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Profiling of gender-regulated gene transcripts in the filarial nematode *Brugia malayi* by cDNA oligonucleotide array analysis

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Abstract

Microarray technology permits high-throughput comparisons of gene expression in different parasite stages or sexes and has been used widely. We report the first use of this technology for analysis of gene expression in filarial male and female worms. The slide array (comprised of 65-mer oligos representing 3569 EST clusters) was spotted with sequences selected from the extensive *Brugia malayi* EST database (http://zeldia.cap.ed.ac.uk/fgn/brugia.php). Arrays were hybridized with Cy dye labeled male and female cDNA. The experimental design included both biological and technical (dye-flip) replicates. The data were normalized for background and probe intensity, and the relative abundance of hybridized cDNA for each spot was determined. Genes showing two-fold or greater differences with $P < 0.05$ were considered gender-regulated candidates. One thousand one hundred and seventy of 2443 clusters (48%) with signals above threshold in at least one sex were considered as gender-regulated gene candidates. This included 520 and 650 clusters up-regulated in male and female worms, respectively. Fifty of 53 (94%) gender-regulated candidate genes identified by microarray analysis were confirmed by real-time RT-PCR. Approximately 61% of gender-regulated genes had significant similarity to known genes in other organisms such as *Caenorhabditis elegans*. Many *C. elegans* homologues of these genes have been reported to have reproductive phenotypes (sterility or abnormal embryo development) by RNA interference. This study has provided the first broad view of gender-regulated gene expression in *B. malayi*; this should lead to improved understanding of reproduction in filarial nematodes. More generally, this approach holds great promise as a means of studying stage-specific or tissue-specific gene expression in parasitic nematodes.
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1. Introduction

Filarial nematodes cause important tropical diseases in humans such as onchocerciasis (“river blindness” caused by *Onchocerca volvulus*) and lymphatic filariasis (“elephantiasis” caused by *Brugia malayi*, *B. timor* and *Wuchereria bancrofti*). The parasites are dioecious, and they exhibit marked sexual dimorphism. Brugia adult worms live in lymphatic vessels. The ovoviviparous females release microfilariae (MF) that are ingested by insects; these are essentially equivalent to the L1 stage of other nematodes. Microfilariae molt twice in competent insect vectors to become infective stage larvae (L3) that are infective to humans. L3 molt twice in the human host and to become adult worms that are reproductively active for years. Obviously, parasite reproduction is necessary for transmission, and transmission is necessary for reproduction. Improved understanding of the reproductive biology of filarial worms may provide new insights into the epidemiology of filariasis and lead to new tools for controlling these diseases. Basic research may identify new

Abbreviations: RT-PCR, reverse transcription polymerase chain reaction; RNA, ribonucleic acid; EST, expression sequence tag; MSP, major sperm protein; PMT, photomultiplier tube

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targets for intervention that interfere with egg, microfilaria, or sperm production [1,2]. Filariasis researchers stand to benefit from extensive functional genomic studies that have been performed on the free-living nematode *Caenorhabditis elegans* [3,4]. Since filarial parasites share many genes with *C. elegans* [5], information available on genes involved in reproduction in *C. elegans* is an important starting point for parallel studies in filarial worms [6]. However, there are important differences between filarial worms and *C. elegans*. For instance, while adult filarial worms exist as males (XY) and females (XX), *C. elegans* has males and hermaphrodites but no true females and no Y chromosome.

The large expressed sequence tag (EST) database and extensive genomic sequence information available for *B. malayi* [7–9] provide a solid foundation for studying the molecular biology of this organism using gene expression profiling and functional genomics approaches [10–13]. We have previously identified a small number of sex-regulated genes in *B. malayi* worms by differential display RT-PCR and real-time RT-PCR [11,12]. One of these genes (microfilaria sheath protein) was recently shown to be essential for normal production of microfilaria by RNA interference (RNAi) [13]. However, the pace of progress with this “molecule by molecule” approach is limited.

Microarrays can be used to rapidly assess and quantitate relative levels of expression of thousands of genes in parallel [14,15]. Early experience with small-scale arrays has demonstrated the potential value of this approach for studying gene expression in parasites [16–19]. We now report production and use of the first large-scale oligonucleotide microarray for a nematode parasite. We chose to first evaluate the array with a study of gender-linked expression because of prior work on this area in *B. malayi* and in *C. elegans*. Our results show that oligonucleotide microarray analysis is a reproducible, rapid, and highly efficient method for profiling gender-associated gene expression in *B. malayi*.

2. Materials and methods

2.1. Parasite materials

Adult *B. malayi* worms were isolated from the peritoneal cavity of infected jirds (*Meriones unguiculatus*) obtained from the NIAID Filariasis Repository (University of Georgia, Athens, GA). Male and female worms were carefully separated by size; broken worms were discarded. The worms were washed and immediately frozen at −80 °C.

2.2. RNA isolation and probe preparation

Worms (usually 30 female and 100 male adult worms per batch) were crushed under liquid nitrogen with a ceramic mortar and pestle and extracted in TRIzol reagent (Invitrogen, Carlsbad, CA) as previously described [12]. The accuracy of worm gender separation was assessed by RT-PCR with *B. malayi* major sperm protein primers (*BmMSP*) and *B. malayi* embryo-associated fatty acid-binding protein primers (*Bm-FAB*-1) [11]. RNA quality was assessed with a model 2100 Bioanalyzer (Agilent, Palo Alto, CA). cDNA was synthesized from 5 to 7 μg each of male and female total RNA samples using 3DNA capture sequence primers (3DNA Array 350 Detection system, Genisphere, Hatfield, PA) and SuperScript II Reverse Transcriptase (Gibco BRL, Gaithersburg, MD) for each probe according to standard protocols. cDNA was concentrated by Microcon YM-100 filter (Millipore) and either used immediately or stored at −80 °C. cDNA was synthesized from two different male and female RNA samples (independently prepared as biological replicates).

A two-step protocol was used for hybridization (3DNA Array 350 Detection system, Genisphere, Hatfield, PA). First, oligo arrays were hybridized to the cDNA probes in 2× SDS based-hybridization buffer and washed in 2× SSC, 0.2% SDS according to the manufacturer’s protocol. Fluorescent Cy3- and Cy5-capture reagents were combined in hybridization buffer and added to each array. The arrays were incubated and washed as above. Each experiment consisted of a pair-wise competitive hybridization of cDNA samples from female and male worms and a reciprocal dye-flip replicate. Since biological duplicates were tested, a total of four DNA microarrays were used for comparison of the two types of cDNA.

2.3. Microarray fabrication

B. malayi clusters for arrays were selected from 8392 clusters generated by the Filarial Genome Project and posted at the website http://zeldia.cap.ed.ac.uk/fgn/brugia.php [7]. These clusters were derived from 15 cDNA libraries that represent the major *B. malayi* life cycle stages. They represent approximately 40% of the total number of predicted genes for *B. malayi* [8]. Clusters with multiple ESTs, or with detectable similarity to proteins in public databases using BLAST [20], and sequence permitting design of a unique 65-mer oligonucleotide were chosen for inclusion on the array. Oligonucleotides were synthesized from the consensus sequence of selected clusters ($n = 3569$) by standard methods by Illumina (San Diego, CA). The oligonucleotides (50 nM in 3× SSC with 0.75 M betaine) were printed in duplicate on MWG Epoxy slides (MWG Bioteche Inc, High Point, NC) by a locally constructed linear servo arrayer (after the DeRisi model, http://derisilabs.ucsf.edu/).

2.4. Data processing and analysis

Slides were scanned immediately after hybridization on a ScanArray Express HT Scanner (Perkin-Elmer, Boston, MA) to detect Cy3 and Cy5 fluorescence at 543 and 633 nm, respectively. Laser power was kept constant for Cy3/Cy5 scans, and photomultiplier tube values were 69 and 60 V, respectively. An additional scan was done for each slide with the PMT set for 54 and 46 V. The high PMT scan was done in order to maximize signal from low intensity spots. Likewise,

the low PMT scan was done in order to characterize differential hybridization for high intensity spots which appeared saturated in the high PMT scan. Griding and analysis of images was performed with ScanArray software Express V2.0 (Perkin-Elmer, Boston, MA). Each spot was defined on a pixel-by-pixel basis, using a modified Mann–Whitney statistical test. The resultant values were background subtracted and Lowess [21] normalized by using GeneSpring 6.1 software (Silicon Genetics, Redwood City, CA). Twenty percent of the data were used to calculate the Lowess fit at each point. Oligonucleotide elements that received "present" (intensity > 200 intensity unit or the signal/background > 2) calls in all four microarrays and displayed > 700 or > 127 background subtracted intensity units (high or low PMT settings, respectively) in two of four channels for either Cy3 or Cy5 were identified, and all others were excluded from the analysis. The $\log_2$ ratio of median dye intensities for each remaining element was averaged across all four microarrays. Data were filtered using the Student's *T*-test function in GeneSpring. Genes with differences with *P*-values <0.05 in either the high or the low PMT scans and that had arithmetic ratios ≥ 2.0 were considered to be significantly gender-regulated. The best *P*-value obtained with high or low PMT scans and its accompanying arithmetic ratio were reported. Multiple testing corrections were not used in order to minimize type-2 error.

2.5. Real-time RT-PCR to confirm selected gender-regulated candidate genes identified by microarray analysis

Selected gender-regulated gene candidates from the microarray analysis were chosen for confirmation studies by real-time RT-PCR as previously described in detail [12]. Briefly, complementary DNA primers (primer sequence information is available upon request) were designed from EST sequences obtained from GenBank with Primer Express software (Version 1.0, PE Applied Biosystems, Foster City, CA). The PCR reactions were carried out in 96 well microtiter plate wells in a 25 μl reaction volume with SYBR Green Master Mix (PE Biosystems, Forster City, CA) with optimized concentrations of specific primers. Experimental design and relative quantification calculations were carried out according to RQ software (Relative Quantification (RQ) ABI Prism 7000 Sequence Detection System). Nicotinamide adenine dinucleotide dehydrogenase subunit 1 (BMC02280, NADH subunit 1) was used as a not gender-regulated internal control [12].

2.6. Homology search of B. malayi genes

A similarity search was performed using WU-BLASTX and WU-TBLASTX (https://blast.wustl.edu); [20] with 1170 gender-regulated cluster consensus sequences as queries versus multiple databases including *C. elegans* proteome (Wormpep version 132) (Wellcome Trust Sanger Institute, unpublished), non-redundant protein GenBank (11/14/04) [22] and nematode nucleotide sequences [23] with *B. malayi* ESTs removed. Homologues were reported for *E*-values less than or equal to 1e − 20.

To identify cases in which *C. elegans* homologues of *B. malayi* clusters have been surveyed for phenotypes in *C. elegans* using RNAi, the clusters with significant matches to *C. elegans* proteins were cross-referenced to a list of 17913 *C. elegans* genes for which information on RNAi-induced phenotypes was available (http://www.wormbase.org/) [24,25]. All matches with similarities with *E*-values less than or equal to 1e − 20 were reported.

3. Results

3.1. Gender-regulated gene transcripts were reproducibly detected by oligo arrays

As our study represents the first use of oligonucleotide microarrays to study gene expression in *B. malayi*, we took great care to ensure the validity of our results. Our first objective was to ensure that similar levels of high quality total RNA was isolated from each sexually mature parasite population. Bioanalyzer 2100 results showed that all RNA samples isolated from male and female worms had equivalent quality and displayed minimal degradation (data not shown). Additional evidence that equivalent quantities of male and female total RNA were used for each hybridization experiment was obtained by examining relative expression levels of internal controls [NADH subunit 1 (BMC02280) and histone H3 (BMC00764)] previously shown to be expressed equally in both sexes by real-time RT-PCR [12]. Expression of these control genes was equal in male and female worms by microarray analysis.

3.2. Expression profiling

A complete list of clusters for the arrays, oligonucleotide sequences, and hybridization data is posted at the NCBI GEO database [26] http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GPL1483. Of 3569 clusters represented on the array, 2443 had hybridization signals above the threshold for at least one sex. One thousand one hundred and seventy (48% of these clusters and 33% of total clusters on the microarray) met our criteria for gender-regulated expression (two-fold or greater differences and *P*-values < 0.05) (Fig. 1). Of 1170 clusters, 455 clusters (39%) had no significant similarity to known proteins in other organisms. This is somewhat higher than the percentage of novel genes represented on the array (1160 of 3569 clusters or 32.5%). The differentially expressed clusters included 520 and 650 clusters up-regulated in male and female worms, respectively. The frequency distribution of higher:lower expression ratios is shown in Fig. 2. Although there were more female up-regulated gene candi-

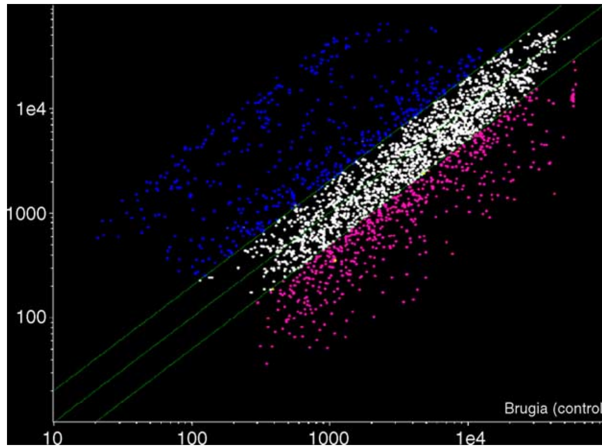

Fig. 1. Expression profiling of clusters from *Brugia malayi*. This is scatter plot of the mean normalized fluorescence intensity values from the male versus female comparison derived from the low PMT scan. Expression values that are ≥2-fold (the flanking green lines) and $P < 0.05$ in male are blue (upper left) and those that are ≥2-fold higher and $P < 0.05$ in females are dark pink (lower right). White spots are less than two-fold unregulated. Spots outside the two-fold line that are not red or blue have a $P > 0.05$.

dates, expression ratios for male up-regulated genes tended to be higher. The median expression ratios for male and female up-regulated gene candidates were 3.88 and 3.01, respectively. The frequency of male up-regulated clusters with expression ratios of 10-fold or greater was much higher (169 of 520, or 32.5%) than that in female-enriched clusters (57 of 650, or 9%) ($P < 0.001$ by χ^2). Functional class information for male up-regulated and female up-regulated clusters with significant similarity to known genes is listed in Table 1. Functional classes for the most highly male or female up-regulated known genes (expression ratios ≥10) in *B. malayi* are shown in Table 2.

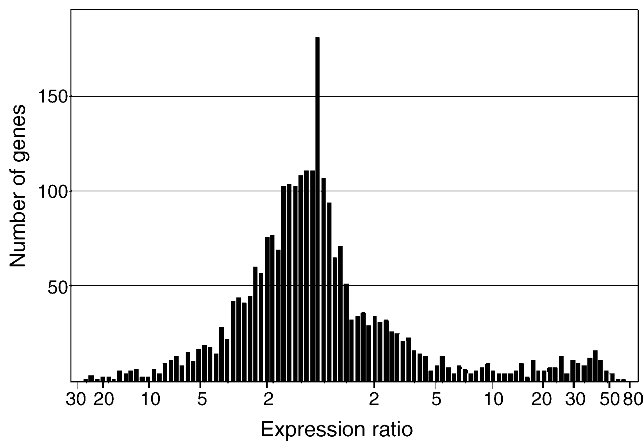

Fig. 2. Frequency distribution of expression ratios obtained from *B. malayi* clusters on a microarray probed with male- and female-specific cDNA. The signal intensity when probed with Cy5-labelled cDNA derived from female worms was divided by that of the Cy3-labelled cDNA derived from males or vice versa and then $\log_2$ transformed. Expression ratios were plotted on the abscissa and frequency was plotted on the ordinate.

Table 1
Number of gender-regulated clusters by functional class

Description	Male	Female
Cytoskeleton	28	0
Muscle	5	2
Major sperm protein	8	0
Oxidation-reduction	11	0
Protease	4	0
AvL3-1	4	0
Zinc-finger proteins	3	0
DNA synthesis	5	0
NADH dehydrogenase subunit 5 and 6	4	0
Protein synthesis	6	27
Phosphatase/kinase	7	15
ATP synthase	0	4
Citric acid cycle enzymes	0	8
Histones	0	6
Egg/embryo development	0	5
Brugia serine proteinase inhibitors (*Bm-spn*-1 and 2)	0	4
Chaperonin/heat shock protein	0	5
GTP-binding/Ras-related	0	6
Receptors (hormone, sperm and nuclear)	0	3
Ion channels	0	2

3.3. Independent confirmation of gender-regulated genes identified by the oligo array with real-time RT-PCR

To verify the gender-regulated genes identified by the arrays, we examined gene expression in adult male and female worms by real-time RT-PCR analysis. Fifty-five gender-regulated gene candidates identified by microarray analysis were selected for real time RT-PCR analysis. Twenty-eight of these genes were up-regulated in female

Table 2
Highly gender-regulated, known proteins in *B. malayi* adult worms

Description	Male	Female
Major sperm protein (MSP)/MSP-like	12	0
Serine/threonine kinase	4	0
Phosphatase	4	0
PDZ/LIM/PAZ-domain	4	0
Tyrosin protein	2	0
Protein phosphatase/kinase	4	0
Zinc-finger	2	0
Trehalase	2	0
Homolog of human gonadotropin release hormone receptor	2	0
Sheath proteins	0	4
PAN-domain protein	0	1
Ion-channel	0	2
Nuclear receptor	0	2
Caveolin/High mobility protein/Glutathione	0	3
Others	24	8
Total	60	20

Expression ratio >10.

Table 3
Real-time RT-PCR to confirm selected sex-regulated candidate genes identified by microarray analysis

	Identified by array	Confirmed by real-time RT-PCR	Identified by real-time RTPCR	Confirmed by array
Female	27	27	9	9
Male	26	23	8	8
Total	53	50 (94%)	17	17 (100%)

worms and 27 were up-regulated in males. Expression ratios for these genes ranged from 2- to 44-fold by microarray analysis. Two genes gave no satisfactory results with the primers used. Fifty of the 53 remaining genes (94%) were confirmed by real time RT-PCR to have gender-regulated expression in the same direction as was observed by microarray analysis (Table 3). We also examined microarray results for clusters previously identified as having gender-regulated expression by real-time RT-PCR [12]. All of the genes with gender-regulated expression by real-time RT-PCR were verified by microarray analysis (Table 3). A strong correlation was observed between fold difference values obtained by microarray analysis and log fold differences by real time RT-PCR (Fig. 3).

3.4. Male up-regulated clusters

Of the 520 candidate male up-regulated clusters, 320 (62%) had significant similarity to known genes in other organisms (Suppl. Table 1). Of these, 210 (66%) had significant similarity to genes in *C. elegans* (Suppl. Table 1). One hundred and sixty-nine of these (80%) have been studied by RNAi in *C. elegans* [24,25] (Suppl. Table 2), of which, 57 (57/169; 34%) had a reproduction phenotype such as sterility.

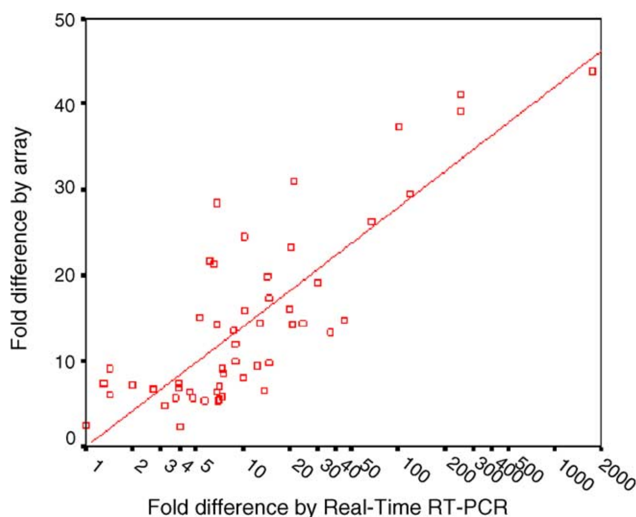

Fig. 3. This semi-log plot shows the relationship between gene expression values (expressed as fold-differences) obtained by microarray and by real-time RT-PCR for selected genes with gender-biased expression by microarray (Spearman rank correlation coefficient, 0.73, $P < 0.001$).

Male up-regulated clusters can be divided into four groups: (1) genes previously reported to be male up-regulated in filarial parasites, such as major sperm proteins (MSPs) [11,12]; (2) genes reported to be male up-regulated in other nematodes, such as a transcription factor (zinc finger, BMC07636 and BMC98240), a malate dehydrogenase (BMC04971), and a serine/threonine phosphatase (BMC10182) [18,27–29]; (3) genes that were newly identified as male up-regulated, such as genes with sequence similarity to muscle and cytoskeleton proteins (e.g. myosin, paramyosin, intermediate filament, troponin and actin) and genes related to cellular and extracuticular extracellular metabolism (e.g. cuticular glutathione peroxidase (BMC02584, BMC06710 and BMC12436), cytochrome oxidases (BMC04127)); (4) novel genes (38% of the total). MSP proteins and MSP-like domain proteins, serine/threonine phosphatases and kinase were most abundant among highly male-biased expressed known genes (expression ratios ≥ 10, Table 2); these accounted for 20 and 10% of the total, respectively. The *B. malayi* homologues (BMC06058 and BMC11125) of *C. elegans* gene coding for F54D7.3 protein (ortholog of the human gene gonadotropin release hormone receptor gene) were also highly up-regulated in males.

3.5. Female up-regulated clusters

Of the 650 candidate female-regulated clusters, 395 (61%) encoded proteins with homologues in other organisms (Suppl. Table 3). Of these, 303 clusters (77%) had a *C. elegans* homologue (Suppl. Table 3). Two hundred and thirty-seven of these genes (237/303; 79%) have been studied by RNAi in *C. elegans* (Suppl. Table 2) [24,25], and 112 (112/237; 47%) of them had phenotypes. Most phenotypes were related to embryogenesis or embryo development. Female up-regulated clusters with significant similarity to known genes fell into four groups: (1) genes previously confirmed as being female up-regulated including microfilarial sheath protein, high mobility protein, fatty acid binding protein and caveolin in filarial nematodes [12,13]; (2) genes previously reported to be female up-regulated in other nematodes. These included genes involved in transcription and translation (e.g. nuclear hormone receptors, RNA polymerase, tRNA synthetase, ribosomal proteins and elongation factor), protein processing/trafficking (heat shock proteins and chaperonin), and genes involved in embryonic development and growth in *C. elegans* (e.g. protein F52B11.3-BMC07645, BO250.1-BMC00336 and F33H1.4-BMC09458); (3) genes newly identified as female-regulated including those involved in nematode reproduction (such as egg receptor for sperm, *B. malayi* serine proteinase inhibitor, collagen, multi-drug resistance factor, and tubulin); (4) novel genes (39% of the total). Known genes with expression ratios ≥ 10 are listed by functional class in Table 2. Female up-regulated genes identified previously such as sheath proteins, caveolin and high mobility protein were also highly female up-regulated in this study.

4. Discussion

This paper presents the first use of oligonucleotide microarrays to profile gene expression in a filarial nematode. We focused on gender in this paper because of the relative ease of obtaining RNA from adult worms and the availability of pilot data on gender-biased genes from our prior work [11,12]. Our results show that gene expression profiles of ∼48% (1170/2443) of clusters that hybridized with cDNA from adult worms were gender-regulated. This is a higher percentage than that reported for *C. elegans* (∼12%) [27] and lower than that reported for *Trichostrongylus vitrinus* (72%) [18]. These differences may be due to selection bias of genes represented on the chips and may not reflect biological differences between these nematodes. The *C. elegans* microarray contained 17,871 genes and represented about 94% of the 18,967 genes annotated, while the *T. vitrinus* microarray contained only 716 ESTs from gender-selected cDNA libraries. There are also biological differences between these nematode species. For example, the uteri of mature *Brugia* female worms are often full of microfilariae, which are essentially equivalent to the L1 stage in *C. elegans* and *T. vitrinus*. Obviously, female up-regulated genes in *B. malayi* will include genes expressed by developing microfilariae.

Our experiments identified 520 male up-regulated genes and 650 female up-regulated genes with expression ratios ≥2 that were significant at the 95% confidence level. Considering that females produce eggs and embryos, we expected to find many more female up-regulated clusters than male up-regulated clusters. The finding that the mean expression ratio for male up-regulated clusters was higher than that for female up-regulated clusters was consistent with results reported for male up-regulated clusters in *C. elegans* [27].

Several lines of evidence support the validity of microarray results obtained in this study. First, reproducibility was good in biological replicate samples. Seventy-nine percent of the genes with gender-regulated expression in one biological sample also had gender-regulated expression in a second biological sample. Second, 94% of selected gender-regulated clusters identified by microarray were confirmed independently by real-time RT-PCR (Table 3). Third, all genes previously reported to have gender regulated expression by real-time RT-PCR [12] were confirmed to have gender regulated expression by microarray, and there was a strong correlation between fold-difference values obtained by these two methods. Fourth, many of the genes identified in our array experiment have reproductive phenotypes in *C. elegans*.

4.1. Gene expression in males

Thirty-eight percent (199/520) of the male-regulated *B. malayi* clusters were novel. Forty percent (210/520) of the male-regulated clusters had a *C. elegans* homologue, and many of these have been reported to be male up-regulated in *C. elegans* when compared with hermaphrodites [27]. Genes involved in reproductive processes (e.g. major sperm proteins), molecules in energy storage and supply, cytoskeleton and muscle-related molecules, and enzymes in cellular metabolism especially in oxidation-reduction (cuticular glutathione peroxidase and cytochrome oxidase) were most notable amongst male up-regulated annotated clusters. The differential expression of these transcripts in male worms is likely to be related to their biological functions in reproduction.

Major sperm proteins (MSPs) are nematode-specific cytoskeleton proteins comprising ∼15% of total sperm protein [30,31]. These are among the most highly represented ESTs in *B. malayi* [8]. MSPs were also highly represented in male-enriched datasets for both *T. vitrinus* and *C. elegans* [3]. MSPs are involved in the movement of the amoeboid nematode sperm [32]. Recent studies show that MSPs also have a signaling role in oocyte production and maturation in *C. elegans* [33,34]. The retention of this MSP signaling function in *Ascaris suum* [34] suggests that this may also be present in filarial nematodes. Thus, the finding that 20% of highly male-up-regulated clusters in our study were either MSPs or homologues of *C. elegans* genes that contain an MSP domain is impressive but not surprising (Table 2).

In addition to MSPs, clusters with significant similarity to *C. elegans* serine/threonine kinases and phosphatases were also common among highly male up-regulated transcripts (Table 2). These genes are also highly expressed in *C. elegans* germ line tissue [3] and in the *T. vitrinus* male-selected cDNA library [18]. These enzymes might play important roles in regulating sperm maturation by post-translational modification [3] and in signaling cascades or protein modification within the oocyte following fertilization [1,29]. Knockouts of the *C. elegans* homologue of a male-enriched serine/threonine protein phosphatase gene *Od-mpp*1 from the parasitic nematode *Oesophagostomum dentatum* caused sterility of hermaphrodites [1,29] through impaired sperm function [35]. The *B. malayi* homologue of this serine/threonine phosphatase (BMC10182) was highly up-regulated in males with expression ratios of 37 by microarray and 101 by real-time RT-PCR.

Several other groups of male up-regulated clusters contained special domains thought to be related to reproductive processes. For instance, *B. malayi* homologues of the *C. elegans* PDZ-domain (C25G4.6, pfam00595) (clusters BMC11959 and BMC04055) were highly male up-regulated (expression ratios ≥20). This is consistent with the findings in the *T. vitrinus* male-selected cDNA library and in *C. elegans* microarray experiments [18,27]. The PDZ domain, a common modular protein-interaction domain, is believed to be involved in diverse signal transduction pathways [36]. RNAi of the *C. elegans* homologue with a PDZ domain (C25G4.6) caused sterility in the treated worms or their progeny, suggesting a role in gamete development [24,35]. In addition to the PDZ domain containing proteins, two other clusters highly expressed in *B. malayi* males had significant similarity to a PAZ domain (pfam02170) containing protein in *C. elegans* (ZK757.3B). This domain is found in the *PIWI*-domain gene

family that is essential for producing and maintaining germline stem cells responsible for gametogenesis in *Drosophila* [37]. *PIWI*-domain gene family members are enriched in the *C. elegans* germline-intrisic group (genes expressed similarly in germ lines making only sperm or only oocytes) [3].

Several cytoskeleton and muscle-related molecules were male up-regulated with ratios ranging from 2 to 7. These included 33 clusters with sequence significant similarity to myosin, paramyosin, troponin and actin (Table 1). Genes encoding tegument-associated molecules such as actin, tropomyosin and dynein light chain have previously been shown to be differentially expressed in adult *Schistosoma mansoni* males [38,39]. The explanation suggested for this was that males are larger (with more tegument and muscle) and more active in copulation than females [40,41]. However, this may not apply to *B. malayi*, since *Brugia* females seem to be as active as males in vitro, and there is no obvious difference in relative muscle content.

Another possible muscle-related male up-regulated *B. malayi* cluster (BMC01860) is a homologue of *C. elegans* gene Y1A5A.1, a male-enriched LIM domain (pfam00412) containing protein with a homologue in *T. vitrinus* [3,18,27]. Onchocerciasis patients and animals vaccinated with irradiated filarial L3 produce strong antibody responses to a related protein cloned from the filarial worm *Acanthocheilonema viteae* (AvL3-1) [42]. LIM domain-containing proteins are involved in diverse biological processes including cytoskeleton organization and cell lineage specification by mediating protein-protein interactions. The reported localization of OvL3-1 (the *Onchocerca volvulus* homologue of AvL3-1) in muscle tissue [43] suggests that this LIM domain protein may interact with muscle proteins.

Several genes encoding enzymes involved in energy production (NADH dehydrogenase subunit 5 and 6, BMC12393, BMC5921) and β-oxidation of fatty acid oxidation (3-ketoacyl-CoA thiolase, BMC07758) were highly expressed in male worms. Enzymes involved in glycolysis and the citric acid cycle were also male up-regulated in *C. elegans* and *T. vitrinus* [3,18]. These findings may reflect the high energy required for sperm motility, since fatty acids provide an efficient energy source. In addition to these enzymes, two *Brugia* clusters with high male expression ratios were homologous to *C. elegans* trehalases. The disaccharide trehalose has been proposed to have essential physiological functions in nematodes as a source of energy and as a protectant against environmental stress [44]. Increased trehalose transcription may reflect an increased energy requirement in male worms or their sperm.

4.2. Gene expression in females

Thirty-nine percent (255/650) of female up-regulated *B. malayi* clusters were novel. Forty-seven percent (303/650) of the female up-regulated clusters had significant similarity to genes from *C. elegans*. Many of these *C. elegans* genes were highly expressed in embryos by microarray experiments [4], and 47% (112/237) of those studied by RNAi had phenotypes such as embryo lethality, growth defects and post-embryonic defects. Many of the annotated female up-regulated clusters in our study were associated with embryonic development (fatty acid binding protein, chaperonin-like protein), eggshell formation (microfilaria sheath protein), factors involved in transcription and translation (RNA polymerase, tRNA-synthetase, ribosomal proteins), and energy supply (ATP synthase and enzymes in the citric acid cycle).

A few of these genes deserve more comment. A gene coding for a lipid-binding protein (*Bm-FAB*-1), has been previously shown to have female up-regulated expression [11,12]. Its localization in perivitelline fluid of developing embryos within the uteri of adult female worms supports the hypothesis that this protein is involved in transfer of fatty acids to developing embryos [45]. The gene coding for microfilaria sheath protein (*Bm-SHP*-1), an abundant eggshell protein, has previously been shown to be female up-regulated in filarial parasites [12], and the inactivation of MF sheath protein by RNAi in adult female *B. malayi* has been reported to inhibit MF release from female worms [13]. Chaperonins such as heat shock protein 70 (Bm-HSP-70) have been reported to have female up-regulated expression in other nematodes [18]. Chaperonins are large, multi-subunit proteins that facilitate the protein-folding process by providing a protected environment. Recent studies show that these molecules play a role in transduction and regulation of cGMP levels [46], and that they are essential for embryonic development in *C. elegans* [47,48]. The notable female up-regulated expression of a heat shock gene (*Bm-shp*-70) and chaperonins in our study suggests that these molecules may play a parallel role in *B. malayi* reproduction and perhaps also in protein synthesis. The fact that other genes required for protein synthesis had female up-regulated expression is consistent with a high requirement for protein synthesis for egg production and larval development in female worms.

Several clusters with significant similarity to genes encoding histones 2 and 4 were expressed in a female up-regulated manner in *B. malayi*. Histones comprise a family of closely related, basic DNA-binding proteins that play a role in gene regulation. Several histones and histone-like proteins are differentially expressed between males and hermaphrodites in *C. elegans* [20]. RNAi of *his*-71, a histone-like gene with a female-enriched homologue in *T. vitrinus*, resulted in embryonic lethality in *C. elegans* [24,25].

Interestingly, genes coding for two *B. malayi* serine proteinase inhibitors (*Bm-spn*-1 and *Bm-spn*-2) had female up-regulated expression by microarray. *Bm-spn*-1 has been reported to be enriched in filarial L3 [49,50], and *Bm-spn*-2 has been reported to be expressed only by microfilariae [51]. Increased expression of *Bm-spn*-2 in female worms suggests that this gene is also expressed in developing larvae and microfilariae within female worms. *Bm-spn*-2 has been suggested to have a role in immune evasion in filarial parasites [52]. Related mammalian proteins have been reported to be involved in remodeling of germ line tissues and

migration of germ cells [53]. Additional studies are needed to establish whether these proteins are necessary for filarial reproduction.

Several clusters with significant similarity to transcription regulatory elements, (including high motility group proteins and Y-box factor), had female up-regulated expression, while clusters encoding transcription factors such as zinc finger proteins (BMC07636, BMC06355) were up-regulated in male worms. These elements may be involved in coordinated expression of gender-regulated transcripts in *Brugia*.

In conclusion, microarray analysis has provided the first broad view of gender-regulated gene expression in *B. malayi*. It is likely that many of these genes are involved in reproduction (gamete production, gamete function, and embryo development). Much more work will be required to understand how the products of these genes function and interact and to explore their potential as new drug targets. However, we believe this work represents an important step toward a deeper understanding of reproduction in filarial worms. More generally, this approach holds great promise as a means of studying stage-specific or tissue-specific gene expression in parasitic nematodes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2005.05.005.

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