sample for injection. Indexed retention time (iRT) standards (Biognosys) were added to each prepared sample prior to the mass spec run at a 3:10 ratio (v/w).

2.3 | High pH reversed-phase HPLC fractionation for spectral library preparation

For the spectral library preparation, samples were resuspended in 15 μ l of buffer A (0.1% FA). All protein derived from fish samples were combined in one tube and topped up with buffer A for a total volume of 85 μ l. For this and all following steps, brain library and liver library preparation were done separately but using the same methods. High pH reversed fractionation was achieved by attaching a XBridge Peptide BEH C18 column (Cat. 186003570; Waters Corp.) to an Accela liquid chromatography system (Thermo Scientific) using the HPLC application. A 135-min gradient at constant 300 nl/min was designed as follows: The gradient was established using mobile phase A (0.1% FA in H₂O) and mobile phase B (0.1% FA, 95% ACN in H₂O): 2.1%–5.3% B for 5 min, 5.3%–10.5% for 15 min, 10.5%–21.1% for 65 min, 21.1%–31.6% B for 13 min, 31.6%–94.7% B for 6 min, 94.7% for 6 min and 4.7% B for 15-min column conditioning. A total of 110 fractions were collected and then reduced to ~50 μ l using a SpeedVac system (Thermo Fisher Scientific). Fractions were pooled into 25 groups by combining different parts of the gradient and dried with a SpeedVac system (Thermo Fisher Scientific). The dried peptides were resuspended in 0.1% FA and 3% ACN in water and protein quantity measured

at A_{280} via NanoDrop (Thermo Fisher Scientific). Concentrations were normalized across all fractions, and iRT (Biognosys) standards were added to each fraction at a 1:10 (v/w) ratio in preparation for the mass spectrometer.

2.4 | LC-MS/MS data acquisition

An Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) was attached to an Ultimate 3000 UHPLC (Thermo Fisher Scientific) for both DDA and DIA analyses. Peptides were injected and eluted through a 50 cm EASY-Spray column PepMap RSLC C18 (Cat. ES803; Thermo Fisher Scientific) with a 135-min gradient at constant 300 nl/min kept at 40°C. The gradient was established using mobile phase A (0.1% FA in H₂O) and mobile phase B (0.1% FA, 95% ACN in H₂O): 2.1%–5.3% B for 5 min, 5.3%–10.5% for 15 min, 10.5%–21.1% for 70 min, 21.1%–31.6% B for 18 min, ramping from 31.6% to 94.7% B in 2 min, maintaining at 94.7% for 5 min and 4.7% B for 15-min column conditioning. For introduction into the MS, an electrospray potential of 1.9 kV was used and the ion tube temperature was set to 270°C. General MS settings included default charge state of 3, application mode set to standard for peptide, and an EASY-IC was used for internal mass calibration in both MS1 and MS2 ions.

For DDA analysis, the MS was set to profile mode with a resolution of 120,000 (at 200 m/z) and a full MS scan (375–1,400 m/z range) was obtained. Other settings included 30% RF lens, 3 s between master scans, ion accumulation time of 100 ms with a target value of 4e5, activation of MIPS (monoisotopic peak determination of peptide) and an isolation window of 1.6 m/z for ions. The ions carrying charges from 2⁺ to 5⁺ and measured above an intensity threshold of 5e4 and were selected for fragmentation using higher energy collision dissociation (HCD) at 30% energy. Dynamic exclusion was used after 1 event for 10 s with a mass tolerance of 10 ppm.

In DIA-MS analysis, a HCD fragmentation method was used with a quadruple isolation window of 25 m/z . Precursor mass range was set to 400–1,200 m/z for each injection. HCD was set to 30%, and the mass defect was 0.9995. MS2 was run with a scan range of 350–1,500 m/z , at a resolution of 30,000, maximum injection time of 100 ms and a target value of 1e6.

2.5 | Spectral library generation

Data-dependent acquisition raw files were processed, and the library generated with Spectronaut Pulsar X (version 12, Biognosys) using default settings (Monroe, Zhang, Schunter, & Ravasi, 2020). A false discovery rate of 0.01 was set at all levels (peptide, protein, and peptide spectrum match). Peptides with a minimum length of 7 and maximum of 52 were allowed, along with a maximum of two missed cleavages. Digest type was set to specific and defined as Trypsin/P. Peptide identity was achieved via sequence alignment against the *A. polyacanthus* proteome with 36,741 entries. Default library filters

in Spectronaut include ion mass to charge from 300 to 1,800 Da, as well as minimum relative intensity of 5%, and a minimum amino acid ion length of two. Three to six fragment ions per precursor peptide were used in the library and iRT calibration required an R-squared minimum of .8. Minimal trypsin contamination was found and removed during the proteome database search.

A second library was generated for both liver and brain by processing all DDA and DIA runs together in Spectronaut Pulsar X (Monroe et al., 2020). All settings were the same as above including the protein database used to search for peptide identities. To create a 'complete' library (combining both tissues) for *A. polyacanthus*, brain and liver libraries were merged using the combine library function in Spectronaut Pulsar X.

2.6 | Quantitative analysis of biological samples

Data-independent acquisition data were then loaded into the Spectronaut Pulsar X (version 12, Biognosys) software and mapped against the generated spectral libraries leading to protein and peptide identification and quantification (Monroe et al., 2020). Default settings of the programme were applied for analysis including: estimation of FDR using a 0.01 q -value cut-off for precursors and proteins; peptide quantity measurement determination using the mean of one-three best peptides; quantitation calculated using the area of extracted ion chromatogram (XIC) at MS2 level; and excluding duplicate assays. Upon completion, peptide quantities for all samples ($n = 47$ liver, $n = 49$ brain) were exported as a data matrix containing all nonredundant protein identifications for further analysis.

Data matrices were loaded into the program Perseus v1.6.2.1 (Tyanova et al., 2016). Data were filtered by using a cut-off of at least 80% valid values in at least one condition. Protein group quantities were then log₂ transformed to establish a normalized distribution, and missing values were inferred using the normal distribution setting in Perseus. Differential abundance between conditions was inferred statistically using a multiple-sample test (ANOVA) with a permutation-based FDR at 0.05 (250 randomizations, no group preservations). Significant differences between each of the four conditions were determined using a Tukey post hoc test (FDR < 0.05) in Perseus. Protein names and functions were identified with the *A. polyacanthus* genome annotation.

2.7 | Data representation

Mass spectrometry DIA and DDA raw data files acquired via the Orbitrap Fusion Lumos have been uploaded to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al., 2019) with the dataset identifier PXD017605 (Monroe et al., 2020). Spectral library files, the proteome FASTA file for *A. polyacanthus*, used to create these libraries, and raw data output from Spectronaut Pulsar X (Version 12, Biognosys) after quantitative analysis can also be found in this repository.

3 | RESULTS

3.1 | Spectral library

The key to the SWATH/DIA-MS analysis is the availability of a high-quality spectral library for the organism in question. To achieve this, we used samples from an experiment covering exposure to elevated temperature and elevated pCO₂ as well as different tissues dissected from our organism *A. polyacanthus*. Equal amounts of protein from each condition and each tissue were used to prevent bias in the library representation. We combined a range of protein preparation protocols to ensure both better quality and quantity peptides prior to mass spectrometry (Figure 1). This included purification via methanol/chloroform to remove nonproteins, an FASP protocol which improved quality and yield, and desalting using cartridges with both c18 filters and R3 material to minimize protein loss. We then used the newer Orbitrap Fusion mass spectrometry platform that has been shown to identify more peptides than other systems (Zhang et al., 2019). Lastly, the new genome developed recently for *A. polyacanthus* gave us a high-quality database of protein coding genes to search against for spectral library generation.

Based on the above optimal workflow, we created six different spectral libraries to test our hypothesis. In each library, we were able to identify a large number of peptides and proteins, and as expected, the highest identification was discovered in the combined tissue library (Figure 2). The resulting spectral libraries contained anywhere from 49,136 to 103,022 proteotypic peptides and 8,273 to 12,553 protein groups which equates to a 22%–35% coverage of all the protein coding genes (*A. polyacanthus* genome, unpublished; Table 1).

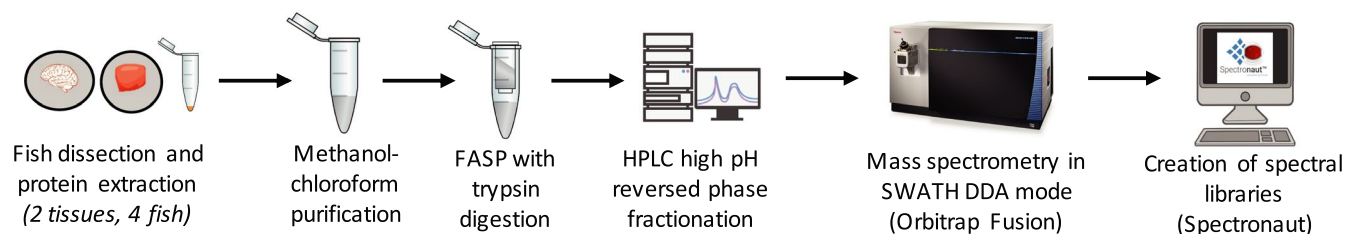
Consistent with full tryptic peptide properties, most identified peptides were 8–18 amino acids in length in all libraries.

3.2 | DIA/SWATH-MS for the discovery of ecologically relevant molecular processes

To determine the utility of these various spectral libraries, we compared the proteomes of liver and brain tissue from *A. polyacanthus* exposed to different environmental conditions against each of the different libraries (Figure S2). Using DDA + DIA libraries resulted in a significant increase in identified precursors and protein groups for both liver and brain samples (Figure 3). Approximately 3,300–4,100 protein groups were identified in brain depending on the library specificity and ~2,900 to 3,100 protein groups identified in liver. A higher data completeness was revealed when using the DDA + DIA libraries versus the DDA libraries correlating to the overall number of protein groups found when using the different libraries (Table 2).

There was little difference between the number of precursors and protein groups identified between the tissue-specific DDA + DIA library and the combined DDA + DIA library in both liver and brain. Furthering the case that a comprehensive species library combined with experiment-specific DIA runs can lead to no significant loss of identification as compared to the tissue-specific spectral library as long as it contains the targeted DIA tissue. By incorporating the DIA samples into the spectral library, we are able to bolster the number of peptides represented in the library that could be found in the sample content which leads to fewer chances of false discovery without limiting the proteome coverage. To measure variability across

Data dependent acquisition (DDA)



Data independent acquisition (DIA)

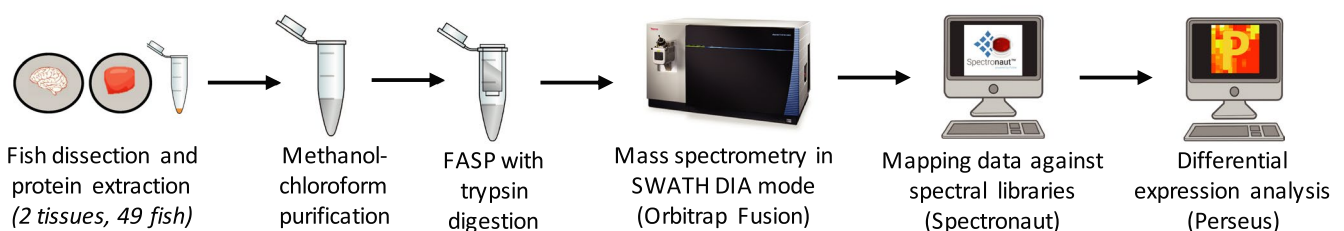


FIGURE 1 Flowchart of the proteomics methodology used in both data-dependent and data-independent acquisition

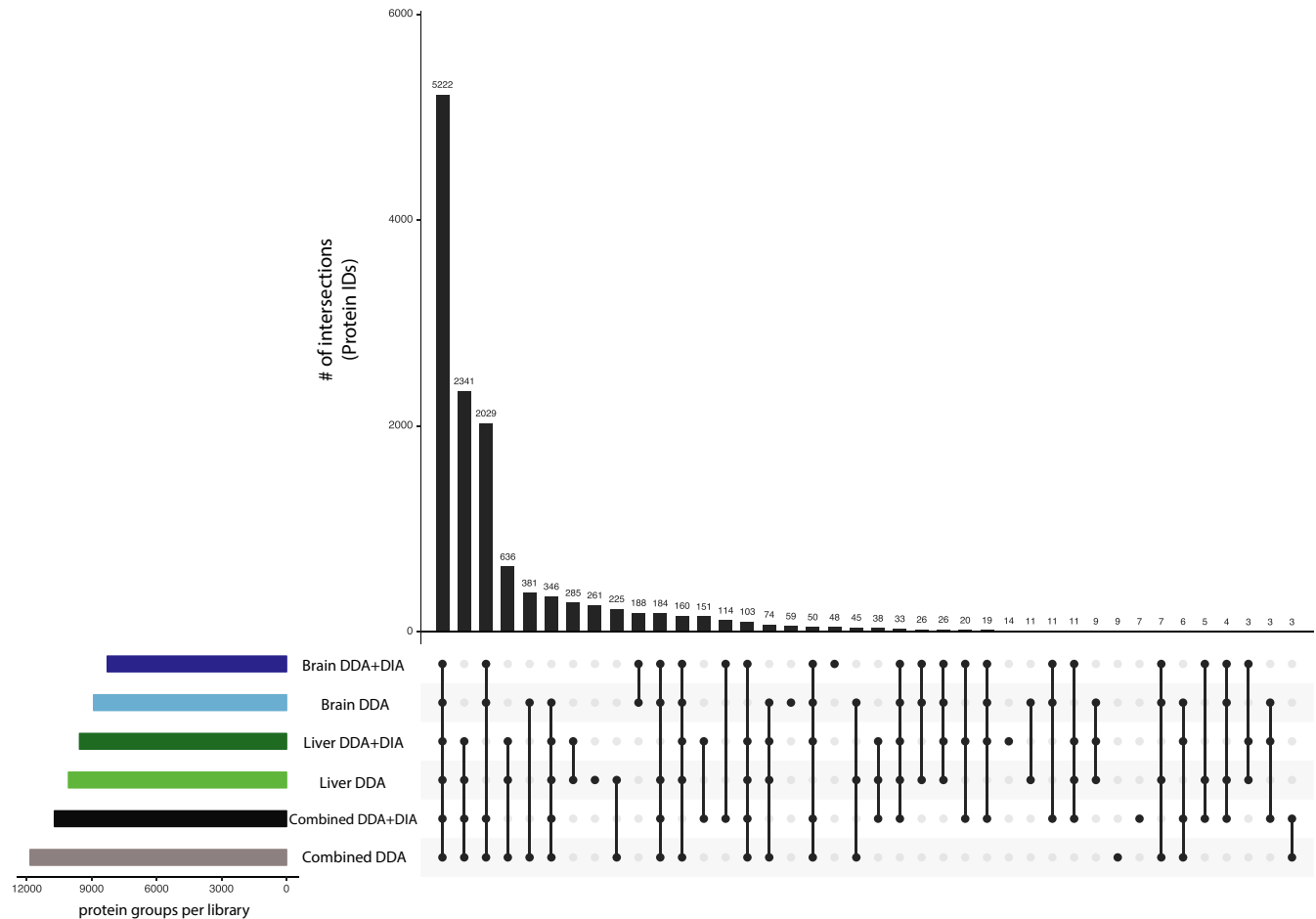


FIGURE 2 Graph of overlapping protein groups between different spectral libraries. The majority of protein identifications are all included in the combined libraries

TABLE 1 Size of the different libraries created. Proteotypic peptides are those that uniquely identify a single protein

	Liver DDA	Brain DDA	Combined DDA	Liver DDA + DIA	Brain DDA + DIA	Combined DDA + DIA
No. of injections	25	25	50	72	74	146
Protein groups	10,825	8,901	12,084	10,541	8,273	12,553
Peptides (proteotypic)	102,888 (82,909)	69,662 (52,385)	130,310 (103,022)	98,475 (79,547)	65,302 (49,136)	126,866 (100,224)
Precursors	134,369	84,599	170,650	131,912	83,214	170,918

Abbreviations: DDA, data-dependent acquisition; DIA, data-independent acquisition.

samples and between replicates, we used the median coefficient of variation (CV) that represents the ratio of the standard deviation to the mean. CVs were higher in the DDA + DIA libraries than the DDA libraries for both liver (~31% vs. 23%) and brain (~20% vs. 15%; Table 2). Liver samples also had a small number of full profiles which refer to protein groups and precursors that are found across all DIA samples queried (Figure 3). Lack of full profiles compared to sparse profiles reveals a high variability between samples and is expected due to the high complexity of our experiment's design.

Tissue specificity might be an issue for the proteome analysis of nonmodel organisms. Notably, when running the targeted DIA samples of one tissue against the spectral library of a different tissue, precursor

identification decreased by an average of 43% in the liver DIA samples and ~55% in the brain DIA samples (Figure 3). This indicates that a species-specific spectral library must contain protein samples from all targeted tissues or risk decreasing protein group identification by up to ~75% depending on the quality of the spectral library.

3.3 | Differential expression and functional analysis

An important function of the spectral library is to examine how well it can identify biologically and ecologically relevant differentially expressed proteins in a DIA-MS targeted analysis. Here, we are able to

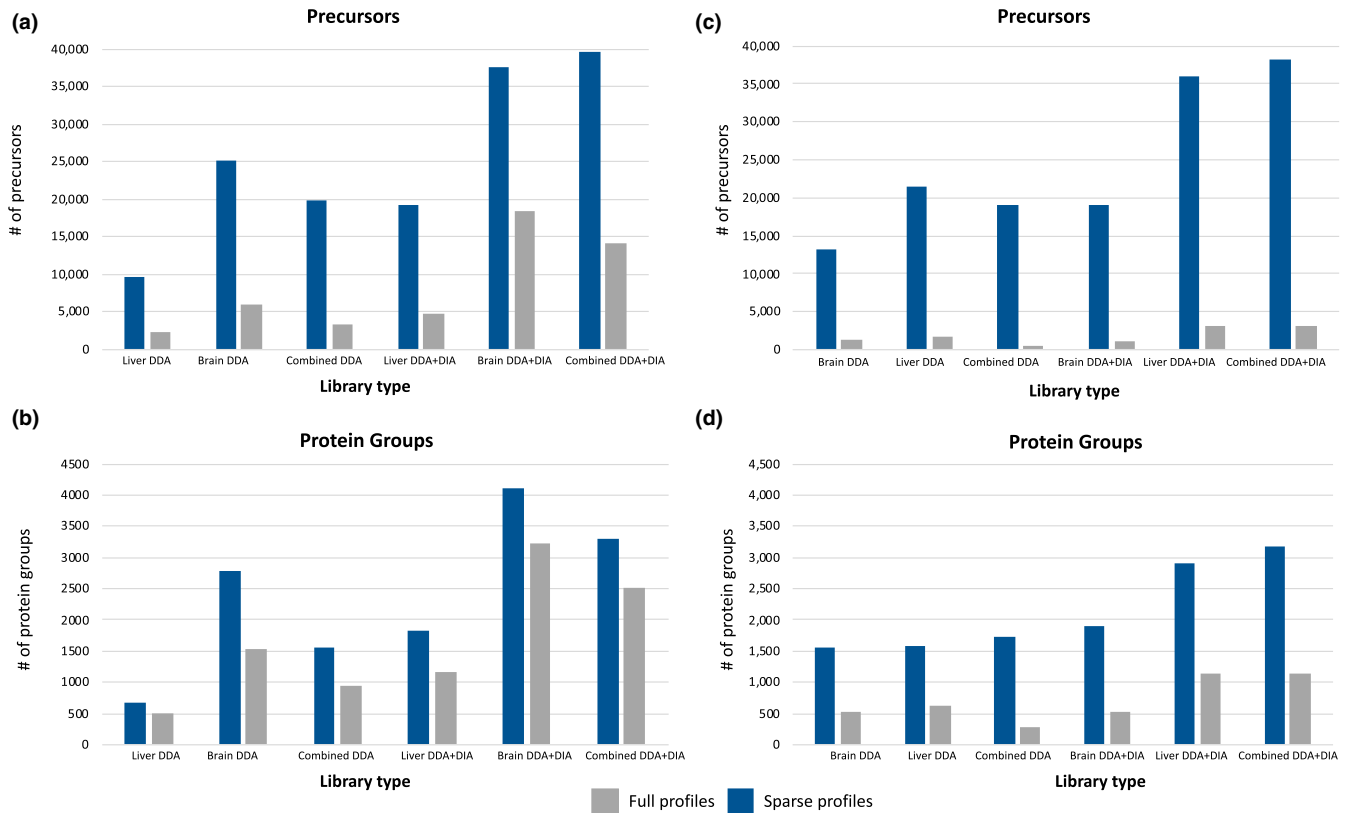


FIGURE 3 (a and b) represent the comparison of brain data-independent acquisition (DIA) samples against different libraries. (c and d) show comparisons of liver DIA samples from different conditions against different libraries. Full profiles indicated in grey represent proteins found across all DIA samples (liver samples are highly variable), sparse proteins represented in blue are those proteins found in at least one DIA sample

TABLE 2 Data completeness and median CV's of both liver and brain DIA runs compared against all library types

	Data completeness (%)	Median CVs (%)
Liver DIA samples		
Liver DDA library	50.60	23.1
Liver DDA + DIA library	62.60	31.4
Brain DDA library	54.50	22.2
Brain DDA + DIA library	55.30	25.6
Combined DDA Library	45.0	22.4
Combined DDA + DIA library	60.40	31.4
Brain DIA samples		
Brain DDA library	57.30	15.2
Brain DDA + DIA library	77.10	21.2
Liver DDA library	56.7	14.8
Liver DDA + DIA library	56.40	16.8
Combined DDA Library	50.10	14.9
Combined DDA + DIA library	67.70	20.9

Abbreviations: CV, coefficients of variance; DDA, data-dependent acquisition; DIA, Data-independent acquisition.

show the definite usefulness of an experiment-specific DIA + DDA library. No unique differentially expressed proteins (DEPs) were discovered using the combined DDA library in any comparison or tissue (Figure 4). Despite having a significant amount of shared identified proteins, using the tissue-specific DDA + DIA libraries and the combined DDA + DIA libraries both identified high amounts of unique proteins (27% and 13%, respectively, for the brain, and 28% and 16% for the liver). This analysis made it clear that using a combined DDA library is insufficient for the identification of DEPs; when analysing highly variable samples, the library must have some specificity (tissue or study related).

Higher numbers of statistically significant differentially expressed proteins were uniquely identified in the tissue-specific DDA + DIA library in both liver and brain analysis (~27.5% of all identified DEPs). One main goal of this study was to determine whether a 'whole organism' library could identify sufficient ecologically relevant DEPs as compared to a tissue-specific library. In order to determine whether the reduced number of unique proteins led to any loss in ecologically relevant protein identifications, we looked deeper at the function of the DEPs identified using all library types (Data S1). As our DIA experiment was designed to examine the stress response of a fish to combined climate change stressors, we examined the significant DEPs for those related to cellular stress in fish. These included proteins related to the heat shock response (HSP),

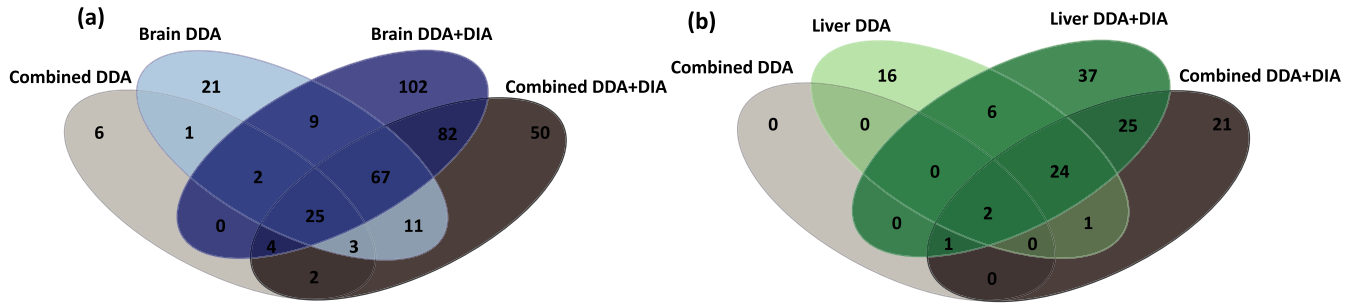


FIGURE 4 Venn diagrams of overlapping and unique differentially expressed proteins identified using different spectral libraries for both brain (a) and liver (b) targeted data-independent acquisition (DIA) analysis. Significant differential expression analysed across all four conditions was calculated via ANOVA (FDR < 0.05)

a well-documented expression change seen in fish exposed to elevated temperatures (Basu et al., 2002), cytochrome related genes involved in oxidative stress and several other previously identified stress response genes in marine fish representing protein degradation and cell death. In Figure 4, we see the combined DDA libraries provided the least identifications of the complex cellular stress response identified by the other libraries. Furthering the necessity of the inclusion of experiment-specific DIA runs in the spectral library, especially when combining tissues. In DDA + DIA libraries, we see little difference between the differential expression in the combined and tissue-specific identifications (Figure 5). This suggests that using a whole organism library (including several tissues or body parts) is sufficient to identify important changes in the entire proteome when a nonmodel organism is exposed to varying environmental conditions.

4 | DISCUSSION

Recent technological advances in proteomics have turned mass spectrometry into a mainstream analytical tool with ecological applications (Aebersold & Mann, 2016). SWATH/DIA-MS is one of the

first to allow for a big data approach to proteomics via its ability to create large data matrices containing accurately measured proteins across various samples with minimal missing values (Aebersold & Mann, 2016). Here, we are able to present one of the first applications of this method to examine ecologically relevant changes in protein expression in a nonmodel organism. As the limitation of this method is the quality of the spectral library, we examined the utility of different library compositions to determine the most useful, both in overall protein identification and in the statistical analysis of differential expression (Krasny et al., 2018). Using eight individuals and multiple tissues to create, these libraries yielded a large number of protein group identifications in both liver and brain libraries (~9,500) that led to ~12,000 protein groups in the combined organism level library. These protein identifications are significantly higher than several other studies using multiple tissues as well as six to eight individuals from varying conditions (Blattmann et al., 2019; Braccia et al., 2018; Tang et al., 2015). We are able to show that while a tissue-specific library approach yields the highest data completion, there is no loss of ecologically relevant information when using an organism level library, as long as that library contains study-specific data. This is especially important when working with small organisms, such as during developmental or larval stages where the biological material

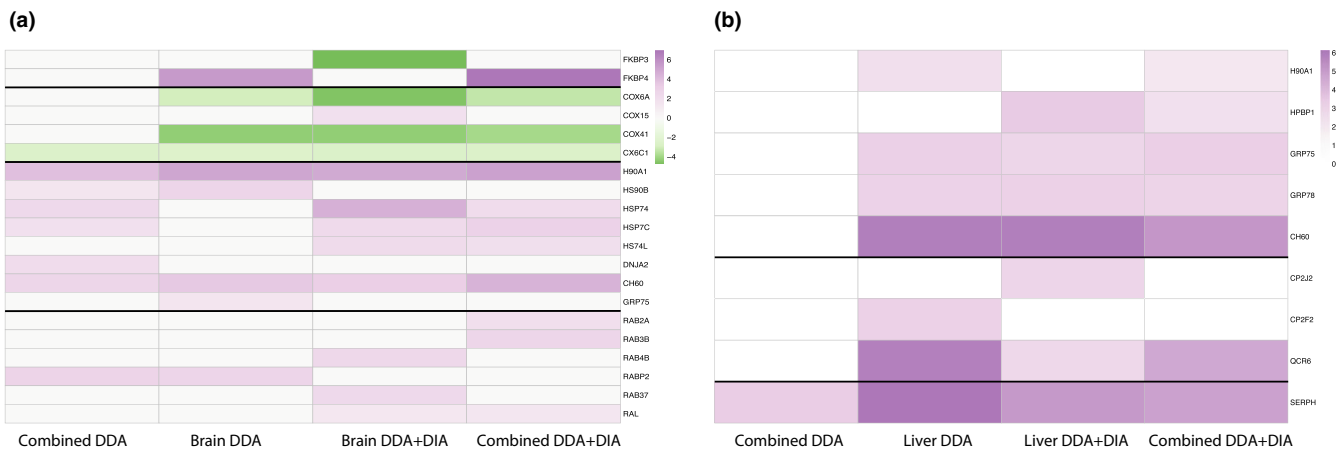


FIGURE 5 Heatmap of differentially expressed stress related proteins in different library comparisons, values refer to differences based on Tukey's post hoc test after an ANOVA test of significance (FDR < 0.05). (a) Those identified in brain data-independent acquisition (DIA) targeted analysis and (b) those identified in liver analysis. Thick lines indicate separations between proteins related to shared functions

is usually limited. Further studies using nonmodel organisms can be confident that a whole organism approach to a spectral library will lead to successful differential expression analysis as well as promote future proteomic studies in the nonmodel species.

The spectral library generation is the most laboratory intensive and time-consuming part of the SWATH/DIA-MS methodology as it requires fractionation prior to mass spectrometry. However, once an organism-specific library is created, future studies can use this reference cutting down on costs and laboratory time. Using a tissue-specific library led to a greater number of identifications at the peptide and protein level when querying samples of that same tissue, but when we compared the liver DIA samples to the brain-specific library or vice versa, we discovered a significant loss in data completeness and protein identification. Therefore, unless one tissue will always be the focus in an organism, a comprehensive species-specific library will be more useful to future proteomic studies than a tissue-specific one.

We also undertook the first study to quantify the usefulness of adding study-specific DIA data to the spectral library in a nonmodel organism. A previous study suggested the utility of this method in laboratory curated cancer cells with positive results in increases of protein identifications (Gandhi et al., 2017). We discovered that the addition of study-specific DIA data in the creation of the spectral library, especially at the whole organism level, increased the identification of protein groups and peptides significantly. Creating a base organism level DDA spectral library then adding DIA specific runs to tailor that library to a specified experiment can lead to the best results of both a study-specific and whole organism library (Gandhi et al., 2017).

To validate the created spectral libraries, we used DIA samples from a complex experimental design with approximately twelve individuals from each of four different conditions. As the experiment was set up to identify the reaction of this fish to the combined climate stressors of ocean acidification and ocean warming, we searched the differentially expressed proteins for those related to common stress responses in fish. Proteins related to cellular stress were found in every brain and liver analysis using different library types. However, a more complex stress response was identified using tissue-specific and study-specific (DIA + DDA) libraries. In particular, we were only able to identify changes between the combined condition and the elevated temperature condition when using DDA + DIA libraries (Data S1). This was a very small change consisting of less than five proteins but when using the DDA libraries the identification of any proteins between these conditions was lost. Proteins identified as part of the cellular stress response included up-regulation of HSPs, a common indicator of stress related to abiotic and biotic factors in fish and other organisms (Basu et al., 2002; Huth & Place, 2016). We also identified several cytochrome proteins involved in oxidative and metabolic processes and associated with thermal stress responses in salmon (Akbarzadeh et al., 2018). *SEPRH*, *FKBP* and *RAS*-related proteins have also been previously identified as significantly expressed in marine fish under stress (Chen, Farrell, Matala, Hoffman,

& Narum, 2018; Evans & Somero, 2009; Liu, Xin, et al., 2016). With these methods, we are able to identify expected changes in relevant molecular pathways previously recognized as being associated with environmental stress responses in fish.

Our in-depth approach into the utility of different spectral libraries provides new insights into the use of SWATH/DIA-MS on nonmodel organisms and in complex experimental designs with high individual variation. Overall, this method achieved good reproducibility and high accuracy across a highly variable sample set. We were able to identify up to 4,000 protein groups and up to 253 differentially expressed proteins using this method. Within the differentially expressed proteins, we successfully identified many proteins related to stress responses in fish, confirming the ability of this method to identify ecologically relevant pathways when examining individuals exposed to varying environmental conditions. We provide the first proof-of-concept for the use of SWATH/DIA-MS in nonmodel organism from a wild, nonlaboratory bred population. The creation of the reference library will furthermore significantly reduce sample preparation time and ~50% of LC-MS time along with the associated costs on future proteomic studies of this fish. These results contribute to our better understanding of proteome changes in our own study organism, *A. polyacanthus*, as well as providing analytical data to encourage the use of this method in further ecological based proteome studies of any nonmodel organisms.

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AUTHOR CONTRIBUTIONS

AAM, HZ, CS and TR conceived of and designed the research. AAM and CS prepared the samples. AAM and HZ performed the experiments and processed the data. AAM analysed the data. AAM wrote the manuscript with input and revisions from HZ, CS and TR. All authors read and approved the manuscript.

DATA AVAILABILITY STATEMENT

The mass spectrometry DDA and DIA proteomics data acquired during this experiment have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017605 (Monroe et al., 2020; Perez-Riverol et al., 2019).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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