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Non-lethal determination of sex and reproductive condition of Eastern oysters *Crassostrea virginica* Gmelin using protein profiles of hemolymph by Proteinchip® and SELDI-TOF-MS technology

Yaqin Li [a,*], Ghazala Siddiqui [b], Gary H. Wikfors [a]

[a] NOAA National Marine Fisheries Service, Northeast Fisheries Science Center, 212 Rogers Avenue, Milford, CT 06460, USA
[b] Centre of Excellence in Marine Biology, University of Karachi, Karachi-75270, Pakistan

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ABSTRACT

As the demand for hatchery-produced oyster seed increases for both aquaculture and restoration purposes, techniques to improve seed production, such as accurate determination of broodstock oyster sex and gonad development, have become more important. In this study, we developed a novel method of determining oyster sex and developmental stage through protein profiling of hemolymph using a relatively new proteomic tool, Proteinchip® and SELDI-TOF-MS. Over 139 peptides/proteins were detected from oyster hemolymph, 62 of which appeared to be involved in reproductive activities. Using the protein-profile information, individual broodstock oysters were categorized successfully into one of five groups: undifferentiated, female developing, female ripe, male developing and male ripe. The accuracy of categorization, confirmed by subsequent, traditional histological methods, was 98.8% ($p<0.05$). Wide application of this method is still limited by cost; however, results of this research open doors for further study to develop more-affordable and portable methods based upon detection of specific hemolymph peptides and proteins.

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1. Introduction

The Eastern oyster *Crassostrea virginica* Gmelin is an important commercial species in aquaculture on the eastern and Gulf of Mexico coasts of the US (Carlton and Mann, 1996). Hatchery-produced seed oysters are grown out in the natural environment to commercial size with a certain degree of care. Seed oysters are also used in oyster restoration projects (e.g., Carlsson et al., 2008). In standard hatchery protocols, broodstock oysters are conditioned, and spawning usually is triggered by thermal stimulation (Loosanoff and Davis, 1963). To be induced to spawn, oysters must be ripe, with gonads in mature developmental stages. The readiness of broodstock oysters for spawning cannot be assessed accurately by visual inspection. The conventional method of determining a population's sex ratio and developmental stage involves sacrificing individuals and making gross-anatomical observations of gonads, or more precisely, dissecting, sectioning, and staining the reproductive organs for microscopic examination (Lango-Reynoso et al., 2000; Arcos et al., 2009). This method provides comprehensive information on gonad development but is very tedious for a hatchery, requires sacrificing some broodstock oysters, and does not provide sex and developmental-stage information on oysters that are not sacrificed but kept for spawning. Alternately, muscle relaxants such as magnesium chloride can be used to induce gaping of shells, which may allow visual inspection of gonad maturation (Norton et al., 1996; Butt et al., 2008; Suquet et al., 2009). Only mature oysters can be recognized according to sex. Further, magnesium chloride may have harmful effects upon oysters in short term treatments (Butt et al., 2008), and in Pacific oysters, secondary toxic effects may exist (Suquet et al., 2009). Arcos et al. (2009) reported that vitellin/vitellogenin-like proteins changed with gonad development in female oysters, *Crassostrea corteziensis,* and suggested that the levels of these proteins may be indicators of female reproductive activities. This method also involves sacrificing analyzed oysters, and no male reproductive information is yielded.

A recently-developed, non-invasive magnetic resonance imaging (MRI) technique is able to differentiate sexes of Pacific oysters through the shells without any physical damage to the broodstock oysters (Davenel et al., 2006; Pouvreau et al., 2006). Two disadvantages remain: 1) it is difficult to differentiate the developmental stages because the technique is able only to discriminate between sexes when the gonad is fully developed. 2), on a practical note, the instrument may be too expensive for most hatcheries (Davenel et al., 2006). A less-expensive method using low nuclear resonance relaxometry (NMR) was able to differentiate sex of *Crassostrea gigas* (Davenel et al., 2009). As with the MRI method, however, the NMR method appeared to be able to differentiate sex only when the gonad was fully developed.

* Corresponding author. Tel.: +1 203 882 6561; fax: +1 203 882 6570.
E-mail address: judy.yaqin.li@noaa.gov (Y. Li).

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Proteins in hemolymph are implicated in bivalve immune functions (Li et al., 2009). Association between lower protein levels and infestation with *Haplosporidium nelsoni* or *Perkinsus marinus* was noted in Eastern oysters (Barber et al., 1988; Chu and La Peyre, 1989; Chu et al., 1993). Change in protein level was also noted in another mollusc, *Milanese obsolete*, when parasitized by larval trematodes (Cheng et al., 1983). Enzymes present in hemolymph have been implicated in pathogen degradation (Cajaraville et al., 1995). Furthermore, seasonal variation in total dissolved protein levels in hemolymph serum has been linked to reproductive cycles in a number of bivalves, including the Eastern oyster *C. virginica* (Fisher and Newell, 1986), the blue mussel *Mytilus edulis* (Mulvey and Feng, 1981), the giant scallop *Placopecten magellanicus* (Thompson, 1977), and the Pacific oyster *C. gigas* (Luna-González et al., 2008; Li et al., 2009). Seasonal, low levels of proteins correspond with the high temperatures of summer (Luna-González et al. 2008) and spawning of oysters (Mulvey and Feng, 1981; Fisher and Newell, 1986; Li et al., 2009). Low values of hemolymph protein content in *C. gigas* in the summer–fall also correspond with high mortality rates (Luna-González et al., 2008).

Little is known about the association between the protein compositions and profiles in hemolymph and the reproductive cycle, although the total amount of protein in hemolymph appeared to vary during gonad development in oysters. A female-specific, vitellin-like protein was detected in the hemolymph of the Pacific oyster *C. gigas* using immunological procedures (Suzuki et al., 1992). Most recently, Arcos et al. (2009) reported the presence of vitellin (Vn) in female hemolymph, detected by antiserum, in the oyster *C. corteziensis*. Vn is a major yolk protein stored in yolk granules of oocytes (eggs). It is a macromolecular protein associated with lipids and carbohydrates and used as a nutrient during embryogenesis. In bivalves, it is not clear whether the synthesis of major yolk protein occurs through an autosynthetic or heterosynthetic pathway, although it appeared more likely that proteins are synthesized within the ovary. Suzuki et al. (1992) suggested that the presence of a vitellin-like protein in hemolymph may originate from degenerated oocytes. Whether or not the presence of this protein and the relative amount in hemolymph relates to gonad development remains unclear.

A relatively new method for protein analysis, ProteinChip® and SELDI-TOF-MS, is a technology that combines chromatography and mass spectrometry. The ProteinChip® array technology (chromatographic chip surface) is utilized to purify and separate proteins on the chip from complex mixtures with high resolution (Weinberger et al., 2001). Mass spectrometry is used to detect proteins that are selectively adsorbed to chip surfaces after impurities are washed away. Differences in the position and amplitude of spectral peaks are used to obtain a "fingerprint" or "signature" of specific proteins present in the sample. Compared to traditional mass spectrometry and 2-D electrophoresis, SELDI-TOF-MS is more sensitive and able to provide information on multiple proteins simultaneously. The advantages of this technology also include high-throughput screening, versatility, ease of use, and speed with comparative low cost (Gomiero et al., 2006).

SELDI-TOF-MS has been used for biomarker discovery in the biomedical field (Wright et al., 1999; Zhang et al., 2002; Mei et al., 2006). The potential application of SELDI-TOF-MS, as well as other proteomic technologies, in aquatic and environmental sciences has been recognized only recently (Lopez, 2007; Nunn and Timperman, 2007). Most aquatic applications reported thus far have involved different profiles of proteins from organisms exposed to different environmental pollutants or stressors, with the objective of finding potential biomarkers for specific environmental conditions (Knigge et al., 2004; Gomiero et al., 2006; Provan et al., 2006). ProteinChip® and SELDI-TOF-MS technology was specifically selected for this study because: 1) it is able to detect multiple peptide/protein signals in samples such as hemolymph known to contain a large number of such materials, 2) it is highly sensitive.

In this study, profiles of proteins in hemolymph during oyster reproductive development were obtained using Proteinchip® and SELDI-TOF-MS technology. We discuss a potentially novel way of differentiating oyster sexes and developmental stages with this protein profiling information.

2. Materials and methods

2.1. Broodstock oyster conditioning

Broodstock oysters between 1 and 2 years of age were obtained from the Frank M. Flower & Sons oyster hatchery in Bayville, New York, USA. Oysters were conditioned at 20–24 °C in 3 well-aerated, 350-L tanks for 8 weeks. Tanks were cleaned and filled with 0.1-μm filtered sea water on alternate days. The 3 different tanks, each containing 100 oysters, were fed separately with cultured microalgae: 100% *Tetraselms chui*, 100% *Chaetoceros neogracile*, or a 50:50 mixture of *T. chui* and *C. neogracile*. The algal strains were obtained from Milford Laboratory Microalgal Culture Collection and grown in 500-l tanks with artificial illumination. The oysters were gravity-fed with 6% of dry tissue weight of oyster in dry weight of algae per day. Ten specimens from each tank were sacrificed at an interval of 15 days from each tank to collect hemolymph samples and subsequently subjected to histological examination to determine sex and gonadal developmental stage. No significant differences in total protein concentration or protein profile in the hemolymph were found between feeding treatments; thus, oysters from the 3 tanks were pooled for protein analyses.

2.2. Hemolymph collection

About 200 μl of hemolymph was drawn from each individual oyster. For extraction of hemolymph, a notch was made in the shell ventral edge. Hemolymph was withdrawn from the adductor muscle using a needle and 1-ml syringe. After hemolymph was drawn, oysters were dissected and examined histologically to determine sex and gonadal developmental stage of each individual. Immediately after hemolymph was withdrawn, hemocytes and other particulates were removed from hemolymph by centrifugation and filtering. Centrifugation was done at 100×*g* (1100 rpm) at 4 °C for 10 min. Supernatants were then transferred to 0.2-μl nylon micro centrifuge filters (Spin-X HPLC, Corning Incorporated) and centrifuged at 100×*g* for an additional 10 min. Filtered hemolymph was stored at −80 °C until analyses (for total protein concentration and SELDI protein profiling).

2.3. Histological determination of oyster sex and developmental stage

The soft tissue of each oyster was fixed in Davidson's Fluid (Shaw and Battle, 1957). Later, a 5-mm piece of oyster tissue was cut with a razor blade along a line extending from the lower corner of the labial palps across the stomach to the posterior end of the body. The tissue was dehydrated, wax embedded, sectioned at 5 μm, and stained using a hematoxylin–eosin staining procedure (Howard et al., 2004).

The sexes were determined on the basis of histological examination of gonads, and the following gonadal developmental stages were defined:

a. Undifferentiated (UD, Fig. 1a)

The few follicles present were contracted or empty, interfollicular connective tissue abundant. Few primordial germ cells presented. Sex undifferentiated.

b. Developing (Fig. 1b and d)

In the early developing stage, follicles were few in number and small in size, with abundant interfollicular connective tissue. Size and number of follicles gradually increased with the development of gonads,

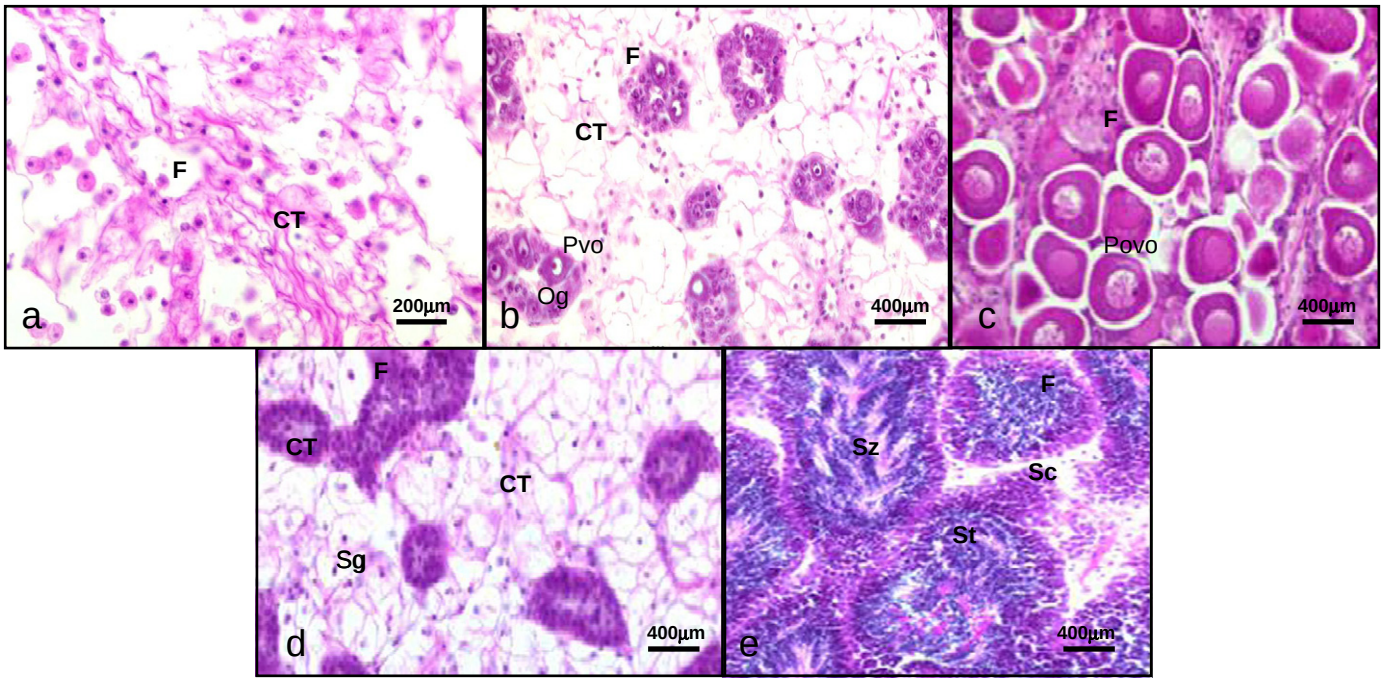

Fig. 1. Sections of gonad tissues showing different sex and gonadal developmental stages. (a) Undifferentiated. (b) Developing female. (c) Ripe female. (d) Developing male. (e) Ripe male. Abbreviations: CT = connective Tissue; F = follicle; Pvo = pre-vitellogenic oocytes; Vo = vitellogenic oocyte; Povo = post vitellogenic oocyte; Sg = spermatogonia; Sc = spermatocytes; St = spermatids; Sz = spermatozoa.

occupying more space, eventually spreading over the digestive diverticula. Germ cells attached close to the walls of the follicles. Male developing stage (MD) was characterized by the presence of mostly speremato gonia and spermatocytes with few spermatids. For female developing stage (FD), mostly oogonia and pre-vitellogenic oocytes were present during early development and later on, with the development of the gonad, a few vitellogenic oocytes were also visible. The diameter for the oocytes was 20.47 ± 4.81 μm for the FD stage.

c. Ripe (Fig. 1c and e)

Follicles completely filled the gonadal area and were highly distended, fully spread over the sides of digestive diverticulae. Connective tissue was nearly absent. For male ripe stage (MR): lumena of the follicles filled with spermatozoa; few spermatocytes presented at the periphery of the follicles. For female ripe stage (FR): mostly vitellogenic and post-vitellogenic and very few pre-vitellogenic oocytes presented. Oocytes were polygonal in shape in the nearly-ripe condition and later on became rounded with the advancement of ripeness. The diameter of oocytes was 49.13 ± 5.57 μm for FR stage.

2.4. Total protein determination

Total protein concentration in hemolymph of each individual oyster was determined following a modified Biuret method using a total protein kit from Sigma (Sigma-Aldrich, Missouri, USA, product codes TP0200 and B 3934). Hemolymph was diluted 5 and 20 times prior to the measurements to ensure that the concentrations of protein in diluted samples were in the linear range of the absorption *vs.* concentration calibration curve. Bovine serum albumin was used as the standard.

2.5. SELDI protein profiling

2.5.1. Proteinchip® preparation and reading

IMAC-30 chips (ProteinChip® IMAC-30, Biorad Laboratories, formerly Ciphergen Biosystems, Fremont, CA, USA) were used for the analysis. IMAC-30 (Immobilized-Metal Affinity Chromatography) chips bind a sub-group of copper-binding proteins. Hemolymph samples were thawed on ice and vortexed briefly to mix. Samples were then diluted 10× by adding 50 μl of sample to 450 μl of binding buffer, 50 mM Tris–HCL, pH 8. Prior to sample loading, chips were assembled in a bioprocessor (Ciphergen Biosystems) for efficiency in chip preparation. Chips were then loaded twice with 50 mM $CuSO_4$, 100 μl per spot, followed by a brief wash with H_2O, 100 μl per spot. One hundred microliters of binding buffer was then added to each chip spot and discarded. One hundred microliters of diluted hemolymph sample was applied to the chip spot and incubated on a shaker for 1 h, allowing protein binding to the chip surface. Spots were then washed twice with the binding buffer, followed by a brief wash with 1 mM HEPES, pH 7.5, to remove interferences and weakly-bound proteins. Chips were disassembled from the bioprocessor and air-dried for 5 min. A matrix or EAM (Energy Absorbing Molecules) solution, saturated sinapinic acid (SPA; Ciphergen Biosystems), was prepared in 50% (v/v) acetonitrile/0.5% (v/v) trifluoroacetic acid according to the manufacture's instructions. One microliter of matrix solution was applied twice to each spot. Spots were air-dried after each application.

After the ProteinChip® arrays had been processed, they were read on a ProteinChip® Biomarker System Model PBS ΠΙ (use of the trade names does not imply endorsement) to measure the masses and relative intensities of the protein peaks. To cover the wide mass range (2000 to 50,000 Da) of peptides and proteins of interest, chips were read at two laser intensities, low and high. For the low-intensity readings, the settings were: laser intensity 220, detector sensitivity 7, positions 20 to 80 with an increment of 5, resulting in 13 different sampling positions. For the high-intensity readings, the settings were: laser intensity 280, detector sensitivity 9, positions 22 to 82 with an increment of 5, resulting in 13 different sampling positions. For both low and high intensities, 2 warming shots per position were fired prior to data collection to remove salts and other contaminants. Five laser shots (transients) were collected subsequently on each position, and a total of 65 shots for each intensity were used to generate an average spectrum for each spot (sample). To ensure the mass accuracy, instrument calibration was done externally with a standard

mixture of peptides and proteins (All-In-One Peptide and All-In-One Protein Standards, BioRad Laboratories).

2.5.2. Data processing using Ciphergen software

Two separate steps were involved in data processing: pre-processing and data analysis. Pre-processing consisted mainly of data normalization, while data analysis consisted of peak labeling and univariate analysis.

Pre-processing was achieved using ProteinChip® software version 3.1.1. Following baseline fitting and baseline subtraction, the data from all spectra were calibrated initially using external and internal calibration protocols. Using histological results, each oyster was assigned to one of the 5 groups detailed above, FD, FR, MD, MR and UD, and normalized using the total ion current (TIC) normalization method with the external coefficient of 0.2. For the low-laser energy data set (molecular weights 2–50 kDa), the TIC started from 2 kDa to 100% of the spectrum size. For the high-laser energy data set (molecular weight >50 kDa), the TIC started from 10 kDa to 100% of the spectrum size. In all cases, the EAM (Energy Absorbing Molecules) or matrix noise were all outside of the normalization range.

Protein peaks were identified using CiphergenExpress™ software 3.0 (CE 3.0). Protein peaks with a signal-to-noise ratio (S/N) of 3 and minimum valley depth of 1 were autodetected. The minimum peak threshold was 20% of all spectra. Basic statistics were obtained for each peak, e.g., mean, standard deviation, median. Differences in the peak intensities among the 5 oyster groups were analyzed with CE 3.0, which employs the Mann–Whitney test. Levels of significance in the differences between the treatments (p values) were obtained.

2.5.3. Test of data reproducibility — calculation of CV

Data reproducibility was tested with standard human serum samples prepared on a chip and read by a Ciphergen ProteinChip® Reader as described above. After all normalization steps, the peaks with a signal-to-noise of 5 or greater were labeled and selected for CV (coefficient of variance) calculation. The average peak intensity of each selected peak and standard deviation were calculated, and the CV for each peak was then calculated by dividing the Standard deviation (SD) by the mean. The pooled CV (CVp) of all peaks was used to indicate the data reproducibility, and calculated following the equation:

$$\mathrm{CVp} = \sqrt{(\mathrm{CV1} * \mathrm{CV1} + \mathrm{CV2} * \mathrm{CV2} + \cdots + \mathrm{CVk} * \mathrm{CVk})/\mathrm{k}}$$

where CVi is the coefficient of variance for each peak used, and k is the number of peaks. For this experiment, the CVp was 6.6%. Examples of spectra showing data reproducibility in the experiment are presented in Fig. 2.

2.6. Statistical analysis

To evaluate the changes in proteins when oysters underwent reproductive development, individual protein peaks of FD, FR, MD, MR were compared to those of UD oysters. The Kruskal–Wallis test with CiphergenExpress™ software (v.3.0) was applied. Those peaks that were significantly up- or down-regulated from levels of UD oysters were identified (Table 1).

To explore the question of whether or not oysters undergoing male or female reproductive development had distinctive protein profiles, a multivariate, discriminant analysis (DA) was performed to describe the protein profiles of the 5 groups of oysters: FD, FR, MD, MR and UD, using StatGraphics Plus statistical software (originally Manugistics, Inc, Maryland, now marketed by StatPoint, Inc, North Virginia). Only those peaks with significant differences ($p \leq 0.01$) among groups, identified by the CiphergenExpress™ software 3.0 (detailed above), were used as variables in DA. These peaks were selected for the DA because they are likely to be related to oyster reproductive development; whereas, other peaks may be involved in other common, physiological functions within the hemolymph.

Discriminant analysis was also performed to describe the protein profiles for the female, male, and undifferentiated oysters, when female oysters include FD and FR oysters, and the male oysters include MD and MR oysters.

ANOVA and a subsequent multiple range test (Statgraphics Plus) were applied to compare the difference in total protein concentration from 5 developmental stages.

3. Results

3.1. Hemolymph total protein

Total protein in hemolymph varied among the different developmental stages (Fig. 3). The sexually-undifferentiated oysters appeared to have lower protein levels than those at other stages. Significant differences, however, were found only between the undifferentiated and female ripe oysters.

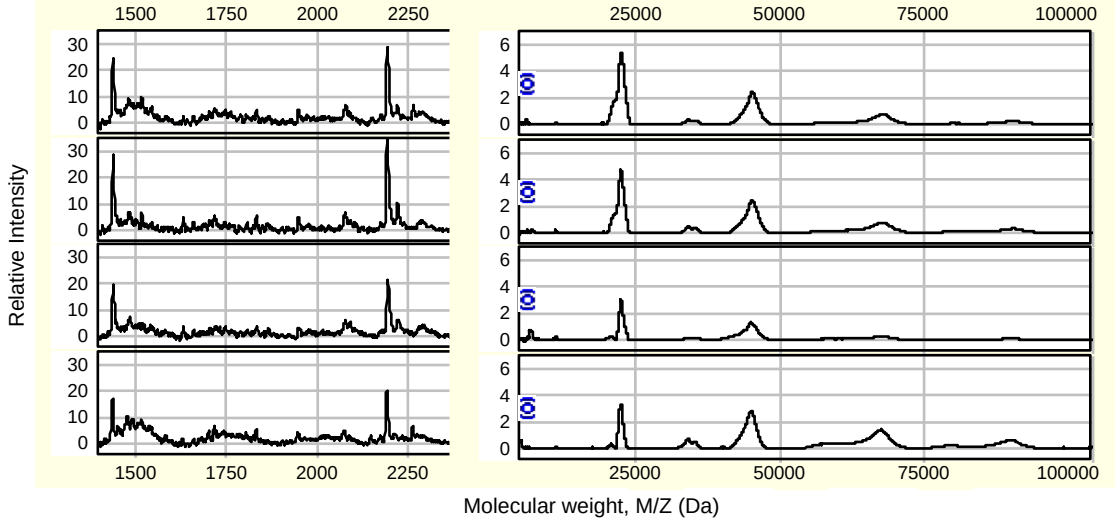

Fig. 2. Segments of protein spectra of individual female oyster hemolymph demonstrating the data reproducibility of Ciphergen Protein Chip® and SELDI-TOF-MS technology.

Table 1
Differentially-expressed peptides and proteins in oyster hemolymph from different sexes and developmental stages.

	Female		Male	
m/z	Developing	Ripe	Developing	Ripe
3253	↓	↓	↓	↓
3328	↓	↓	↓	↓
3373	↓	↓	↓	↑
3412	↑	↑	↑	↑
3488	↑	↑	↑	↑
3832	↓	↓	↑	↑
3911	↓	↓	↑	↑
3942	↓	↓	↑	↑
4028	↓	–	↑	↑
6345	↓	↓	↑	↑
7223	↑	↑	↑	–
18996	↓	↓	↓	↓
19238	↓	↓	↓	↓
19632	↓	↓	↓	↓
20380	↓	↓	↓	↓
21841	↓	↓	↓	↓
22293	↓	↓	↓	↓
42987	↓	↓	↓	↓

↑, ↓, –, indicates up-regulated, down-regulated or no alteration compared to undifferentiated oysters, respectively. Only the highly significantly-different peaks ($p \leq 0.01$) are listed. *m/z* = mass-to-charge ratio.

3.2. Protein profiles

A total of 139 peptide/protein peaks were detected from oyster hemolymph with the mass range of 2503–44,869 Da. Among those, 62 peaks showed statistically significant differences ($p \leq 0.01$) among the 5 groups of different sexual development stage. Thus, these 62 peaks are likely involved in sexual development and reproductive activities, while other 77 are likely carrying out immune and other physiological functions in the hemolymph regardless of sexual development and reproduction.

More proteins were down-regulated than up-regulated when sexually-developing and mature oysters were compared to undifferentiated individuals (Table 1). Nine peaks were down-regulated in all of the 4 groups, but only 2 peaks were up-regulated. Down-regulations were particularly prominent in female oysters wherein two additional peaks were down-regulated only in female oysters but up-regulated in males.

3.3. Protein profiles among the 5 oyster groups — discriminant analysis

The above analysis showed that there were differences in the individual proteins among the 5 groups of oysters in different developmental stages. To determine if protein profiles (when all proteins were considered) differed between the 5 groups, and if the protein profiles can be used to classify oyster groups, we used a discriminant analysis. All of the 62 proteins that showed significant differences among the groups were used as independent variables, and a total of 84 oyster-hemolymph samples were used in the analysis. The results showed a highly-significant difference among the groups (Fig. 4). Discriminant function 1 and function 2 (both statistically significant with $p<0.05$), together explained 82.5% of the model variance. Among the 84 samples used to fit the model, 83 or 98.8% were correctly classified. The proteins weighted most highly in the model were those with *m/z* of 20,664, 21,841, 19,238 and 2699.

As stated in the introduction, one of the advantages of the SELDI-TOF-MS technology is the capability of detecting large number of proteins simultaneously. With the number of proteins detected, we were able to discriminate oyster groups based upon hemolymph protein profiles. To determine if the oyster groups could still be discriminated if fewer proteins were available for use in the analysis, we picked 50 and 35 proteins from the 62 proteins with highest relative quantities in hemolymph. Discriminant analyses were performed with 50 and 35 proteins.

When 50 proteins were used in the discriminant analysis, the model showed significant differences among the groups ($p<0.05$) (Fig. 5a). Functions 1 and 2 (both significant with $p<0.05$) together explained 69.5 % of the total variance. Among the 84 samples used to fit the model, 80 or 95.2% were correctly classified.

We also performed the discriminant analysis with the 35 proteins as independent variables. The model still produced two statistically significant ($p<0.05$, but >0.01) discriminating functions, functions 1 and 2 (Fig. 5b). These two functions, however, together only explained 64% of the total variance in the model. Among the 84 samples used to fit the model, 71 or 84.5% of the samples were classified correctly.

3.4. Protein profiles from male, female and undifferentiated oysters — discriminant analysis

When the discriminant analysis was performed on only 3 groups of oysters, female (including FD and FR), male (including MD and MR) and UD, the model produced overall more-robust, significant discriminant functions compared to analyses with oysters in 5 groups. When all 62 peaks were considered in the model, function 1 ($p<0.0001$) and function 2 ($p<0.001$) explained 57.29% and 42.71% of variance, respectively. The two functions explained almost 100% of the variance in the model. The model correctly classified 100% of samples into the 3 groups specified. The results indicated that protein

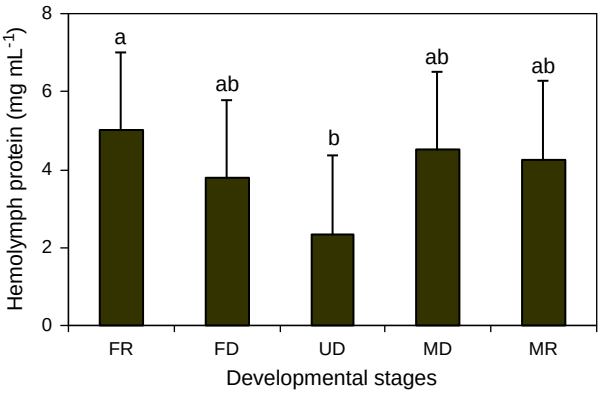

Fig. 3. Variation in hemolymph total proteins among different stages (mean ± SE). FR = femal ripe, FD = female developing, UD = sexually undifferentiated, MD = male developing and MR = male ripe. Different letters by the error bars indicate significant difference between stages using multiple range test ($p<0.05$).

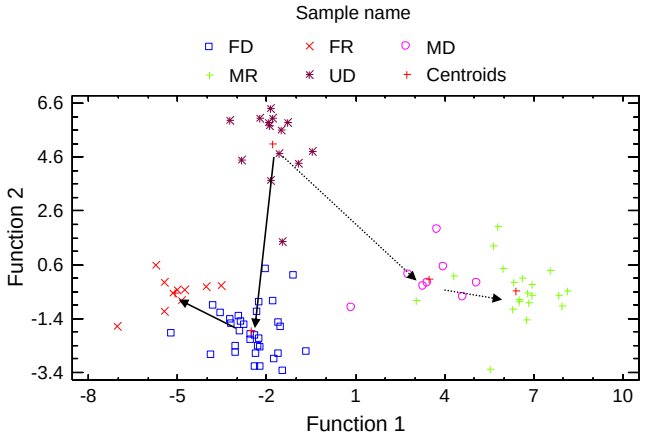

Fig. 4. Discriminant analysis comparing different oyster sex and developmental stages. All of the 62 proteins showing difference among the groups were used as independent variables. FR = femal ripe, FD = female developing, UD = sexually undifferentiated, MD = male developing and MR = male ripe.

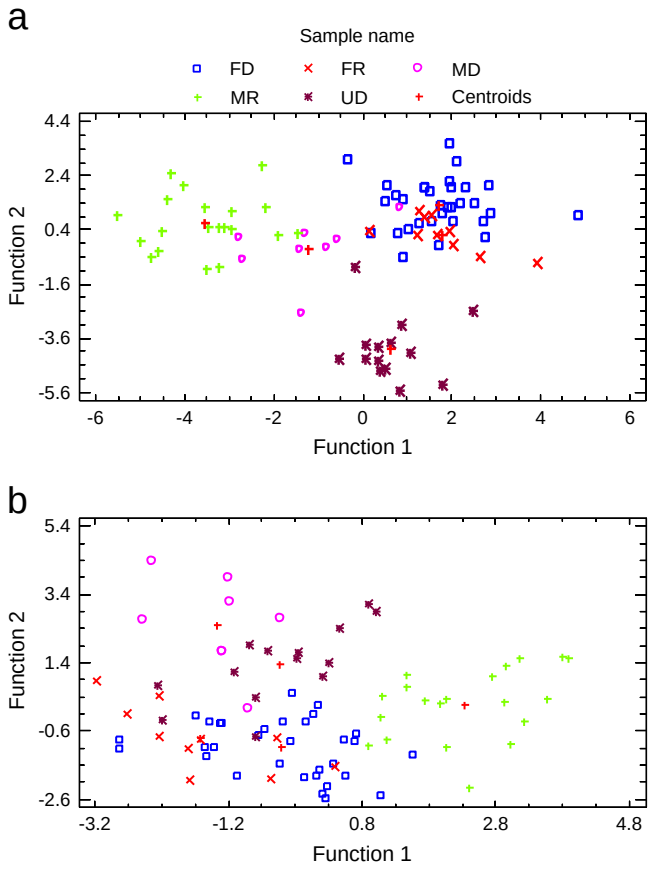

Fig. 5. Discriminant analysis of comparing oysters with different sex and developmental stages. 50 and 35 proteins included in the analysis as independent variables for panel a and b, respectively. See text for details on the selection of proteins. FR = femal ripe, FD = female developing, UD = sexually undifferentiated, MD = male developing and MR = male ripe.

profiles of hemolymph are distinctively different in female and male oysters. The differences are much greater between different sexes than those between developmental stages.

When 50 peaks were included in the analysis, the model still produce very robust, significant discriminating functions ($p<0.01$ for both functions). The two functions together explained nearly 100% of the variance. The model successfully classified 81 of 82 samples into the correct group.

When 35 peaks were included in the analysis, the model failed to produce significant discriminating functions ($p=0.10$ and 0.19 for functions 1 and 2, respectively). This model successfully classified 69 of 82 samples or 82% under this confidence level.

4. Discussion

Results of this study showed that the analysis of Eastern oyster hemolymph protein profiles from broodstock oysters can determine the sex and gonad developmental stages with great accuracy using the Proteinchip® and SELDI-TOF-MS technology. Broodstock oysters can continue to live and spawn after hemolymph is drawn. Thus, the broodstock oysters need not be sacrificed, and unambiguous results are obtained for individual oysters that can be spawned subsequent to analysis. This technique has great potential as demand for hatchery-produced seed oysters increases, both for aquaculture and oyster restoration purposes. The advantage of this method over the recently-developed, non-invasive MRI and NMR methods is that it is able to not only differentiate sexes but also the gonad developmental status which the latter methods are not able to differentiate (Davenel et al., 2006, 2009).

The protein-profile method of differentiating sexes and developmental stages of oysters relies on a large number of peptides/proteins detected by Proteinchip® and SELDI-TOF-MS technology. Two main disadvantages of this method remain: it is very expensive, and it is not user friendly (requires highly-trained technical personnel), thus it may not be very practical for direct hatchery application. These limitations may be overcome when the Proteinchip® and SELDI-TOF-MS technology becomes more affordable and more widely used in aquatic sciences and in general.

Proteinchip® and SELDI-TOF-MS technology is suitable for this study because it is sensitive enough to detect a large number of protein signals in a single sample of small volume. The multivariate discriminant analysis applied showed that a lower number of protein peaks would compromise the predictive power of the model in catagorizing oysters into the appropriate developmental stages.

This study demonstrated that the Proteinchip® and SELDI-TOF-MS technology is a suitable tool to study molluscan hemolymph protein profiles. The sample preparation is straightforward with raw hemolymph or hemolymph diluted in buffers. A large number of proteins can be detected at the same time. In this analysis, >139 peptides/proteins were detected by IMAC-30 chips, which binds only a subgroup of metal-binding proteins. More proteins may be captured by manipulating the binding conditions and by applying other available chips designed to bind specific subsets of proteins.

Results of this study have opened up many perspectives for future research. Among them, a more practical method of differentiating sex and gonad development may be developed by combining SELDI-TOF-MS technology with other techniques such as ELISA — enzyme-linked immunosorbent assay. A first step in developing such techniques would involve narrowing the number of proteins related to gonad development by manipulating the conditions of SELDI-TOF-MS analysis. Once a limited number of proteins which relate to gonad development are identified, specific antibodies to these proteins could be developed. Other applications of Proteinchip® and SELDI-TOF-MS technology in mollusc biology could be to relate profiles of hemolymph proteins to the immunological function and status when oysters are subjected to various challenges, such as pathogens, diseases, harmful algae, or extreme physical and chemical conditions (temperature, toxin etc). Subsequently, Proteinchip® and SELDI-TOF-MS analysis of hemolymph may be able to identify biomarkers for environmental stresses that the bivalves experience.

The female-specific, vitellin-like protein with molecular weight of ~500 kDa reported by Suzuki et al. (1992) in the hemolymph of *C. gigas* was not captured by SELDI in our *C. virginica* samples. These proteins were likely broken down by the laser desorption/ionization process as it is well known that this laser can break some of the weak molecular bonds, such as the interactions between subunits of large, multiunit protein molecules. Vitellins and vitellogenins in crustaceans are, indeed, macromolecules consisting of heterogeneous subunits with lipid and carbohydrate inclusions (Quinitio et al., 1989; Suzuki et al., 1992; Arcos et al., 2009). These proteins denatured into multiple heterogenic fragments through SDS-polyacrylamide gel electrophoresis (Suzuki et al., 1992; Arcos et al., 2009). Of the large number of peptides/proteins detected in this study, some of them may be fragments of the vitellins and vitellogenins. Until further study of protein sequencing is done, however, it is not possible to match the fragments with the actual vitellins and vitellogenins.

Our results showed that the female, ripe oysters had higher protein concentrations in the hemolymph than undifferentiated oysters. Male, ripe oysters and undifferentiated or other intermediate-stage of oysters had no significant difference in total protein concentration in hemolymph. Previous research showed a decrease in hemolymph protein content in spring for Eastern oysters coincident with the oyster reproductive cycle (Fisher and Newell, 1986; Chu and La Peyre, 1989). Flat oyster hemolymph also showed seasonal variation, with low levels occurring in March in Dutch, French and Irish costal waters (Cronin et

al., 2001). It has been suggested that the decrease in hemolymph protein level could be related to gametogenic development (Fisher and Newell, 1986; Chu and La Peyre, 1989). Our results showing a higher protein level in mature female oysters appeared to contradict these findings. The seasonal decreases in protein levels in these studies, however, may have been influenced by increases in temperature and related increases in metabolic rate. In our study, oysters were kept under constant temperature, and thus the change in hemolymph protein may reflect reproductive activities alone.

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