Proteomic Analysis of Eggs from *Mytilus edulis* Females Differing in Mitochondrial DNA Transmission Mode*\[S\]

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Many bivalves have an unusual mechanism of mitochondrial DNA (mtDNA) inheritance called doubly uniparental inheritance (DUI) in which distinctly different genomes are inherited through the female (F genome) and male (M genome) lineages. In fertilized eggs that will develop into male embryos, the sperm mitochondria remain in an aggregation, which is believed to be delivered to the primordial germ cells and passed to the next generation through the sperm. In fertilized eggs that will develop into female embryos, the sperm mitochondria are dispersed throughout the developing embryo and make little if any contribution to the next generation. The frequency of embryos with the aggregated or dispersed mitochondrial type varies among females. Previous models of DUI have predicted that maternal nuclear factors cause molecular differences among unfertilized eggs from females producing embryos with predominantly dispersed or aggregated mitochondria. We test this hypothesis using females of each of the two types from a natural population. We have found small, yet detectable, differences of the predicted type at the proteome level. We also provide evidence that eggs of females giving the dispersed pattern have consistently lower expression for different proteasome subunits than eggs of females giving the aggregated pattern. These results, combined with those of an earlier study in which we used hatchery lines of *Mytilus*, and with a transcriptomic study in a clam that has the DUI system of mtDNA transmission, reinforce the hypothesis that the ubiquitin-proteasome system plays a key role in the mechanism of DUI and sex determination in bivalves. We also report that eggs of females giving the dispersed pattern have higher expression for arginine kinase and enolase, enzymes involved in energy production, whereas ferritin, which is involved in iron homeostasis, has lower expression. We discuss these results in the context of genetic models for DUI and suggest experimental methods for further understanding the role of these proteins in DUI.

Proteomics has made a rapid progress when applied to model species where genomic databases are well developed, but recent reviews have pointed to the growing interest in applying proteomics to nonmodel species in areas such as evolutionary ecology (1), aquatic toxicology (2), aquatic pollution (3), aquaculture (4), and marine biology (5). In the marine mussel genus *Mytilus*, an unusual system of mtDNA inheritance occurs characterized by the presence of two mitochondrial genomes (called F and M) one of which is inherited maternally and the other paternally (6–9). This phenomenon, called doubly uniparental inheritance of mtDNA (DUI),\[1\] suggests a connection between mtDNA inheritance and sex determination. DUI also occurs in other species belonging to three bivalve orders (10, 11). Since the 1990s, DUI has been under intensive investigation, but the molecular mechanism for DUI and how it relates to sex determination, is still to be elucidated (for review, see 12–15).

Cytological studies of fertilized eggs using fluorescently labeled sperm mitochondria from species with DUI have identified two types of embryos (16, 17). In one type, the sperm mitochondria migrate independently and disperse among the blastomers. It is not clear whether or not these mitochondria are later degraded. In the second type of embryo the sperm mitochondria migrate in a close aggregate, which is partitioned to only one blastomere in subsequent cell divisions. In females producing mainly daughters the first pattern is much more common, whereas the second type is more common in females that produce predominantly sons. This observation suggests that fertilized eggs showing the dispersed sperm mitochondrial pattern develop into female embryos and eggs with the aggregation develop into males. It has been sug-

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\[1\] The abbreviations used are: DUI, doubly uniparental inheritance; ANOVA, analysis of variance; BH, Benjamini and Hochberg; 2-DE, two-dimensional electrophoresis; FDR, false discovery rate; Ime, linear mixed effects; mtDNA, mitochondrial DNA; REML, restricted maximum likelihood; SFiFisher, sequential combined probability test of Fisher; SGoF, sequential goodness of fit.
gusted that the aggregated mitochondria are finally delivered into the primordial germ cells in male embryos (18), thus accounting for the paternal transmission of the M genome. Similar observations have been made in another species with DUI, the clam *Ruditapes philippinarum* (19, 20). The aggregation phenomenon was not observed in the oyster *Crassostrea gigas* (21) a species in which DUI has not been reported. The precise role of the F and M genomes in mitochondrial inheritance and sex determination in species with DUI has not yet been established. The two genomes are substantially different in DNA sequence in coding regions, with divergence ranging from 20 to 40% depending on species (13). Many candidate functional differences have been discovered including rearrangements in the control region (*e.g.* 22–25), a second copy of the cytochrome oxidase II (COII gene) in the M genome of the mytilid *Musculista senhousia* (25), F and M specific open reading frames (26), and an M genome insertion extending the COII gene in unionid bivalves (27, 28). In *M. galloprovincialis*, the M genome is transcribed in spermatagonia and spermatocytes, though not in somatic tissue (29). In unionids, the M genome COII protein is expressed on the outer sperm mitochondrial membrane (30). The F genome COII protein is strongly expressed in the outer vitelline layer of mature eggs (31).

In *Mytilus*, the association among maleness, sperm mitochondrial aggregation, and presence of the M genome on the one hand, and femaleness, sperm mitochondrial dispersal, and absence of the M genome on the other, have been established by breeding experiments (32) and the aforementioned cytological studies. In pair-matings, mothers can be divided into three types: those that produce daughters almost exclusively, those that produce a very high frequency of sons, and those that produce intermediate frequencies of daughters and sons (33, 34). Fathers do not show this marked variation in progeny sex ratio. From the viewpoint of genetics, these observations are characteristic of a maternal effect in which the phenotype of an individual is determined not by its own genotype but by the genotype of its mother. In DUI the phenotype refers to sex, sperm mitochondrial behavior and possession of the F or M genome. Maternal effects are often mediated through the effect of the mother on the developing oocyte or egg. The study of the molecular basis of DUI might thus be advanced by transcriptomic and proteomic studies of these cells. The potential for such maternal effects are supported by evidence that maternal factors deposited in the oocyte are entirely responsible for its development and that of the early embryo (35). Oocytes show a high level of transcriptional activity to provide mRNAs required for growth of the embryo, including a network of transcriptional regulators (36). RNA transcripts for over 7000 transcribed genes have been identified in human metaphase II oocytes (37). However transcript levels do not necessarily reflect protein levels because of measurement errors and biological factors such as protein turnover rates and variable half-life (38, 39). Thus proteomics approaches are important to complement transcriptomic studies in oocytes (35, 40). For example, comparison of germinal vesicle and metaphase II murine oocytes using 2-DE and silver staining successfully revealed 12 (41) and 63 (42) protein expression differences, many known to be associated with meiosis and murine maturation.

In the current study, we tested a prediction of the DUI maternal effect hypothesis. This is that molecular differences at the proteome level exist between the eggs of females that produce embryos with sperm mitochondrial aggregation and the eggs of females that produce embryos with sperm mitochondrial dispersal. We observe such differences and relate them to specific genetic models of sex determination and DUI, including a new model we propose here. We also consider a hypothesis that pertains to the precise molecular basis of differences between the eggs from females producing embryos with aggregated and dispersed sperm mitochondria. Ubiquitination of sperm mitochondria may play an important role in the tagging of sperm mitochondria for destruction in mammals that exhibit standard maternal mtDNA inheritance (43, 44) resulting in degradation in the proteasome pathway (45). The possibility that this ubiquitin-proteasome system is involved in DUI has been previously referred to in studies considering a model for DUI (12, 15, 17, 34, 46). An earlier proteomics study on *Mytilus* (47) and a recent transcriptomics study of the clam *Ruditapes philippinarum*, which also has DUI (46), have identified proteasome proteins as showing expression differences between a male sex ratio biased family and female sex ratio biased family. These studies taken together provide an *a priori* hypothesis, which we test here. This predicts that eggs from females producing embryos with the dispersed and aggregated patterns should also show consistent proteasome protein expression differences. In the present study we provide evidence for expression differences in line with the prediction using replicated females of each type taken from a natural population. We also report and discuss consistent expression differences for some other proteins potentially involved in DUI including arginine kinase and enolase, which are involved in energy production and ferritin, which is involved in iron homeostasis. We also make suggestions for further experimental work on the involvement of these proteins in DUI.

**EXPERIMENTAL PROCEDURES**

*Mussel Collection, Spawning, and Fertilization—* Mussels were collected in Lamèque New Brunswick and grown out at Mahone Bay, Nova Scotia, Canada (44.44° North 64.38° West). Although the population is predominantly *Mytilus edulis*, *M. trossulus* have been reported in the region, and introgression occurs between the two sibling species. Mussels were not chosen for spawning if they showed any morphological features characteristic of *M. trossulus*. It is known from previous pairing studies in mussels from this area (32, 34) that mothers producing a high frequency of sons also produce a high proportion of embryos showing the sperm mitochondria aggregation pattern.

Mussels were reared in a hatchery in the same holding tank at the Bedford Institute of Oceanography, Dartmouth, Canada. For spawning, mussels were cleaned and rinsed, and placed in individual 500 ml
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plastic cups on the day of spawning. The water in the cups was the same as that in the holding tank but heated to 20 °C to induce spawning. To minimize contaminants, the water was filtered (at 1 μm), UV treated and changed frequently to remove feces. All mussels were reared and spawned under similar environmental conditions. After spawning, gametes were collected and used immediately for crosses to assess mitochondrial behavior. The eggs to be used for proteomics were placed in a refrigerator (3–4 °C) and allowed to settle. Then 15 μl of eggs from each female, obtained by centrifugation for 3 min at 13,000 x g, were resuspended in preservation medium (10% glycerol in 0.9 M NaCl). The eggs in preservation medium were snap frozen in a dry ice/ethanol bath and stored at −80 °C before analysis.

Fluorescent Labeling of Sperm and Microscopic Examination of Embryos—The techniques used for fluorescent labeling of sperm have been described previously (16, 17), and are summarized here. Sperm from spawned males were stained with the mitochondrial-specific dye MitoTracker Green FM and used to fertilize eggs from spawned females. Embryos were examined with a Nikon E800 fluorescence microscope to track the position of the labeled sperm. At the four-cell stage, the cells are designated A, B, C, D. It is assumed that the germ cells originate from cell D, which is also the largest of the four (18). If all sperm mitochondria were present in cell D or close together in the cleavage furrow associated with cell D, a larva was classified as having the aggregated pattern. If the mitochondria were not close to each other and scattered in various cells, a larva was classified as having the dispersed pattern.

Experimental Design—From each of 40 female mussels successfully spawned, a sample of eggs was fertilized and 16 embryos were examined and classified according to whether they exhibited the dispersed or aggregated mitochondrial DNA pattern. The females were then classified into three types.

1 Females in which all 16 of their embryos showed the dispersed pattern were classified as “Female-biased” type (meaning the sex ratio among their progeny would be highly biased in favor of females and that progeny carrying the paternal genome in their gonads would be missing or very rare).

2 Females in which at least 12 of their embryos had the aggregated pattern were classified as “Male-biased” type (meaning the sex ratio among their progeny would be biased in favor of males and that progeny carrying the paternal genome in their gonads would be in the majority).

3 Other Females were classified as being of the “Mixed” type (meaning that the dispersed and aggregated patterns occurred with equal frequency).

Four female mussels of each of the three types (Female-biased, Male-biased, Mixed) were used for the proteomic analysis. Thus there were 12 female mussels employed in the experiment (three types x four biological replicates), each providing a pool of unfertilized eggs from which a protein extract was obtained and analyzed separately using 2-DE electrophoresis. For five mussels (one of the Female-biased type, two of the Male-biased type, and two from the Mixed type) the 2-DE electrophoresis was repeated on the extracted protein to provide technical replicates to allow estimation of experimental variation associated with the technique.

Protein Extraction and 2-DE Electrophoresis—The experimental procedures for proteomics are given in full as supplemental experimental procedures. These followed closely those used previously for mussels (47). In brief, proteins were extracted from unfertilized eggs, solubilized by sonication on ice, and then quantified and cleaned using commercial kits. The first dimension isoelectrofocusing (IEF) was carried out with immobilized pH gradient strips (3–10NL) with a horizontal IEF apparatus. The second dimension of gel electrophoresis was carried out with precast 12.5% polyacrylamide gels using an Ettan Dalt six electrophoresis system. Protein spots were visualized by silver staining. SameSpots software was used to align and analyze gel images and for automatic spot detection, manual filtering, and spot volume measurement. Further details are given in the supplemental experimental procedures.

Variable Transformation and Normalization—For analysis, 244 spots were used (see RESULTS). Transformation and normalization procedures for spot volume followed those used in an earlier study (47). For each spot, spot volume (intensity) was first normalized by dividing by the total spot volume for the gel from which the spot came. For convenience, the normalized values were then multiplied by the total volume, summed over all 244 spots, for the gel in the data set having the lowest total volume. These normalized values were used for analysis of fold values. Fold is defined for a spot as the ratio of the normalized value for the Female-biased type (with dispersed mitochondria) divided by the normalized value for Male biased type (with aggregated mitochondria). However for other analyses, the normalized values were transformed by taking log to base 2. The mean of these log2 values was calculated for each gel. This mean was then subtracted from each of the 244 individual log2 values for the gel to renormalize on the log scale. The resulting variable is called log2norm, the name indicating that the spot volumes were both normalized and log transformed. Tests for fit to normality were carried out on log2norm separately for the data for each biased type. In all cases there was a good visual fit to the normal distribution with a straight line in the Q-Q plot, and a nonsignificant result in the Kolmogorov-Smirnov test. No significant outliers were detected using Grubbs’ test (48). Statistical analysis on log2norm was carried out using Excel and SPSS. In addition, the R language and environment (49) was used to carry out restricted maximum likelihood analysis (REML) using the linear mixed effects (lme) model function from the nlme package (50).

Protein Identification and Mass Spectrometry Analysis—The experimental procedures for protein identification are given in full as supplemental Experimental Procedures. In brief, spots of interest were excised with an Ettan Spot Picker and digested with trypsin using an Ettan Digerester. Recovered peptides were analyzed using HPLC-MS/MS with a nanoelectrospray ion trap. Protein spots were identified from selected peptide spectra using Mascot search engine (Mascot Daemon 2.4.1, Matrix Science, London, UK) which compared the spectra produced against two customized databases. The first database consists of a total of 67,990 EST sequences from four Mytilus species deposited in NCBI. The second database is from an unpublished RNA-seq project (Diz and others, manuscript in preparation) on male mature gonad samples from Mytilus edulis and M. galloprovincialis that provides a consensus transcriptome of 49,713 sequences. The full sequences from the customized databases, which gave significant peptides matching the spectra, were then used in a Blastp search against a nonredundant (nr) protein sequence database of all organisms to ascertain final protein identifications. Use of the full sequences from the Mytilus EST and RNA-seq for the Blast search of course provide more information for searching than the shorter peptide sequences matching the spectra.

RESULTS

Protein Spots Identified by 2-DE—The number of spots observed on the gels analyzed using SameSpots software was usually over 1000, in line with expectation for the technique (51) and a previous study on mussel eggs (47). After filtering (see supplemental Experimental Procedures), 244 spots were retained for analysis. An example of one of the gels analyzed is given in Fig. 1. Silver staining shows a linear response range over about two orders of magnitude (52, 53), but at higher protein concentrations the response slope flat-
tens. This also occurs with other stains such as SYPRO Ruby but is rather more marked for silver staining (54, 55). The spots used in the present study did not have a staining intensity beyond this response range and had intensity well below the point at which stain saturation appears at the center of spots. Loading 100 μg of protein does not permit detection of proteins of very low abundance (51).

Biological Variation Is Significant When Compared With Technical Variation—Technical replication involves repeating the 2-DE electrophoresis and analysis on a given biological replicate (egg sample from a given female mussel). Technical replicates allow the estimation of the experimental variation inherent in the 2-DE technique, for comparison with the biological variation, which in this study is the difference between the egg proteome of the four female mussels within each of the biased types. The relative magnitude of the technical and biological variation was assessed for the Male-biased and Mixed types each of which had two technical replicates for two of the four biological replicates. The technical variation for these replicates was assessed over all 244 spots using ANOVA. The added variance components for the interactions “Mussels × Spots” (which measures biological variation across spots) and “(Technical Replicates within Mussels) × Spots” (which measures the technical variation) were calculated. For the Male-biased type, biological and technical variances are 33.2% and 66.7%, respectively, and for the Mixed type, the values are 38.7% and 61.2%. That the biological variation is less than the technical variation is simply an indication that the former is relatively small in this case. The coefficient of determination calculated from the correlation between technical replicates over all 244 spots ranges between 0.66 and 0.82 for those mussels having two technical replicates, comparable with an earlier proteomics study where the range was 0.73–0.87 (56). The mean square for biological variation was highly significantly greater than that for technical variation for both Male-biased and Mixed type ($p = 0.000$). It is important to note that the significance of any treatment effect among the Female-biased, Male-biased, and Mixed types, is assessed against the biological variation not the technical variation. Because the biological variation is statistically significant, any failure to detect a treatment effect would have been the unfortunate consequence of the biological variation being too large compared with the treatment effect, given the scale of the experiment, rather than to the technical variation inherent in the technique itself.

Biased Types Differ in Protein Expression: Spot by Spot Analysis—The differences in expression in log$_2$norm between the Female (standing for Female-biased), Male (standing for Male-biased), and Mixed types were assessed on a spot by spot basis using REML with a linear mixed effects model (57) including all biological and technical replicates. Two models were used. The first fits female Mussels as a random factor (biological variation), the second fits Mussels as random and Types as a fixed treatment factor, thus testing whether Types explains a significant proportion of the variation not explained by the biological variation. A significant $p$ value in the comparison of the models indicates a significant treatment effect because of Types. This analysis was carried out for each spot comparing Female versus Male, Female versus Mixed, Male versus Mixed, and Female versus Male versus Mixed simultaneously. The $p$ values obtained are given for all individual spots in supplemental Table S1, listed from lowest to highest for each biased type comparison. These $p$ values are properly called a-priori $p$ values and are not adjusted for multiple hypothesis testing. Supplemental Table S1 also highlights $p$ values that can be declared significant using a number of different multiple hypothesis testing methods (see 58, for applications of these methods in proteomics). The first method is the false discovery rate method (FDR) (59) controlled at values of BH 5%, BH 20%, and BH 50%, indicating that respectively 5%, 20%, or 50% of those spots declared significant by the method are expected to be false positives, the remainder true positives. The second method (SGoF) (60) uses the binomial theorem sequentially to test whether the observed number of significant $p$ values in the list above a threshold is greater than expected. The third method (SFisher) applies the Fisher combining probability test (61, 62) to the points above a threshold. For all methods, spots above the threshold are declared significant by the method used. The $q$-value (63), which gives the expected proportion of false positives above any threshold drawn in the list, is also shown. Table I gives the number of spots declared significant using the different methods shown in supplemental Table S1. There are 244 spots, thus 0.05 × 244 = 12.2 are expected to give an a priori $p$ value significant at the 5% level just by chance. The actual number is in excess of this for all the comparisons, suggesting an effect of biased type. For the Female versus Male comparison, the excess of significant $p$ values is equal to 26 (a priori observed) − 12.2 (expected) = 13.8, an estimate of the expected number of true positives. For BH 50% the expected number of true positives is 50% of 31 = 15.5. The number of significant spots for SGoF and SFisher is 7 and 14.
respectively. The \( q \)-value corresponding to the last of the 26 spots that are significant \( a \) priori (spot 97) is 0.358, suggesting that \( 26/H_11003 \times (1 - 0.358) = 16.7 \) are true positives. These different approaches present a consistent picture of between 10 and 20 true positives. Similar results showing evidence for true positives are obtained for Male versus Mixed and Female versus Male versus Mixed, whereas for Female versus Mixed there is no strong evidence of an effect. Support for differences among the biased types also comes from the distribution of \( p \) values. Under the null hypothesis of no effect a uniform distribution is expected (63). However, there is a tendency for an excess of lower \( p \) values as shown in supplemental Fig. S1.

**TABLE I**

|                | Female versus male | Female versus mixed | Male versus mixed | Female versus male versus mixed |
|----------------|--------------------|---------------------|-------------------|--------------------------------|
| \( p \) value significant \( a \) priori (0.05) | 26 | 16 | 30 | 25 |
| BH 5%          | 0 | 0 | 0 | 0 |
| BH 20%         | 0 | 0 | 1 | 0 |
| BH 50%         | 31 | 0 | 90 | 31 |
| SGoF (0.05)    | 7 | 0 | 11 | 6 |
| SFisher (0.05) | 14 | 6 | 35 | 27 |
| \( q \)-value (for \( a \) priori significant \( p \) values) | 0.358 | 0.424 | 0.231 | 0.188 |

**TABLE II**

| Comparison                      | Component                     | \( p \) value | Added variance component % |
|---------------------------------|-------------------------------|---------------|-----------------------------|
| Female versus male:             | (Female vs Male) × spots      | 0.002         | 7.4                         |
|                                 | (Mussels within types) × spots|               | 92.6                        |
| Female versus mixed:            | (Female vs Mixed) × spots     | 0.976         | -4.8*                       |
|                                 | (Mussels within types) × spots|               | 104.8                       |
| Male versus mixed:              | (Male vs Mixed) × spots       | 0.027         | 4.8                         |
|                                 | (Mussels within types) × spots|               | 95.2                        |

* Negative value indicates absence of an effect of types.

**Magnitude of Expression Differences Among Biased Types Assessed Globally**—To ascertain the magnitude of the Types effect globally over all spots two methods were used. In the first method, two two-way ANOVA analyses were carried out over all spots. The first had Mussels (averaging technical reps) and Spots as the two factors. The second had Types and Spots as the two factors. The results of these two two-way ANOVAs were then combined into a nested ANOVA. The results are given in Table II. The component (Mussels within Types) × spots measures the biological variation, whereas the second component, e.g. (Female versus Male) × spots, measures the Types effect. For the Female versus Male, and Male versus Mixed comparisons the added variance component for the Types effect is 7.4% and 4.8% respectively, whereas it has a computed negative value for the Female versus Mixed comparison suggesting no Types effect. Thus over all spots, the Female and Mixed expression patterns are similar but different to the Male expression pattern. The largest difference is for the Female versus Male comparison.

The second method determines whether the expression pattern of the Mixed type is closer in value to the Female or Male type using a model from quantitative genetics to measure dominance (64) in which the difference in value between two homozygotes, e.g. \( A_1A_1 \) and \( A_2A_2 \), is defined as \( 2a \) and the deviation of the heterozygote \( A_1A_2 \) from the midpoint between the two homozygotes (set at zero) is defined as \( d \). An analogy between the Female, Male and Mixed types and three genotypes \( A_1A_1, A_2A_2, \) and \( A_1A_2 \) is justified by reference to hypothesized models for DUI, which envisage a maternally acting nuclear gene with two alleles (see DISCUSSION). This representation of dominance is shown in Fig. 2. With normalized gene expression, the absolute difference between Mixed and Male (or Mixed and Female) can be the same irrespective of whether Male has greater expression than Female, and this is taken into account in the analysis. The average values of \( \log_2 \)norm for Female, Male, and Mixed were calculated for each of the spots in the list with spots ranked from low down to high \( p \) value for the REML analysis for the Female versus Mixed comparisons.
Male versus Mixed comparison (supplemental Table S1). The average values are plotted as deviations from the midpoint as accumulated averages starting with the spot with lowest p value (Fig. 3). The second plotted value is the average of log2norm for the two spots with lowest two p values and so on until log2norm is averaged for all 244 spots in the final plotted point to the right of the graph. For the 10–20 spots showing the lowest p values, Female and Mixed are close in value. Thereafter as more spots are included (those less likely to show an effect) the difference among the three biased types stabilizes but still with closer expression of Female and Mixed types. This analysis is in agreement with the ANOVA analysis in suggesting closer similarity in expression of the Female and Mixed types.

**Specific Proteins Differ in Expression Between the Female and Male Biased Types**—Spots with fold values >1.4 in any comparison between the Female-biased, Male-biased and Mixed types were analyzed by mass spectrometry. There is obviously not an exact correlation between fold values and REML p values, the latter depending on the magnitude of the biological variation as well as the difference among biased types. For example for the Male versus Female comparison the product moment correlation (r) between fold and p values over all 244 spots is −0.68. Of the total of 56 protein spots analyzed, 23 were identified by mass spectrometry and are given in supplemental Table S2 with measurements and statistics pertaining to MS/MS and their positions are marked on the gel photo (Fig. 1).

We focus on proteins differing in expression between the Female-biased and Male-biased types. These show the largest proteome difference globally and the comparison of these types is of greatest interest in relation to DUI. Following a strategy described previously to assess proteomics data (58) using multiple hypothesis testing methods, we have further focused on those spots significant at BH 50% to provide potential candidates for future study. All of these have *a priori* p values below or close to the 5% significance level. Of the 23 protein spots identified, eight spots are significant at BH 50% between the Female-biased and Male-biased types. These are shown in bold in Table III with fold values and p values for the expression differences between Male-biased and Female-biased types. Significance at BH 50% for the eight spots is used as a further criterion for inclusion in Table III of additional identifications of these specific proteins. In addition, two identifications from an earlier study (47) are included. The multiple occurrences of four proteins, arginine kinase, enolase, ferritin-like protein, and proteasome subunits add confidence to the protein identifications. Predicted peroxiredoxin is included as a match for thioredoxin but these two spots do not follow the same pattern in relation to fold and p values, and no clear link with DUI can be discovered and thus these spots are not considered further. Of particular note is the consistency in direction of expression as indicated by the fold values for arginine kinase, enolase, ferritin-like protein, and proteasome subunits. For example, for arginine kinase all four values are

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**Fig. 3. Accumulated average expression for the Female, Male, and Mixed types plotted against spot number.** The plotted values begin on the left of the abscissa with the value of log2norm for the spot with the lowest REML p value. The second plotted value is the average of log2norm for the two spots with lowest two p values and so on until the final plotted value to the right of the abscissa is the average of log2norm for all 244 spots.
TABLE III
Protein spots identified by MS/MS with fold and p values

| Spot code | Protein name                  | Organism*          | Mytilus database* | Fold values (female/male)$^f$ | p value female vs male$^f$ | Fisher combined p value$^f$ |
|-----------|-------------------------------|--------------------|-------------------|-------------------------------|--------------------------|--------------------------|
| 49        | Arginine kinase               | Aplysia kurodai    | EST; RNA-seq      | 1.337                         | 0.073                    |                          |
| 72        | Arginine kinase               | Aplysia kurodai    | EST; RNA-seq      | 1.920                         | 0.024                    |                          |
| 79        | Arginine kinase               | Aplysia kurodai    | EST; RNA-seq      | 1.494                         | 0.130                    |                          |
| 167       | Arginine kinase               | Aplysia kurodai    | EST; RNA-seq      | 1.022                         | 0.072                    | 0.032                    |
| 160       | Enolase                       | Doryteuthis pealeii| RNA-seq           | 1.883                         | 0.026                    |                          |
| 29        | Ferritin-like protein         | Mytilus edulis     | EST; RNA-seq      | 0.746                         | 0.056                    |                          |
| 78        | Ferritin-like protein         | Mytilus edulis     | EST; RNA-seq      | 0.706                         | 0.042                    | 0.017                    |
| 57        | Heterogeneous nuclear         | Crassostrea gigas  | EST; RNA-seq      | 1.704                         | 0.033                    | 0.033                    |
| 123       | Ribonucleoprotein A2-like protein 1 | Membrana gigas | EST; RNA-seq      | 0.706                         | 0.015                    | 0.015                    |
| 86 (48)$^ac$ | Proteasome subunit alpha type-2 | Xenopus laevis    | RNA-seq           | 0.675                         | 0.079                    |                          |
| 156       | Proteasome subunit alpha type-3 | Crassostrea gigas | EST; RNA-seq      | 0.703                         | 0.042                    |                          |
| 67 (109)$^d$ | Proteasome subunit beta type 3 | Osmerus mordax    | EST               | not applicable$^e$            | 0.019 (0.000)            |                          |
| 85        | Thioredoxin peroxidase        | Cristaria plicata  | EST; RNA-seq      | 0.635                         | 0.014                    |                          |
| 146       | Predicted: Peroxiredoxin-6-like | Amphimedon queenslandica | EST            | 1.193                         | 0.761                    | 0.058                    |

* Spots in bold are significant at BH 50%.
$^a$ Spots in brackets (48 and 109), from reference 47.
$^b$ Spot 48 was observed and identified in reference 47 and matched spot 86 in the current study (this spot is not marked in Fig. 1).
$^c$ The full EST or transcript sequences giving the matching peptides were used to search a database of all non-redundant protein sequences (nr).
$^d$ Searches in reference 47 were made against a non-redundant database of all organisms.
$^e$ Searches in reference 47 were made against a non-redundant database of all organisms.

The data for the earlier study (47) in brackets in Table III and the current study are both significant. The results thus confirm the prediction of proteasome expression differences among the different biased types. The potential involvement of the proteasome and the other proteins in Table III, in DUI and sex-determination is elaborated on below.

DISCUSSION

This study supports the prediction of the maternal effect hypothesis. It reveals significant differences in protein spot expression among the three types of unfertilized eggs from Female-biased, Male-biased, and Mixed type females. In addition, several proteins have been identified as differing in expression between eggs from the Female-biased and Male-biased females. The prediction that proteasome subunits might be identified has been confirmed. To evaluate the significance of these findings, we will first briefly review genetic models of DUI and sex determination and consider the quantitative proteomic differences among eggs from the three types of females in relation to these models. We present a new model to account for sperm mitochondrial behavior and sex determination, and explain how this is consistent with the current results and with the results of breeding experiments in Mytilus. We then discuss the proteins showing expression differences between eggs from the Female-biased and Male-biased types of female, and assess their importance in relation to DUI and sex determination. We conclude with some suggestions for further work.

Egg Proteomic Differences, Maternal Effects and Models for DUI and Sex-determination—A model for DUI and sex-determination incorporating several genetic factors (12), built on earlier discussion (33), assumes that a Factor W produced during spermatogenesis specifically labels sperm mitochondria. Factor X produced during oogenesis interacts with W to cause elimination or dispersal of sperm mitochondria in the egg after fertilization (Fig. 4A). The W/X system is assumed to be the mechanism that acts against sperm mitochondrial transmission in animals not possessing DUI. In DUI, a nuclear
Gene Z expressed during oogenesis, with two alleles, Z (produces factor Z) and z (inactive) is involved. The Z factor binds to and inactivates X with the result that sperm mitochondria are not dispersed but retained as an aggregate for later partitioning to the primordial germ cells (Fig. 4A). Either the aggregate itself or factor Z causes masculinization of the embryo. Mothers that are zz produce female offspring with dispersed mitochondria: mothers that are ZZ produced male offspring with the aggregated pattern (Fig 4B). Mothers that are zZ heterozygotes produce an intermediate amount of factor Z, which varies among eggs and has a distribution that straddles a threshold. Below the threshold the dispersed pattern results with a female embryo: above the threshold the aggregated pattern results with a male embryo. Zz heterozygotes can thus produce an intermediate frequency of female (with dispersed mitochondria) and male (with aggregated mitochondria) offspring.

A key feature of this model is that the Z factor is produced from the mother during oogenesis. It is thus a maternal effect model, in which the phenotype of the progeny is determined not by their own genotype but by the genotype of the mother. To mediate their different effects, the alleles Z and z should have different molecular consequences. These could be detectable at the proteome level, even though a single gene is involved. For example, a single gene mutation was identified as cause of the differential expression of 155 proteins between a colorectal cancer cell line and a control line (70). Also a single base pair substitution in Pseudomonas fluorescens caused changes in 46 proteins in specific metabolic pathways involved in fitness effects of the mutation (71). On this basis, factor Z) and Z (high expression of factor Z). Notional expression levels of factor Z are shown. The amount of factor Z in the egg is determined during oogenesis by the diploid genotype of the mother. This is thus a maternal effect model. A low level of factor Z results in a female with mitochondrial dispersal; a high level of factor Z results in a male in which the sperm mitochondria are retained as an aggregate (see A above). zZ heterozygous mothers produce eggs showing variation in the level of factor Z, and both types of progeny are produced depending on whether the level is above or below a critical threshold. C. Model with an additional gene S for sex determination. Factor Z produced during oogenesis switches on gene S that codes for a factor S, which is involved in sex determination. Notional expression levels of factors Z and S are shown. Factor S could be produced before or after fertilisation but would be active in sex determination in the zygote after fertilisation. Allele Z causes higher expression of gene S than does allele z. Two copies of gene S in diploid eggs doubles the dose of factor S. zZ heterozygous mothers produce eggs showing variation in the level of factors Z and S, and the progeny depend on whether the levels are above or below the thresholds. Dashed arrows indicate possibilities that might vary depending on the thresholds, but it is assumed that the expression of the Z and S genes are closely correlated. Thus the possibility that haploid eggs from a zZ mother could develop into females (factor S is low dose) with aggregated mitochondria (factor Z high dose) is excluded.
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the model predicts proteomic differences among the three genotypes \(zz\), \(zZ\), and \(ZZ\), which could thus correspond to the three types of female studied here, Female-biased, Mixed and Male-biased. In observing proteomic differences among these types (Tables I and II and Fig. 3) the results of the current study thus provide some support to the maternal effect model. A further observation requiring explanation is the closer similarity of Female-biased and Mixed types. In relation to the model this would indicate closer similarity between \(zz\) and \(zZ\) than between \(zZ\) and \(ZZ\) implying some degree of dominance of allele \(z\) at the proteome level. This does not necessarily imply dominance of allele \(z\) in relation to the level of factor \(Z\). This is because there may be a nonlinear relationship between the level of factor \(Z\) and the quantitative proteomic differences as measured in the current study.

The above maternal effect model has difficulty in accounting for a number of observations. That triploid mussels are all male has been long established (72). A particular problem for DUI models is that mothers that normally produce 100% diploid daughters carrying the \(F\) genome, produce 100% triploid sons all carrying only the \(F\) genome when eggs were treated with cytochalasin B (13, 32), rather than the \(M\) genome characteristic of males. This conflicts with the expectation that because the \(Z\) gene is maternally acting, a \(zz\) mother should produce 100% daughters carrying the \(F\) genome irrespective of whether they are diploid or triploid. Another difficulty is that in pure species \(x\) hybrid crosses a high frequency of diploid sons and daughters are produced all carrying the \(F\) genome (32). A way to accommodate these exceptional observations is to postulate additional genetics factors. One possible model is shown in Fig. 4C. Factor \(Z\) produced during oogenesis switches on an additional gene \(S\) which codes for a factor \(S\), which is involved in sex determination. Factor \(S\) is active in sex determination in the zygote after fertilization. The \(Z\) and \(S\) genes from sperm are repressed and make no contribution. Allele \(z\) has a higher expression level of factor \(Z\) than allele \(Z\) and thereby causes higher expression of gene \(S\). In diploid eggs there are two copies of gene \(S\), which doubles the dose of factor \(S\). The level of factor \(Z\) in diploid and haploid eggs of mothers of the same \(Z\) genotype are the same. The model can account for males being triploid and having dispersed mitochondria. The hybrid data could be explained if in hybrid crosses there is some relaxation of the repression of the \(S\) gene from sperm. This could cause a female-biased mother to produce some male progeny without causing aggregated mitochondria. The model with gene \(S\) remains a maternal effect model through the action of gene \(Z\), and thus is consistent with the results of the current study, which demonstrate differences among unfertilized eggs from females of the different biased types. An alternative model has also been proposed (13) in which the increased expression of factor \(S\) in male biased mothers is achieved by a duplication of gene \(S\). Discrimination between and testing of such models would require a means to study gene expression after fertilization with the aim of identifying the expression of sex determining genes such as gene \(S\). This could be achieved by genomics studies of fertilized larvae from females of the different biased types.

It is difficult to see how the differences among eggs from Female-biased, Male-biased, and Mixed type females could be consistent with a model in which the differences in mitochondrial behavior and sex are caused by a genetic switch residing on a cytoplasmically inherited factor in the egg such as the \(F\) genome. This is because distinct maternal lines would be expected in populations showing consistent inheritance of sex and mitochondrial behavior down the generations. This is not observed, for example some mothers produce 100% daughters, but some of these daughters do themselves produce sons (34). However given a genetic switch, the \(F\) and \(M\) genomes could play a role in the molecular mechanism involved in sperm mitochondrial behavior and fate, and in the determination of sex. Many candidate functional differences have been discovered (see INTRODUCTION). When considering genetic models for DUI and sex determination it should be borne in mind that knowledge of sex determination in mollusks is not well advanced and a great variety of mechanisms have been reported (73). A distinction can be drawn between sex-ratio and sex-determining genes (73). The maternal effect genes would be an example of the former and gene \(S\) (Fig. 4C) an example of the latter. A reanalysis of published data for Mytilus has suggested a paternal effect on sex-ratio (74). However the heterogeneity of the pooled data from different studies and the smallness of the alleged effect strongly suggest that further evidence is needed before a paternal effect on sex determination in muscles can be considered as established.

Proteins with Potential Involvement in DUI and Sex Determination—The current study has confirmed the result of a previous proteomics study (47) in demonstrating higher expression differences of proteasome protein subunits in eggs from Male-biased than Female-biased females using replicated females taken from a natural population. It is also in agreement with a substantial transcriptomics study on the clam Ruditapes philippinarum in which 17,186 transcripts were analyzed using body tissue from clams (46). Two families were compared, one with a sex ratio biased toward males and the other with a female bias. Of reproductive genes (according to Biological Process ontology), five were reported to show significant expression differences between families, one of which was an E3 ubiquitin protein ligase, which had four times higher expression in the male biased family. For genes involved in the ubiquitination process three other significant family biased genes were identified including proteasome subunit alpha 6 (two times higher expression in the male biased family) and an ubiquitin E1 activating enzyme (nine times higher expression in the male biased family). The two proteomics studies and this transcriptomics study thus provide consistent mutually reinforcing results in demonstrating...
higher expression of ubiquitination process genes in association with male bias. It is interesting that proteasome subunit alpha 6 was identified as being more highly expressed in male biased families in both the earlier proteomics study (47) and the clam transcriptomics study (46). If the proteasome itself participates in processes influencing mitochondrial behavior, concomitant increase in all its composite subunits might be expected. This fits in with the results of Table III where three different proteasome subunits are shown all with fold values in the same direction. Given a role for the proteasome in DUI, the higher level of expression in eggs from the Male-biased type is perfectly in accord with the X, W, Z model if the proteasome is considered analogous to the Z factor, which inactivates factor X in the model (Fig 4A), thus allowing retention of the sperm mitochondria and aggregation. For example, the proteasome has deubiquitinating enzymes (75), so a higher expression of proteasome proteins could also result in the removal of the ubiquitin tag factor X, favoring aggregation.

The remaining proteins in Table III can be considered candidates for involvement in DUI even though, unlike proteasome, there are not a priori hypotheses to work from. Identification of the protein is obviously not in itself sufficient reason for the assumption of a functional role in DUI particularly for genes involved in basic cellular function. The evidence for involvement is strengthened for the proteins in Table III. The p values comparing Female-biased and Male-biased types are significant at BH 50%, the a priori p values are significant in the Fisher combined probability test. Also for arginine kinase, ferritin, and enolase, the fold values are in the same direction for the repeated occurrences of the same protein. However significant differences in expression among biased types do not necessarily imply a role as causal agents in the mechanism of sex determination or mitochondrial behavior. They might instead be an indirect and downstream molecular consequence of DUI mechanisms. Of the two proteins with one occurrence in Table III, heterogeneous nuclear ribonucleoprotein is involved in RNA processing (alternative splicing) and transport. Involvement in sex determination in Drosophila (76) and the loggerhead turtle (77), and also in the regulation of mitochondrial transcripts (78) has been reported. This enhances its interest as a candidate for investigation in relation to DUI. The literature on phosphoenolpyruvate phosphomutase is scarce and its possible involvement in DUI unclear. Of the proteins with multiple occurrences, arginine kinase is a phosphotransferase involved in supplying ATP to cellular processes including growth and motility (79). It has been reported in sea-urchin Paracentrotus lividus eggs (80) and high levels observed during oocyte development of a penaeid shrimp suggests an important role in meeting energy demands for cell growth and proliferation (81). It may be an important target for natural selection in invertebrates, for example in the periwinkle Littorina, where clinal variation suggestive of directional selection has been observed (82). Enolase is an enzyme of the glycolytic pathway and its presence in oocytes is an indication of change of energy metabolism following fertilisation (83). Enolase is highly expressed in Xenopus laevis during oogenesis and is involved in changes in the growth properties of a range of cell types (84). It is interesting that all six occurrences of arginine kinase or enolase in Table III show higher expression in the eggs from the Female-biased type. Given the known function of these enzymes these results are consistent with a greater energy need for the processes associated with mitochondrial dispersal and female sex-determination. This provides an a priori hypothesis for future investigation of these or other enzymes of similar metabolic function. Ferritins are involved in iron storage and transport and protection against iron toxicity. Different expression levels have been observed in different stages of oogenesis in in the Coho salmon Onchorhyncus kisutch (85). Another connection between oogenesis and ferritin is the observation that the bacterium Wolbachia is necessary for completion of oogenesis in the wasp Asobara tabida (69). Wolbachia influences iron metabolism and ferritin is up-regulated when Wolbachia is absent, which may lead to apoptosis during oogenesis (86). Of interest also is that in Schistosoma mansoni one ferritin isoform is present at about 15-fold excess in females compared with males (87). The observation that ferritin like protein has higher expression for both occurrences in eggs from Male-biased females is thus of general interest given the endosymbiotic origin of mitochondria. Of the proteins in Table III, only proteasome is observed in common with differentially expressed transcripts in a female and male biased family in the study of the clam Ruditapes philippinarum (46). The difference between the studies could be a function of the differences in tissue used, the clam study used bodies of mature animals whereas eggs alone were used in the current study. However the clams were reproductively mature and transcripts associated with DUI should have been expected a priori in the cDNA libraries prepared from the two families. Given the variety of sex determining mechanisms in mollusks (73), it should not be assumed that the genetic and molecular mechanisms of DUI and sex determination are the same in mussels and clams even if there is a common phylogenetic origin.

The Ubiquitin–Proteasome System, DUI, and Further Work—The possibility that the ubiquitin-proteasome system is involved in DUI has been frequently referred to in studies considering a model for DUI (12, 15, 17, 34, 46), for example that factor W may correspond with the ubiquitination of sperm. In DUI it would be envisaged that the proteasome fails to act against the sperm mitochondria in male embryos thus allowing aggregation and paternal transmission. As stated above, the results of the current study with higher expression of proteasome proteins in eggs from the Male-biased type is more consistent with the proteasome corresponding to factor Z, which eliminates factor X (Fig 4A). Much work needs to be done to establish a mechanism, but it is promising that there is evidence for the importance of the ubiquitin-proteasome
system in oocyte development generally. Four proteins detected by 2-DE as being differentially expressed among different oocyte stages in mice were in the proteasome pathway (42). Proteasome function is required for normal oocyte development in Drosophila (88), and the evidence of marked changes in the localization of the proteasome during the course of meiosis in yeast (89) resonates with the spatial nature of the mitochondrial aggregation phenomenon in DUI. Further experimental work could involve knockdown or use of inhibitors. There are many examples of the use of inhibitors to establish function of proteasome components in developing oocytes (e.g. 88, 90). The degradation of sperm mitochondria was blocked in fertilized porcine oocytes treated with specific ubiquitin-proteasome pathway inhibitors (45). In Mytilus, similar experiments would be feasible. For example treatment of eggs with an inhibitor before or at fertilisation from females known to produce a high proportion of embryos with aggregated mitochondria is predicted to produce a shift toward dispersed embryos, and lack of the M genome in older larvae, with concomitant proteomic changes. Inhibitors might also be used to explore the role of arginine kinase and enolase. Other environmental interventions and manipulations might also be applied, for example the use of supplements as in the study of the effect of iron on the expression of Wolbachia bacteriofer-ritin (86). Mussels held in the aquarium could be treated with inhibitors or supplements, and biochemical and molecular effects measured on eggs or fertilized larvae and assessed together with cytological observations and breeding data.

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