RESEARCH ARTICLE

Proteomic analysis of proteins associated with body mass and length in yellow perch, *Perca flavescens*

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The goal of commercial yellow perch aquaculture is to increase muscle mass which leads to increased profitability. The accumulation and degradation of muscle-specific gene products underlies the variability in body mass (BM) and length observed in pond-cultured yellow perch. Our objective was to apply a combination of statistical and proteomic technologies to identify intact and/or proteolytic fragments of muscle specific gene products involved in muscle growth in yellow perch. Seventy yellow perch randomly selected at 10, 12, 16, 20, and 26 wk of age were euthanized; BM and length were measured and a muscle sample taken. Muscle proteins were resolved using 5–20% gradient SDS-PAGE, stained with SYPRO® Ruby and analyzed using TotalLab™ software. Data were analyzed using stepwise multiple regression with the dependent variables, BM and length and proportional OD of each band in a sample as a potential regressor. Eight bands associated with BM ($R^2 = 0.84$) and nine bands with length ($R^2 = 0.85$) were detected. Protein sequencing by nano-LC/MS/MS identified 20 proteins/peptides associated with BM and length. These results contribute the identification of gene products and/or proteolytic fragments associated with muscle growth in yellow perch.

Keywords:
Muscle / *Perca flavescens* / Proteomic fingerprint / Yellow perch

1 Introduction

A major challenge in commercial aquaculture is to produce fast growing fish while maintaining a high level of meat quality. One barrier to accomplishing this undertaking is an insufficient understanding of the role of genes underpinning the cellular and molecular mechanisms responsible for muscle growth and development in fish species. Determining the gene expression events giving rise to the proteins underlying muscle growth in fish species will provide valuable information for brood stock selection strategies and improved muscle yield for fish producers. The growth of a muscle is the result of a balance between protein accumulation (anabolism) and degradation (catabolism). Currently there is a distinct lack of information on events during the growth of fish muscle.

In fish species, muscle growth has commonly been shown to be the result of a combination of hyperplasia, an increase in myofiber number throughout life, and hypertrophy, an increase in myofiber size [1]. This is in contrast to muscle growth in land animals in which muscle growth, after birth, is predominantly due to hypertrophy. Individual fish species exhibit unique combinations of hypertrophy and hyperplasia during postnatal muscle growth. For example, the teleost, sea bream, is a relatively large fish that grows predominantly by hypertrophy, whereas zebrafish, a relatively small fish, grows predominantly by hyperplasia. These fish exhibit these same differences in muscle hyperplasia

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Abbreviations: BL, body length; **BM,** body mass; **PSE,** pale, soft, and exudative

versus hypertrophy during muscle regeneration [2–5]. The as yet unknown mechanisms responsible for hyperplastic muscle growth are proposed to reside in the differences in satellite cell proliferation [6, 7], as well as the involvement of undefined subpopulations of myogenic cells [8]. Myosin isoform expression has also been shown to be associated with different muscle growth patterns [9–11].

Genetic selection has the potential to increase the growth rate of yellow perch. However, the accompanying changes in gene expression related to increased muscle growth in fish are not fully understood and may be accompanied by concomitant negative meat quality characteristics, such as those observed in other animals of agricultural interest that have been selected for increased muscle growth. For instance, selection for increased lean muscle growth in swine and poultry has resulted in pale, soft, and exudative (PSE) meat. Some strains of pigs have a genetic susceptibility to stress that is negatively correlated with the overall quality of the processed meat derived from those animals [12, 13]. A PSE-like anomaly has also been observed in turkey muscle [14–18]. These anomalies have a significantly negative economic impact on the agriculture industry. Although the gene products associated with PSE-like syndrome in poultry have yet to be fully elucidated, Updike *et al.* [19] used a proteomic approach based on 1-D gel electrophoresis to identify three metabolic proteins in fast glycolytic muscle associated with processed meat quality in turkeys genetically selected for rapid muscle growth.

In this study, our objective was to apply a combination of statistical and proteomic technologies to identify intact and/or proteolytic fragments of muscle specific gene products involved in muscle growth in yellow perch. Here, we report the use of a proteomic sequence tag technology using LC-MS/MS in combination with stepwise linear regression to associate individual electrophoretically separated protein/peptide bands from whole muscle of yellow perch body mass (BM) and body length (BL). This evaluation of yellow perch skeletal muscle provides an excellent system to identify the effects of genetic selection on developmental proteomics in fish species.

2 Materials and methods

2.1 Experimental animals

Seventy yellow perch randomly selected from ages 10, 12, 16, 20, and 26 wk were collected at the Ohio Center for Aquaculture Development, Piketon, OH. Yellow perch were humanely euthanized by immersion in MS-222 according to the manufacturer's instructions. Fish were preserved by immersion in a storage buffer (100 mM KCl, 20 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM ethylene glycol-bis[β-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM NaN_3, pH 6.8, with 50% glycerol), and then frozen at −20°C. Fish were thawed, rinsed in 70% ethanol, and blotted dry. Individual BM and BL were recorded and 50 mg of muscle tissue was collected from the epaxial muscle in the region below and cranial to the dorsal fin, above the lateral line, using a new sterile scalpel for each fish.

2.2 Protein extraction

Frozen muscle samples were quickly minced and placed into 1.5 mL of a urea/thiourea buffer containing 8 M urea, 2 M thiourea, 75 mM DTT, 50 mM Tris, 3% SDS, and 0.004% bromophenol blue, pH 6.8. Muscle proteins were extracted in this buffer for 30 min on ice and then centrifuged at $10\,000 \times g$ for 10 min. The extracted protein samples in the supernatant were then stored at −20°C until gel electrophoresis.

2.3 SDS PAGE

Muscle proteins were resolved by SDS-PAGE according to Updike *et al.* [19], using a 1.5 mm × 12 cm × 14 cm polyacrylamide slab gel consisting of 5–20% gradient resolving gel (30:0.8, acrylamide, *N*,*N*′-bis-methylene acrylamide) and a 3% stacking gel. Both resolving and stacking gels contained 0.1% SDS. Twenty-five micro-liters of muscle protein extract (83 μg protein) were loaded in each lane of a 15 lane gel. Each gel was loaded with a broad range molecular weight standard (BioRad Laboratories, Hercules, CA). Proteins were separated by applying a constant voltage of $10\ V \cdot cm^{-1}$ for 2 h 30 min. After electrophoretic separation, gels were stained overnight with gentle agitation in SYPRO® Ruby Protein Stain (BioRad Laboratories) and destained according to the manufacturer's protocol. Briefly, gels were rinsed in 10% methanol, 7% acetic acid for 60 min. This rinse step decreases background fluorescence. Gels were then rinsed in distilled water for 10 min before image analysis.

2.4 Image analyses

Fluorescent gel images were acquired and digitized employing UV light (302_{nm} excitation, 595_{nm} emission) using FluorChem Imaging System software v.2.0 and a Multi Fluor-s Imager with a CCD camera (Alpha Innotech, San Leandro, CA). Raw images were subsequently imported into TotalLab™ 1D software (Nonlinear Dynamics, Newcastle upon Tyne, UK) and analyzed. The following user-defined parameters were employed: background subtraction–rolling ball, radius = 300; band detection: minimum slope = 30; noise reduction = 3; % maximum peak = 1.0; Gaussian volumes fitted to peaks–no advanced fitting; R_f calibration aligned with no lane, and use curve lines and snap R_f lines to bands were checked. Because the densities of the myosin heavy chain and actin bands were not within the dynamic range of the image analysis software, their contributions were not included in the model. Band matching was performed with matching by R_f–vector = 100. Relative mobility

(M_r) and percent contribution of individual bands in each lane were recorded. Identifiable bands between 5 and 200 kDa were selected for statistical analyses.

2.5 Data analysis

Statistical analysis of these data was modified from previous studies [19]. Prediction models of BM and BL were developed separately using a stepwise multiple regression using the STEPWISE option of PROC REG in SAS v.9.1 [20, 21]. The BM or BL values were regressed on percentage contribution of each band, according to the following linear model

$$Y_i = \beta_0 + \beta_j X_{ij} + \varepsilon_i$$

Where Y_i is the BM or BL measurement of the ith fish, ($i = 1,\ldots, 70$), β_0 the intercept, β_j the regression parameter associated with the jth band, ($j = 1,\ldots, 48$), X_{ij} the percentage contribution for the jth band of the ith sample, andε_i is the residual error term.

Independent variables in the model (X_{ij}) were added sequentially using the STEPWISE option of the REG procedure of SAS v.9.1, with a threshold probability of 0.05. The procedure used 14 iterations to identify the terms (i) in the final model for BM and seven iterations to identify the terms (i) in the final model for length. The data were checked for observations that were outliers with leverage and one outlier was subsequently removed from the analysis for BM. All observations were used for the dependent variable length.

A bootstrap analysis was performed to validate the models and their associated parameter estimates [22]. The specific bootstrap used in our study was the bias-corrected and accelerated bootstrap [23]. In short, a bootstrap of 70 observations selected with replacement at random from the original set of 70 observations was first constructed. Parameter estimates of the regression coefficients were then estimated using least squares. This process was iterated 1000 times, and the parameters, β_j, of the bootstrap were calculated as the simple means of the 1000 repetitions.

2.6 Proteomic analysis

Those bands identified as significant in the linear prediction models were excised from the SDS-PAGE gel and subsequently analyzed by mass spectrometry, which was performed at the Campus Chemical Instrument Center MS and Proteomics Facility of The Ohio State University using established methods. Gels were digested with sequencing grade trypsin from Promega (Madison, WI) using the Montage In-Gel Digestion Kit from Millipore™ (Bedford, MA) following the manufacture's recommended protocols. Briefly, bands were trimmed as close as possible to minimize background polyacrylamide material. Gel pieces were then washed in 50% methanol/5% acetic acid for 1 h. The wash step was repeated once before gel pieces were dehydrated in ACN. The gel bands were rehydrated and incubated with a DTT solution (5 mg/mL in 100 mM ammonium bicarbonate) for 30 min prior to the addition of 15 mg/mL iodoacetamide in 100 mM ammonium bicarbonate solution. The gel bands were incubated with iodoacetamide in the dark for 30 min before bands were removed. The gel bands were washed again with cycles of ACN and ammonium bicarbonate (100 mM) in 5 min increments. The gel bands were dried in a speed vac and then protease was driven into the gel pieces by rehydrating them in 50 μL of sequencing grade modified trypsin at 20 μg/mL in 50 mM ammonium bicarbonate for 10 min. Twenty micro-liters of 50 mM ammonium bicarbonate were added to the gel bands and the mixture was incubated at room temperature overnight. The peptides were extracted from the polyacrylamide with 50% ACN and 5% formic acid several times and pooled together. The extracted pools were concentrated to ~25 μL in a speed vac.

2.7 MS–LTQ

Capillary Nano-LC/MS/MS was performed on a Thermo Finnigan LTQ mass spectrometer equipped with a nanospray source operated in positive ion mode. The LC system was an UltiMate™ Plus system from LC-Packings A Dionex (Sunnyvale, CA) with a Famos auto sampler and Switchos column switcher. Solvent A was water containing 50 mM acetic acid and solvent B was ACN. Each sample (5 μL) were first injected on to the trapping column (LC-Packings A Dionex) and washed with 50 mM acetic acid. The injector port was switched to inject and the peptides were eluted off the trap onto the column. A 5 cm, 75 μm I-D ProteoPep II C18 column (New Objective, Woburn, MA) packed directly in the nanospray tip was used for chromatographic separations. Peptides were eluted directly off the column into the LTQ system using a gradient of 2–80% B over 50 min, with a flow rate of 300 nL/min. Total run time was 60 min. The MS/MS was acquired according to standard conditions established at the Campus Chemical Instrument Center MS and Proteomics Facility of The Ohio State University. Briefly, a nanospray source operated with a spray voltage of 3 KV and a capillary temperature of 200°C was used. The scan sequence of the mass spectrometer was based on the TopTen™ method; briefly the analysis was programmed for a full scan recorded between 350 and 2000 Da, and a MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive instrument scans of the ten most abundant peak in the spectrum. The CID fragmentation energy was set to 35%. Dynamic exclusion was enabled with a repeat count of 30 s, exclusion duration of 350 s and a low mass width of 0.5 Da and high mass width of 1.50 Da.

Sequence information from the MS/MS data was processed by converting the raw data files into a merged file (.mgf) using MGF creator (merge.pl, a Perl script). The resulting mgf files were searched using MASCOT Daemon by Matrix Science (Boston, MA). Data processing was performed following the guidelines described by Wilkins and

coworkers [24]. Assigned peaks had a minimum of ten counts (S/N of 3). The mass accuracy of the precursor ions was set to 2.0 Da given that the data were acquired on an IT mass analyzer and the fragment mass accuracy was set to 0.5 Da. Considered modifications (variable) were methionine oxidation and carbamidomethyl cysteine. Protein identifications were checked manually and proteins with a MASCOT score of 40 or higher with a minimum of two unique peptides from one protein having *a*, *-b* or *-y* ion sequence tag of five residues or better were accepted.

3 Results

Electrophoretic and statistical analyses were used to associate individual protein/peptide bands from whole muscle of yellow perch with BM and BL. Figure 1 shows two representative lanes from a 5% to 20% SDS-PAGE of the whole muscle of yellow perch. Lane BL is representative of a lane used to identify bands associated with the BL of yellow perch. Lane BM is representative of a lane used to identify bands associated with the BM of yellow perch. The molecular weight standards are presented at the left of the figure for reference. The nine-protein/peptide bands, solid circles (●), associated with BL were band numbers 2, 16, 20, 24, 26, 36, 38, 40, and 44. The eight-protein/peptide bands associated with BM, solid squares (■), were band numbers 17, 25, 26, 36, 38, 39, 44, and 46. The four protein/peptide bands associated with both BM and BL, represented by the solid square and circle (● and ■), were bands 26, 36, 38, and 44.

Forty-eight bands were subjected to stepwise linear regression analysis and 13 were found to be significant in relation to BM and BL) of yellow perch. Eight-protein/peptide bands were subsequently identified as being associated with BM ($R^2 = 0.84$; Table 1). Nine of the protein/peptide bands were associated with length ($R^2 = 0.85$; Table 1). Four of the protein/peptide bands were commonly associated with both BM and BL (Table 1). The estimated regression parameters for each protein/peptide band, as identified in the stepwise linear regression, are summarized in Table 1. A negative regression parameter indicates that the presence of that band is associated with a smaller or shorter fish, and conversely, a positive regression parameter indicates that band is associated with a heavier or longer fish. Also in Table 1, Gaussian MLE refers to standard Gaussian maximum likelihood estimates of parameters and their associated standard errors, whereas "bootstrap analysis" lists the bootstrap estimates and their 95% lower confidence bounds (95% LCB) and higher confidence bounds (95% HCB).

All selected bands are displayed on the electropherogram (Fig. 2). The electropherogram is from cathode (left) to anode (right). The relative pixel density is presented on the *Y*-axis and the molecular weights (kDa) are on the *X*-axis. The nine-protein/peptide bands associated with length, solid circles (●), were band numbers 2, 16, 20, 24, 26, 36, 38, 40, and 44. The eight protein/peptide bands associated with BM, solid squares (■) were band numbers 17, 25, 26, 36, 38, 39, 44, and 46. The four-protein/peptide bands associated with mass and length, solid square, and circle (● and ■) were bands 26, 36, 38, and 44.

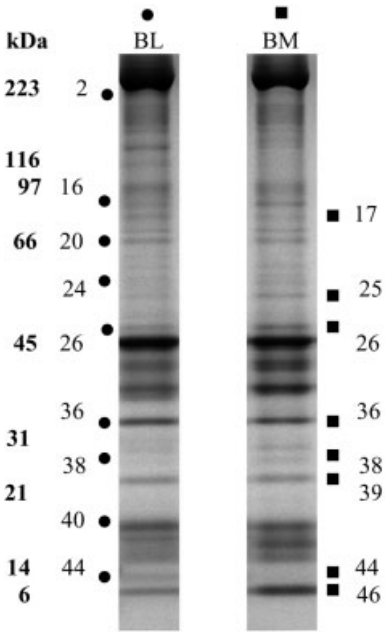

Figure 1. Representative lanes from a 5 to 20% SDS-PAGE of the whole muscle of yellow perch showing the region of the gels in which bands were associated with yellow perch BL and BM of yellow perch. The molecular weight standards are presented at the left of the figure. Symbols represent the eight-protein/peptide bands (■) significantly associated with BM; the nine-protein/peptide bands significantly associated with BL (●) and the four-protein/peptides were associated with both BM and BL (● and ■).

Table 1 shows the results of bootstrap analysis documenting the validation of the proteomic model used to identify bands associated with muscle growth in yellow perch. Analysis of the selected bands by nano-LC/MS/MS and protein identification of the peptide fragments using MASCOT revealed 26 proteins that were determined to be associated with changes in BM and/or BL. The proteins identified from each band are tabulated in Tables 2–5.

4 Discussion

The objective of this study was to identify the muscle derived protein/peptides in electrophoretically separated bands that are both positively and negatively associated with fish muscle growth in commercially produced yellow perch. Using a combination of 1-D gradient gel electrophoresis, imaging technology, multiple linear regression and bootstrap analysis, and protein sequencing demonstrates the feasibility of this method as a first step toward the identification of proteins/peptides associated with the cellular mechanisms of

Table 1. Linear regression coefficients and goodness-of-fit statistics for the analysis of 48 protein bands for the prediction of BM (g) and BL (cm) of yellow perch between 10 and 26 wk of age, using whole muscle proteins resolved on 5–20% gradient SDS-PAGE

Band no.	Molecular weight (kDa)	Dependent variable (estimate ± SE)		Bootstrap analysis					
		BM (g)	BL (cm)	BM (g)			BL (cm)		
				Mean	95% LCB	95% HCB	Mean	95% LCB	95% HCB
2	197.2		−0.26 ± 0.10				−0.25	−0.46	−0.05
16	87.9		0.78 ± 0.23				0.74	0.30	1.22
17	83.5	0.32 ± 0.16		0.37	0.06	0.70			
20	73.4		−0.08 ± 0.04				−0.13	−0.21	−0.05
24	56.4		0.79 ± 0.22				0.78	0.34	1.22
25	53.8	0.69 ± 0.23		0.66	0.22	1.14			
26[a)]	49.8	1.55 ± 0.27	0.62 ± 0.07	1.50	0.98	2.06	0.61	0.47	0.75
36[a)]	28.7	2.13 ± 0.79	1.26 ± 0.27	2.12	0.57	3.77	1.30	0.76	1.84
38[a)]	25.8	−9.78 ± 2.83	−3.02 ± 1.05	−9.78	−15.63	−4.38	−3.07	−5.22	1.04
39	23.3	76.92 ± 17.96		76.95	42.41	114.04			
40	22.5		2.96 ± 1.47				2.93	0.04	5.97
44[a)]	16	0.87 ± 0.25	0.42 ± 0.09	0.89	0.41	1.40	0.44	0.27	0.62
46	10.8	4.33 ± 1.40		4.29	1.59	7.19			
	Intercept	−1.77 ± 0.84	4.48 ± 0.22						
	Standard error	3.95	1.12						
	R-square	0.84	0.85						
	Adjusted *R*-square	0.81	0.83						

LCB = lower confidence bounds.
HCB = higher confidence bounds.
a) Designates bands significantly associated with both BM and BL.

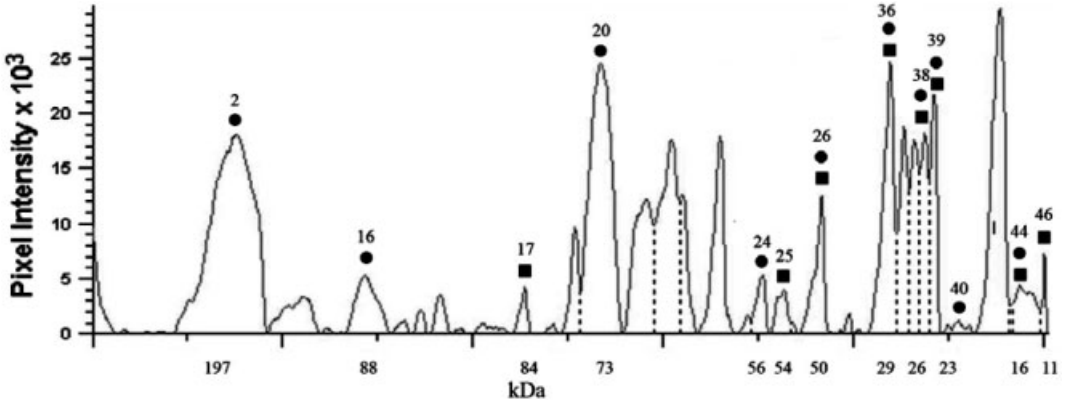

Figure 2. Electropherogram showing eight-protein/peptide bands (■) significantly associated with BM and nine-protein/peptide bands significantly associated with length (●). Four-protein/peptides were associated with both BM and BL (● and ■).

muscle growth in fish. This combination approach generates a reproducible and high throughput method capable of analyzing a large number of samples simultaneously.

Current 2-DE technologies can resolve many more protein/peptide spots than the number of protein/peptide containing bands of current 1-D technologies; however, it is generally accepted that 2-DE suffers from potential difficulties in reproducibility. In addition, the number of 2-DE gels required to analyze the 70 samples in the current study would prevent them from being easily used in a high throughput study. It would be difficult to reproduce 70 comparable 2-DE gels for the subsequent image, statistical, and primary sequence analyses. The use of muscle tissue from multiple animals of a single species exhibiting different muscle growth characteristics allows the use of the multiple linear regression model, which generates statistical power much greater than that of ordinary proteomic analysis, which generally involve fewer sample numbers.

Table 2. Proteins from 5 to 20% gradient SDS-PAGE and multiple linear regression analysis that are predicted to be positively associated with BM (g) in yellow perch between 10 and 26 wk of age

Band no.	M_r[a)]	Identification	Mowse score[b)]	% Cov.	Peptide matches	Mass[c)]	Species	Acc. no. (gi)
17	83.5	Alpha actin	595	39	11	41 922	Fish	47217173
		Myosin heavy chain	588	6	10	222 040	Fish	9971579
		Adenosine monophosphate deaminase	221	7	5	66 375	Fish	57547482
		Muscle-type creatine kinase CKM2	164	7	4	42 980	Fish	21694043
		Aldolase A	138	5	2	40 214	Fish	22671688
		Creatine kinase M1-CK	135	6	3	42 980	Fish	4027925
25	53.8	Phosphoglucose isomerase-2	747	26	11	62 165	Fish	20067651
		Skeletal α-actin type-2b	504	29	10	42 193	Fish	30268605
		Pyruvate kinase	412	16	7	58 572	Fish	74096033
		Desmin	243	9	5	51 582	Fish	33186832
		Phosphoglucose isomerase-1	208	6	3	62 102	Fish	20067653
		Nuclease diphosphate kinase B	174	34	4	17 214	Fish	10121713
		Myhc 4 protein	159	2	2	125 167	Fish	28278650
		Creatine kinase M1-CK	104	4	2	42 980	Fish	4027925
		Aldolase A	73	3	1	40 214	Fish	22671688
		Dehydrogenase, glyceraldehyde phosphate	73	4	1	35 979	Lobster	229256
26	49.8	Skeletal muscle actin	676	45	13	42 276	Fish	32250994
		ATP synthase β chain	499	26	9	55 327	Fish	47605558
		Enolase 1, (alpha)	401	15	5	47 386	Fish	37590349
		Creatine kinase M1-CK	100	4	2	42 980	Fish	4027925
		Aldolase A	74	3	1	40 214	Fish	22671688
		Parvalbumin β	71	10	1	11 673	Xenopus	131120
36	28.7	Nuclease diphosphate kinase B	4376	47	6	17 214	Fish	10121713
		Myosin light chain 2, isoform B	278	28	5	19 213	Fish	21322382
		Parvalbumin beta	66	10	1	11 673	Xenopus	131120
39	23.3	Myosin light chain 2, isoform B	302	35	6	19 213	Fish	21322382
		Adenylate kinase	239	22	5	21 401	Fish	125160
		Fast skeletal myosin light chain 3	114	30	3	16 794	Fish	1339869
		Nuclease diphosphate kinase B	56	11	1	17 214	Fish	10121713
44	16	Adenylate kinase	405	35	8	21 401	Fish	125160
		Myosin light chain 1	358	32	7	21 880	Fish	7678752
		Ribosomal protein L18	185	18	3	21 632	Fish	7595809
		Fast skeletal myosin light chain 3	145	43	7	16 976	Fish	5852836
		Myosin light chain 2, isoform B	105	25	5	19 213	Fish	21322382
		Nuclease diphosphate kinase B	97	28	3	17 214	Fish	10121713
		Glyceraldehyde 3-phosphate dehydrogenase	68	9	2	36 241	Fish	25989185
46	10.8	Chain D, met-perch hemoglobin	283	33	4	16 171	Fish	56967212
		β-Globin	95	7	1	16 215	Fish	1431593
		Muscle actin	80	6	2	42 325	Sea squirt	10111

a) Mass was predicted from gel using TotalLab™ software, Nonlinear Dynamics.
b) Score calculated using MASCOT.
c) Mass prediction from protein identification in NCBI BLAST protein database.

The use of a bootstrap analysis allowed us to validate the models and their associated parameter estimates [22]. Prior to the discovery of the bootstrap as a method of validating statistical estimates [25], it would have been customary to divide the data in half and use one-half to develop the models and the other half to validate and compare them. The problem with this approach was in how one should select the two halves. That is, the results were largely dependent on the selection of the two halves. In addition, valuable information was not used for model construction, because half of the data were ignored during this step. The specific bootstrap used in our study was the bias-corrected and accelerated bootstrap [23]. In short, a bootstrap sample of 70 observations selected with replacement at random from the original set of 70 observations was first constructed. Parameter estimates of the regression coefficients were then obtained using least squares. This process was iterated 1000 times and the bootstrap of the parameters β_j was calculated as the simple means of the 1000 repetitions reported in Table 1.

Table 3. Proteins from 5–20% gradient SDS-PAGE and multiple linear regression analysis that are predicted to be negatively associated with BM (g) in yellow perch between 10 and 26 wk of age

Band no.	M_r[a)]	Identification	Mowse score[b)]	% Cov.	Peptide matches	Mass[c)]	Species	Acc. no. (gi)
38	25.8	Glyceraldehyde 3-phosphate dehydrogenase	878	26	5	36 241	Fish	25989185
		GAPDH protein	490	19	4	38 546	Fish	63101249
		Adenylate kinase	339	26	6	21 401	Fish	125160
		Tropomyosin	227	33	10	32 767	Fish	12082279
		Myosin light chain 2, isoform A	82	22	3	19 235	Fish	21322384
		Myosin light chain 2, isoform B	79	43	6	19 213	Fish	21322382
		Nuclease diphosphate kinase B	77	34	4	17 214	Fish	10121713
		Fast skeletal myosin light chain 3	67	11	2	16 794	Fish	1339869
		Fast skeletal myosin light chain 1a	66	15	3	21 172	Fish	1339865

a) Mass was predicted from gel using TotalLab™ software, Nonlinear Dynamics.
b) Score calculated using MASCOT.
c) Mass prediction from protein identification in NCBI BLAST protein database.

Table 4. Proteins from 5 to 20% gradient SDS-PAGE and multiple linear regression analysis that are predicted to be positively associated with BL (cm) in yellow perch between 10 and 26 wk of age

Band no.	M_r[a)]	Identification	Mowse score[b)]	% Cov.	Peptide matches	Mass[c)]	Species	Acc. no. (gi)
16	87.9	Actin, alpha 1, skeletal muscle	798	48	12	42 288	Fish	18858249
		Myosin heavy chain	791	8	14	222 040	Fish	9971579
		Sarcoplasmic/ER calcium ATPase 1A	433	9	7	110 193	Fish	1546051
		Muscle-type creatine kinase CKM2	276	16	6	42 980	Fish	21694043
		Creatine kinase M1-CK	249	14	5	42 980	Fish	4027925
		AMP deaminase 3	91	2	2	89 122	Mouse	2494043
24	56.4	Pyruvate kinase	478	26	15	58 572	Fish	74096033
		Myosin heavy chain	131	3	7	222 040	Fish	9971579
		Sarcoplasmic/ER calcium ATPase 1A	104	3	3	110 193	Fish	1546051
		Actinin alpha 3	96	3	4	104 072	Fish	32766313
		Nuclease diphosphate kinase B	62	20	2	17 214	Fish	10121713
26	49.8	Skeletal muscle actin	676	45	13	42 276	Fish	32250994
		ATP synthase beta chain, mitochondrial precursor	499	26	9	55 327	Fish	47605558
		Enolase 1, (alpha)	401	15	5	47 386	Fish	37590349
		Creatine kinase M1-CK	100	4	2	42 980	Fish	4027925
		Aldolase A	74	3	1	40 214	Fish	22671688
		Parvalbumin beta	71	10	1	11 673	Frog	131120
36	28.7	Nuclease diphosphate kinase B	4376	47	6	17 214	Fish	10121713
		Myosin light chain 2, isoform B	278	28	5	19 213	Fish	21322382
		Parvalbumin beta	66	10	1	11 673	Frog	131120
40	22.5	Fast skeletal myosin light chain 3	546	52	9	16 794	Fish	1339869
		Myosin light chain 2, isoform B	292	45	8	19 213	Fish	21322382
		Myosin light chain 1	265	32	8	21 343	Fish	16117365
		Glyceraldehyde 3-phosphate dehydrogenase	148	26	5	36 241	Fish	25989185
		Nuclease diphosphate kinase B	99	34	5	17 214	Fish	10121713
		GAPDH protein	84	19	4	38 546	–	63101249
44	16	Adenylate kinase	405	35	8	21 401	Fish	125160
		Myosin light chain 1	358	32	7	21 880	Fish	7678752
		Ribosomal protein L18	185	18	3	21 632	Fish	7595809
		Fast skeletal myosin light chain 3	145	43	7	16 976	Fish	5852836
		Myosin light chain 2, isoform B	105	25	5	19 213	Fish	21322382
		Nuclease diphosphate kinase B	97	28	3	17 214	Fish	10121713
		Glyceraldehyde 3-phosphate dehydrogenase	68	9	2	36 241	Fish	25989185

a) Mass was predicted from gel using TotalLab™ software, Nonlinear Dynamics.
b) Score calculated using MASCOT.
c) Mass prediction from protein identification in NCBI BLAST protein database.

Table 5. Proteins from 5 to 20% gradient SDS-PAGE and multiple linear regression analysis that are predicted to be negatively associated with BL (cm) in yellow perch between 10 and 26 wk of age

Band no.	M_r[a)]	Identification	Mowse score[b)]	% Cov.	Peptide matches	Mass[c)]	Species	Acc. no. (gi)
2	197.2	Myosin heavy chain	1548	16	25	222 040	Fish	9971579
20	73.4	Myosin heavy chain	706	6	12	222 040	Fish	9971579
		Actin 2	289	19	7	42 221	Prawn	3907622
		Nuclease diphosphate kinase B	65	16	2	17 214	Fish	10121713
		Novel protein similar to human calsequestrin 1 (CASQ1)	61	1	1	66 819	Fish	33284873
		Fast skeletal myosin light chain 1a	59	4	1	21 172	Fish	1339865
38	25.8	Glyceraldehyde 3-phosphate dehydrogenase	878	26	5	36 241	Fish	25989185
		GAPDH protein	490	19	4	38 546	Fish	63101249
		Adenylate kinase	339	26	6	21 401	Fish	125160
		Tropomyosin	227	33	10	32 767	Fish	12082279
		Myosin light chain 2, isoform A	82	22	3	19 235	Fish	21322384
		Myosin light chain 2, isoform B	79	43	6	19 213	Fish	21322382
		Nuclease diphosphate kinase B	77	34	4	17 214	Fish	10121713
		Fast skeletal myosin light chain 3	67	11	2	16 794	Fish	1339869
		Fast skeletal myosin light chain 1a	66	15	3	21 172	Fish	1339865

a) Mass was predicted from gel using TotalLab™ software, Nonlinear Dynamics.
b) Score calculated using MASCOT.
c) Mass prediction from protein identification in NCBI BLAST protein database.

Use of multiple regression analysis, which is the statistical component of this method, allows for the identification of multiple proteins associated with differential growth, not just the "all or none" results that are normally reported by classical proteomic techniques such as 2-DE or DIGE. Use of this statistical analysis also adds to the power of the overall proteomic methodology. Other statistical methods, such as principal component analysis, circumvent possible colinearity among the predictor variables (bands) by creating new variables that are orthogonal linear functions of all of the bands in an experiment. A principal component analysis also lacks biological meaning and deletions of bands from the principal component analysis are completely arbitrary. Stepwise regression could suffer from colinearity (*i.e.*, high correlation) among predictor variables, but this was not the case in our application judging from the relatively small standard errors of the parameter estimates.

The method described herein has many unique aspects that make it a powerful tool for proteomic analysis. Current use of 2-DE and other proteomic methods, such as multidimensional protein identification technology (MudPIT), are very complex and have both high technical difficulty and cost; they are also associated with highly time-consuming data analysis [26]. In addition, many experiments using 2-DE use a much lower number of analytical and/or biological replicates than those reported in this study [26]. Our methodology uses whole muscle of yellow perch without prior fractionation of the muscle tissue and allows for numerous replicates of biological samples in a short period of time and with less expense. Compared to 2-DE, the 1-D gradient gel electrophoresis method used in our analysis is more repeatable and allows for high throughput of samples. The resolution of proteins using 1-D gradient gel electrophoresis and 2-DE technology both suffer from comigrating proteins in the bands and spots [27]. However, with the use of nano-LC-MS/MS, we are able to accurately sequence the tryptic peptide fragments, and therefore, identify individual proteins in the bands. While we are actively working on a method to quantify individual protein changes within a band across multiple individuals, the methodology reported here has identified the bands containing muscle proteins/peptides that can be further characterized by other biochemical methodologies to determine which of the specific proteins/peptides are responsible for growth of fish muscle. A specific method of determining the differential protein expression from individual proteins within a single 1-D band is currently under investigation by our laboratory. The results herein are an initial step toward defining the cellular and molecular regulation of myogenic growth and development in yellow perch by protein expression profiling.

Variability in muscle growth in fish is common and understanding the genotypic and phenotypic causes of variation of muscle growth is a main interest of the aquaculture industry [28]. Muscle growth can be influenced by multiple factors such as fish strain or species [29], exercise [30], and nutrition [31]. A problem with understanding causes of variation is the lack of information available about the gene products and/or proteolytic fragments of muscle specific gene products associated with the mechanisms of muscle growth and development in aquaculture species reared under commercial conditions [28]. The results of this study have contributed to this understanding by identifying struc-

tural, contractile, and metabolic proteins that may be associated with differences in muscle growth in yellow perch.

To our knowledge there is no reported method to determine the concentration(s) of the individual proteins/peptides within each of the bands. Our ongoing research is attempting to develop a method to quantify the individual protein/peptides within bands. Although their individual contributions to the mechanisms of muscle growth are not known, the glycolytic enzymes, aldolase, enolase, creatine kinase, and pyruvate kinase were found in the bands associated with differences in BM and BL in yellow perch. The identification of aldolase is consistent with findings of Merritt and Quattro [32], who identified different isozymes of aldolase in zebrafish muscle *via* creation of tissue specific cDNA and primers. Enolase has been described previously by Morzel *et al.* [33] in trout by 2-DE and MALDI-MS. These investigators showed differences in the expression of this protein in exercised and rested trout. Bosworth *et al.* [34] previously identified differences in the expression levels of enolase and creatine kinase using analytical and preparative 2-DE in zebrafish muscle. Creatine kinase has also been characterized in other species such as carp by cloning [35] and tilapia using RT-PCR [36]. Pyruvate kinase has also been identified, cloned, and expression levels documented in globefish, *Takifugu rubripes*, skeletal muscle [37].

A proteolytic fragment of the myofibrillar protein, skeletal myosin heavy chain, as well as myosin light chains, actin, and α-actinin were also present in bands associated with muscle growth. The identification of myosin light chains relating to muscle growth are consistent with results of multiple investigators [38–41]. The differential expression of the skeletal muscle myosin heavy chain isoforms in fish species under different environmental conditions affecting muscle growth have been previously reported in carp [39] and puffer fish [40]; however, the role of proteolytic fragments of these proteins in the mechanisms of muscle growth remains unresolved. Similar studies in carp have focused on the skeletal muscle myosin light chains isoforms. It is important to note that, in carp, light chain 1 and light chain 3 proteins are encoded by two different genes and this is different than the method of expression in higher vertebrates where they are encoded by a single gene and post translational regulation by alternative RNA splicing [38, 41]. Moutou *et al.* [42] reported that thyroid hormones, which are generally accepted to have an affect on muscle growth, regulated the expression of myosin light chain isoforms 2 and 3 in sea bream, *Sparus aurata*. Other researchers have reported that myosin light chain expression is affected by environmental factors such as temperature in carp [38, 41], and gene manipulation in both salmon [43] and zebrafish [44]. These observations are important, because the specific gene regulation events associated with myosin light chain expression may be related to the unique combination of hyperplasia and hypertrophy by which fish muscle grows throughout their life. This is in contrast to mammals, in which muscle hyperplasia is restricted largely to embryonic and postnatal periods [45].

Our research used a reproducible, high throughput methodology to identify individual electrophoretically separated protein/peptide bands from whole muscle of yellow perch that are associated with BM and BL. Identification of these bands associated with rapid growth in yellow perch is a first step in developing breeding strategies to select for rapid muscle growth while selecting against deleterious gene products in the brood stock, and thus, avoiding the negative economic impacts previously observed with selection for increased muscle growth in swine and poultry. By combining proteomics methodology and genetic selection based on knowledge of the genes associated with positive traits, while limiting those genes associated with low quality and slow growth in fish muscle, it is possible that breeders will optimize the economic value of yellow perch, while maintaining the gene products and/or proteolytic fragments of muscle specific gene products positively associated with the phenotype of rapid muscle growth. This research is a first step toward identifying these proteins that are involved in the cellular and molecular regulation of muscle growth and development in yellow perch. This work will lead into further categorization of myogenic events in yellow perch through future growth factor studies and the changes in protein expression related to accelerated growth. We are actively developing methodologies to identify specific events that affect myogenesis and provide a more detailed model of muscle growth in yellow perch. Ultimately, genetic brood stock selection based on an understanding of well-defined gene expression patterns will allow for more focused selection stratagems that optimize fish growth and meat quality. If producers are able to make breeding decisions based upon a thorough understanding of the mechanisms of myogenesis, then it could be expected that larger, faster growing fish will be produced. It is anticipated that these improvements would lead to a significant increase in overall yields and increases in fillet dress-out percentages, making yellow perch aquaculture a more feasible and economical enterprise. Our future research goals are to develop a methodology to evaluate individual proteins in 1-D electrophoretic bands and determine changes in these proteins in order to evaluate whole muscle proteomic fingerprints.

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