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Phenotypic and molecular analysis of the effect of 20-hydroxyecdysone on the human filarial parasite *Brugia malayi*

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Abstract

A homologue of the ecdysone receptor has been identified and shown to be responsive to 20-hydroxyecdysone in *Brugia malayi*. However, the role of this master regulator of insect development has not been delineated in filarial nematodes. Gravid adult female *B. malayi* cultured in the presence of 20-hydroxyecdysone produced significantly more microfilariae and abortive immature progeny than control worms, implicating the ecdysone receptor in regulation of embryogenesis and microfilarial development. Transcriptome analyses identified 30 genes whose expression was significantly up-regulated in 20-hydroxyecdysone-treated parasites compared with untreated controls. Of these, 18% were identified to be regulating transcription. A comparative proteomic analysis revealed 932 proteins to be present in greater amounts in extracts of 20-hydroxyecdysone-treated adult females than in extracts prepared from worms cultured in the absence of the hormone. Of the proteins exhibiting a greater than two-fold difference in the 20-hydroxyecdysone-treated versus untreated parasite extracts, 16% were involved in transcriptional regulation. RNA interference (RNAi) phenotype analysis of *Caenorhabditis elegans* orthologs revealed that phenotypes involved in developmental processes associated with embryogenesis were significantly over-represented in the transcripts and proteins that were up-regulated by exposure to 20-hydroxyecdysone. Taken together, the transcriptomic, proteomic and phenotypic data suggest that the filarial ecdysone receptor may play a role analogous to that in insects, where it serves as a regulator of egg development.

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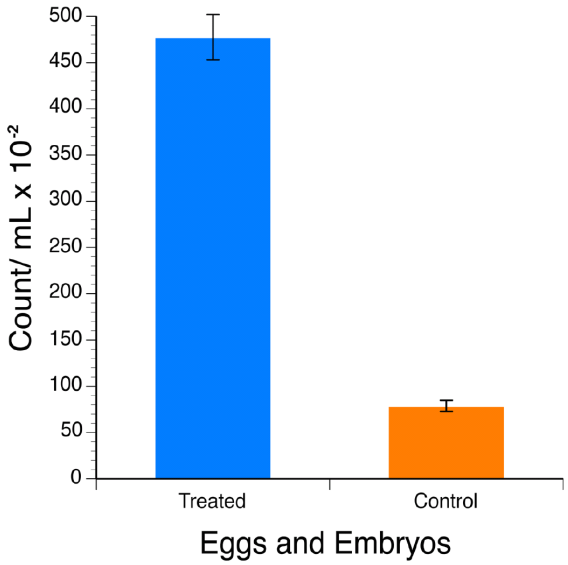
Graphical abstract



Keywords

Filariasis; Elephantiasis; Transcriptomics; Proteomics; Nematode; RNA-seq

1. Introduction

Human filarial parasites cause diseases that inflict significant morbidity upon a large proportion of the poorest people on the planet (World Health Organization (WHO), 2010). Lymphatic filariasis (caused by infection with *Brugia malayi*, *Brugia timori* or *Wuchereria bancrofti*) and onchocerciasis (caused by infection with *Onchocerca volvulus*) together result in the loss of 5.7 million disability adjusted life years (Mathers et al., 2007). As a result, these diseases have been identified by the international community as two of the five Neglected Tropical Diseases (NTDs) worldwide, and both have been targeted for elimination by the international community in the London Declaration on Neglected Tropical Diseases (Turner et al., 2014).

Elimination programs targeting both onchocerciasis and filariasis have been implemented at the national and international levels. All rely primarily upon a strategy of mass drug distribution to interrupt transmission and thereby eventually locally eliminate the parasite (Cupp et al., 2011). However, these programs rely upon the small arsenal of drugs that must be given over a long period of time (i.e., years). This leaves the programs vulnerable to failure in the face of developing resistance. Furthermore, the prolonged treatment courses necessary for effective elimination present substantial logistical difficulties resulting from the need to maintain high drug coverage rates over a long period of time (i.e., years). The current drug regimens used by these programs face limitations in deployment in many areas. For example, diethylcarbamazine (DEC), a drug commonly used to treat lymphatic filariasis, produces severe ocular and systemic complications when given to individuals infected with

O. volvulus (Awadzi, 2003). This precludes the use of DEC in much of Africa, where lymphatic filariasis and onchocerciasis are co-endemic. Similarly, treatment of onchocerciasis using ivermectin is complicated in areas that are co-endemic for the eye-worm *Loa loa*, as severe adverse events have been documented to occur in individuals treated with ivermectin that are heavily infected with *L. loa* (Twum-Danso, 2003). For these reasons, there is an urgent need to develop alternative therapeutic interventions to augment the efforts of the elimination programs.

Ecdysteroids have long been known to play a central role in controlling the development of various invertebrates. They have been best characterized in insects. These hormones exhibit their effects through the activity of ecdysteroid receptors, which act as master transcriptional regulators (Koelle et al., 1991; Baehrecke, 1996). In insects, juvenile hormone and ecdysone regulate both egg development and the molting cycle. As juvenile hormone levels decrease, there is a surge in ecdysone levels leading to molting (Riddiford, 1993). This effect is mediated through a heterodimer of the ecdysone receptor (EcR) and the retinoid X receptor (RXR), two members of the nuclear hormone receptor family of transcriptional regulators (Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1993). The fact that molting and the receptors controlling this process are not found in vertebrates makes this process an attractive potential chemotherapeutic target.

A homolog of the EcR has been identified and shown to be active in *B. malayi* (Tzertzinis et al., 2010). However, its physiological role in controlling the developmental processes in this parasite remains unclear (Mendis et al., 1983; Tzertzinis et al., 2010). In an attempt to decipher the physiological role of the *B. malayi* ecdysone receptor (*Bma*EcR), we have conducted transcriptomic, proteomic and phenotypic studies of the effect on 20-hydroxyecdysone (20E) on gravid adult female *B. malayi* worms.

2. Materials and methods

2.1. Phenotypic studies of the effect of 20E on fecund adult female worms

Gravid adult female parasites were obtained from the Filariasis Research Reagent Resource Center (FR3) at the University of Georgia, USA. A total of five worms per well were cultured in a 6-well plate using 3 ml of CF-RPMI media (RPMI 1640 supplemented with 25 mM HEPES buffer, 2 mM glutamine, 100 U/ml of streptomycin, 100 μg/ml of penicillin, 0.25 μg/ml of amphotericin B, and 10% heat-inactivated FBS). Experiments were designed to consist of two replicates of parasites treated with 20E and two replicate control cultures. When the parasites were received, they were allowed to acclimatize and monitored for any decrease in motility for 24 h (day 0). The 20E was added to the media of the two experimental wells at a concentration of 10 μM on day 1. Control wells received ethanol (the vehicle of 20E). Media (either containing or lacking 20E) were changed every 24 h, and the parasites cultured for an additional 3 days (days 2 - 4). Three aliquots of 20 μl of media were removed from each biological replicate every 24 h and the life stages released into media were counted, resulting in three technical replicates for each biological replicate for each time point. The life stages observed were counted and classified as microfilariae, pre-microfilariae or eggs/embryos. The experiment was repeated five times over a period of 1 year, with parasites isolated from different infected animals.

2.2. RNA extraction

Fecund adult females, cultured as described in Section 2.1, were used to prepare RNA for the transcriptomic analysis. Total RNA was isolated from the two biological replicates of five worms each (two wells each of treated and untreated parasites) after 2 days culture with and without 20E. The worms were flash frozen in liquid nitrogen. RNA was extracted from the worms using Trizol LS (Invitrogen, Carlsbad, CA, USA) (Griffiths et al., 2009; Choi et al., 2011). The biological replicates of the 20E-treated and untreated samples were lysed individually using TissueLyse II (Qiagen, Valencia, CA, USA) followed by chloroform extraction, isopropanol precipitation and elution in 0.1×TE buffer (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). The samples were treated with DNase I (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The RNAs were subjected to drop dialysis using 45 nm Millipore membranes (EMD Millipore, Billerica, MA, USA) against 0.1×TE buffer at 4°C for 2 – 4 h and the RNA was then collected from the membranes. Purity of the samples was assessed using a NanoDrop apparatus (Thermo Scientific, Waltham, MA, USA). The quantity of RNA was determined using a Qubit apparatus (Thermo Fisher, Carlsbad, CA, USA). The purified RNA was stored at −80°C.

2.3. RNA library preparation

The NEBNext poly(A) mRNA Magnetic Isolation Module (New England Biolabs Inc., Ipswich, MA, USA; # E7490) was used to isolate intact poly(A)+ RNA from each previously isolated total RNA preparations. The eluted RNA was used for first and second strand cDNA synthesis using NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA; # E7530). The double stranded cDNA was size selected and the fractions between 250 - 550 bp in size were isolated using 1.8x Agencourt AMPure XP beads. End repair and dA tailing of cDNA library was performed, immediately followed by adaptor ligation. NEBNext Multiplex Oligos for illumina were used for adaptor ligation. The cDNA libraries were PCR amplified and purified. Quality control was performed using a Bioanalyzer (Agilent, Santa Clara, CA, USA) to analyze the quality and size selection of the cDNA. The samples were again subjected to Qubit to quantify the yield of cDNA.

2.4. Transcriptome sequencing (RNA-seq)

RNA-seq was performed on an Illumina MiSeq at the Core facility, Smith College, Northampton, MA, USA as part of the FR3 Repository, following the manufacturer’s protocol. In brief, the cDNA library (at a concentration of 4 nM) was denatured using 0.2 N sodium hydroxide. The library was diluted to 20 pM in Illuminia hybridization buffer, and the template strands hybridized to the adaptors attached to the flowcell surface as previously described (Choi et al., 2011). The library was diluted to 9 pM with Illumina HT1 buffer. The PhiX control was used as an internal standard. The PhiX standards were denatured and diluted using the same protocol as the sample library. The libraries were heated at 96°C for 2 min followed by immediate cooling. Samples were loaded into an Illumina cartridge and single-end reads produced. The raw reads from the RNA-seq experiment are available in the National Center for Biotechnology Information (NCBI) short read archive accession **SRP064921**.

2.5. Data analysis

The Tuxedo suite of programs was used to process and analyze the data (Trapnell et al., 2012). Bowtie2 was used to build indices of the *B. malayi* reference genome from Wormbase (v. WS245) (Langmead et al., 2009). RNA-seq reads from each sample were aligned to the *B. malayi* genome using TopHat (v. 1.4.1) (Trapnell and Salzberg, 2009). A maximum of one mismatch per read was allowed. The mapped reads from TopHat were used to assemble known transcripts from the reference and their abundances were estimated using Cufflinks (Trapnell et al., 2010). Cuffdiff is very conservative when identifying differentially expressed genes compared with edgeR and DESeq (Seyednasrollah et al., 2015). Thus, edgeR was used to analyze the data using the counts generated for the four samples with Cuffdiff. A heatmap was created using Bioconductor pheatmaps R software comparing the expression of individual transcripts in the 20E-treated and untreated parasites. Gene Ontology (GO) annotations were assigned using Wormbase (v. WS245) (Gentleman et al., 2004). Orthologs of the differentially expressed genes from the transcriptome were identified in the *Caenorhabditis elegans* genome using WormBase (v WS249). RNA interference (RNAi) phenotypes of the identified *C. elegans* orthologs were assigned using the RNAi tool available at WormBase. A hypergeometric test was performed to determine the significance of the relative frequency of the RNAi phenotypes that appeared to be over-represented in the genes found to be up-regulated in 20E-treated parasites compared with the frequency of these RNAi phenotpyes in the *C. elegans* RNAi dataset as a whole.

2.6. Real-time quantitative reverse transcriptase-PCR (qRT-PCR) validation

Selected genes were chosen based on the GO annotations for qRT-PCR validation as depicted in Supplementary Table S1. The gene encoding NADH dehydrogenase subunit 4 (ND4) was utilized as an internal control, as its expression was equivalent in 20E-treated and control parasites, based upon the transcriptome analysis described in Section 2.5. Three independent biological replicates of control and 20E-treated parasites were prepared as described above. Total RNA was extracted from each replicate using the protocol described in Section 2.2. Primers for qRT-PCR were designed to span exon-intron boundaries, eliminating the possibility of amplification of contaminating genomic DNA in the sample (Ye et al., 2012). The primers and probes used for the study are shown in Supplementary Table S1. SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Thermo Fisher, Carlsbad, CA, USA) was used in the amplification reactions to validate the primers, using the conditions provided by the manufacturer. Taqman chemistry was applied using iTaq Universal Probes One-Step Kit (Bio-Rad, Hercules, CA, USA) for the qRT-PCR. The efficiency of the PCRs using these conditions was calculated to be 95%. Each biological replicate was analyzed using triplicate technical replicates. Relative quantification measuring gene expression in each sample was calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

2.7. Preparation of protein extracts

Adult female worms cultured in the presence and absence of 20E (as described in Section 2.1), were homogenized in 150 μl of a buffer containing 125 mM Tris-HCl (pH 8.5), 4% SDS, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 100 mM DTT. A total of three

biological replicates of 20E-treated and untreated worms were used for the study. Protein concentrations were determined using the Pierce 660 nm Protein Assay supplemented with Ionic Detergent Compatibility Reagent supplementation (Thermo Fisher, Carlsbad, CA, USA). The samples were processed using Filter-aided sample preparation (FASP) for MS-based proteomic analysis. Raw MS files were processed with MaxQuant with a 1% False Detection Rate (FDR). The normalized ratios of each biological replicate obtained from the MaxQuant analysis were input into the Perseus processing suite (Bell-Temin et al., 2013). An unpaired student's t-test with a significance value of $P < 0.05$ used to test for statistical significance of the quantitation. A total of 6050 proteins were detected, which included multiple isoforms. Proteins with multiple isoforms were considered as a single entity for further analysis, resulting in 3991 unique proteins. An empirical filter of proteins with more than two peptides identified, a P value < 0.05 and a greater than two-fold change of differential expression, was applied to the data. The Bonferroni correction was applied to correct the P value for the proteins analyzed. A total of 359 proteins were identified to be significantly more abundant in the extracts of parasites cultured in the presence of 20E when compared with control extracts. GO terms were assigned to the differentially abundant proteins using WormBase and ParaSite.

2.8. RNAi phenotypic analysis of C. elegans orthologs

Orthologs and corresponding RNAi phenotypes of proteins that were significantly more abundant in parasites treated with 20E were identified as described in Section 2.5. A hypergeometric test was performed to determine the frequency of the RNAi phenotypes that were significantly enriched in the orthologs of the more abundant proteins of the 20E-treated *Brugia* dataset compared with their overall occurrence in the *C. elegans* RNAi dataset.

2.9. GO enrichment analysis of the C. elegans orthologs

The WEB-based GEne SeT AnaLysis (WebGestalt) Toolkit was used for GO enrichment analysis of the *C. elegans* orthologs of the more abundant proteins in the 20E-treated *B. malayi* extracts (Zhang et al., 2005). A hyper-geometric test with a Bonferroni correction was used with a cutoff of $P < 0.001$ to identify GO terms that were significantly over-represented in the orthologs of the abundant proteins of the *Brugia* dataset compared with their overall frequency in the *C. elegans* GO term dataset.

A separate analysis with the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 was conducted for functional annotation clustering of the *C. elegans* orthologs of the proteins that were highly enriched in the 20E-treated proteomic dataset compared with their overall representation in the *C. elegans* dataset (Huang et al., 2008). The DAVID analysis provided enriched GO term clusters that represent a particular function or a pathway.

3. Results

To aid in delineating the role played by the EcR in the filaria, fecund adult female parasites were cultured in vitro in the presence and absence of 20E as described in Section 2.1. Five biological replicates of the experiment were performed, all of which gave similar results. A

representative experiment is shown in Fig. 1. Parasites cultured in the presence of 20E exhibited a significant increase ($P < 0.001$) in the production and expulsion of both microfilariae and eggs/embryos compared with control parasites (Fig. 1). These data implicated 20E in the control of microfilarial development and embryogenesis, and provided a phenotype that could be used to monitor the effect of 20E in subsequent experiments.

To identify the genes participating in the ecdysone responsive pathways, female *B. malayi* parasites were cultured in the presence and absence of 20E and harvested after 48 h. This is the time point at which enhanced microfilariae production and expulsion of immature stages from the treated worms were at their peak (Fig. 1). RNA was then prepared from two independent biological replicates and subjected to transcriptomic analysis. The biological replicates were highly consistent in their Fragments Per Kilobase of Transcript per Million Mapped Reads (FPKM) values (Fig. 2A, B). Differentially expressed genes were identified using the EdgeR Negative Binomial Distribution as having a minimum of a two-fold change in expression between the control and treated worms with a P value <0.01. A total of 44 differentially expressed genes were identified in the 20E-treated parasites compared with control parasites cultured in the absence of the hormone; 30 genes were up-regulated and 14 genes were down-regulated (Fig. 2C).

GO analysis was then performed on the 44 differentially expressed genes. A total of eight of 44 (18%) genes were involved in regulation of transcription, while five of 44 (11%) genes participated in cellular processes and one gene (2%) was involved in cellular differentiation (Fig. 3). Of the 30 up-regulated genes, a total of 18 orthologs were identified in the *C. elegans* genome. The RNAi profile of these genes revealed that almost all the orthologs exhibited phenotypes consistent with an involvement in embryonic developmental processes (Table 1). These phenotypes were significantly over-represented in the dataset of genes up-regulated in *B. malayi* cultured in the presence of 20E compared with their overall abundance in the *C. elegans* RNAi phenotype dataset ($P < 0.001$; hypergeometric test).

To evaluate the quantitative robustness of the transcriptome study, four genes were chosen for validation using qRT-PCR. These genes were chosen from among the transcriptional regulators identified in the GO analysis and represented a range of their predicted up-regulation from five-fold to 392-fold between the treated and untreated parasites. The degree of up-regulation seen in the qRT-PCR assays were found to correspond closely to those calculated from the FPKM analysis of the transcriptome data in all cases (Fig. 4, Table 2). A Pearson correlation coefficient of 0.99 was observed when the degree of up-regulation observed in the transcriptome and qRT-PCR data were compared.

Although the transcriptomic analysis demonstrated excellent reproducibility with biological replicates and qRT-PCR validation, it identified a relatively small number of genes whose transcripts were up-regulated in response to 20E. However, because protein levels may also be regulated through post-transcriptional mechanisms, the effect of 20E on the female proteome was also explored. To accomplish this, total protein extracts from adult 20E-treated worms and control worms were subjected to FASP digest and LC/MS/MS spectroscopy, as described in Section 2.1. A total of 3,991 unique proteins were identified (Supplementary Data S1). Of these, 359 unique proteins were found to exhibit a greater than

two-fold increase in abundance in the 20E-treated parasite extracts when compared with the control worm extracts, while 164 proteins exhibited a greater than two-fold decrease in abundance in the treated extracts. The proteins whose abundance was increased were then identified using the annotations provided in WormBase release 240. An overlap of 11 genes was observed between the transcriptomic and the proteomic data. The majority of the proteins that were more abundant in the 20E extracts had an uncharacterized function (Fig. 5). However, a similar proportion to what was seen in the transcriptome data, 56 of 359 (15%) of the more abundant proteins in the 20E extracts were involved in regulation of transcription. Another 15 (4%) proteins were involved in signal transduction, 12 (3%) proteins in translation, 10 (3%) proteins in chromosomal regulation and cell cycle regulation and 80 (22%) proteins in cellular processes (Fig. 5). A number of structural proteins were also more abundant in 20E-treated extracts. The other categories that were more abundant in the treated extracts included protein kinase activity, tyrosine phosphatase activity and metal ion binding.

A total of 345 protein orthologs of the 359 significantly more abundant proteins from the study above were identified in the *C. elegans* genome. RNAi phenotypes were identified for 268 of these orthologs. As was seen in the more limited transcriptome dataset, a large number of orthologs displayed phenotypes that were involved in developmental process and embryogenesis (Supplementary Table S2). These phenotypes were significantly over-represented in the dataset compared with their overall abundance in the *C. elegans* RNAi phenotype dataset as a whole ($P < 0.001$; hypergeometric test).

A GO enrichment of the *C. elegans* orthologs of the over-abundant *B. malayi* proteins was performed using WebGestalt, as described in Section 2.9. Fig. 6 shows the GO categories (highlighted boxes) that were significantly over-represented in the *C. elegans* orthologs of the over-abundant *B. malayi* proteins compared with their overall representation in the *C. elegans* dataset (Bonferroni corrected $P < 0.001$; hypergeometric test) (Zhang et al., 2005). Most of the over-represented proteins participated in the growth and developmental pathways. A separate DAVID analysis based on GO term clustering was performed to identify the specific gene clusters that were enriched in the proteomic dataset (Supplementary Table S3). The most enriched gene clusters were similar to the WebGestalt analysis, with those participating in embryological events clustering together with the highest enrichment score of 11.13.

4. Discussion

The data presented above suggest that ecdysone may play an important role in the development and birth of microfilaria in *B. malayi*. Worms cultured in the presence of 20E exhibited an increase in expulsion of microfilariae, pre-microfilariae and eggs/embryos into the culture medium for 2 days. While expulsion of immature stages is commonly seen in adult female worms cultured in vitro (c.f. Fig. 1) as well as in adult females in i.p. infected gerbils (A. Moorhead, unpublished data), the number of immature progeny expelled was significantly greater in parasites cultured in the presence of 20E than in parasites cultured in the absence of 20E. These data suggest that 20E-induced abortion of immature life cycle stages from the gravid females, resulting in a depletion of immature parasites from the

uterus by the third day in culture. Although the microfilariae expelled into the media in the worms cultured in the presence of 20E appeared morphologically normal, it was not possible to determine if they were reduced in their ability to infect mosquitoes, as we found that microfilariae produced by gravid females cultured in the absence of 20E were also not infectious to mosquitoes (data not shown). Studies measuring the effect of 20E on infectivity of microfilariae will need to be conducted in a system in which the microfilariae produced are normally infectious (e.g., in infected animals rather than in culture). Additional studies will also be needed to assess whether the phenotypic effect seen is a result of interference with microfilarial development, or on uterine function in the adult female. The potential role that 20E (and by extension the *Bma*EcR) may play in molting will also need to be investigated.

A total of 44 differentially expressed genes were identified in the ecdysone-cultured parasites compared with those cultured in the absence of the hormone. Although few mRNAs were identified as regulated by exposure to 20E, the data were robust, showing high correlation between biological replicates. The majority of the genes that were differentially expressed were of uncharacterized and/ or unannotated function. However, orthologs of many of the differentially expressed *B. malayi* genes were identified in the *C. elegans* genome. As orthologs retain the same functionality during the course of evolution, the RNAi phenotype of the *C. elegans* orthologs could be used to infer the function of the proteins encoded by the mRNAs found to be up-regulated in the *B. malayi* transcriptome. Almost all of the orthologs revealed RNAi phenotypes that suggested involvement in various embryological processes. The RNAi phenotype analysis thus supports the hypothesis that the *Bma*EcR is involved in regulating embryological development in *B. malayi*.

To confirm the veracity of the transcriptomic analyses, a series of representative genes whose expression levels were changed by exposure to 20E were examined using qRT-PCR. The mean fold change of the three biological replicates of all genes as measured by qRT-PCR corresponded closely with the mean FPKM values of RNA-seq replicates. This suggested that the FPKM values observed in the transcriptomic analysis accurately reflected changes in the levels of the various transcripts.

The proteomic data revealed 359 unique proteins whose levels were more abundant in parasites exposed to 20E. Of these a total of 11 were found to coincide in the transcriptome and proteome studies. This is similar to other studies in the systems biology literature, which have generally observed few proportional correlations between transcript and protein expression (Chen et al., 2002; Pascal et al., 2008; Ghazalpour et al., 2011; Yeung, 2011; Darby et al., 2012). The differences in the transcriptome and proteome datasets could be attributed to different translational efficiencies post-translational regulation and differential protein stability (Grossman et al., 1985; Hargrove and Schmidt, 1989; Cho et al., 1998; Greenbaum et al., 2003; Gustafsson et al., 2004; Yeung, 2011, Haider and Pal, 2013). However, the RNAi phenotypes of *C. elegans* orthologs of the more abundant proteins in the 20E-treated parasites displayed a similar pattern as derived from the transcriptomic data. Again, phenotypes indicative of involvement in embryological development were common. The GO enrichment analyses supported this finding, with many of the genes identified participating in the developmental pathway leading to embryogenesis. Taken together, the

RNAi phenotypic analysis and the GO enrichment study support the hypothesis derived from the transcriptome data that the *Bma*EcR is a regulator of genes participating in embryological development.

In summary, the data presented here demonstrate that 20E has demonstrable effects on gravid adult female *B. malayi*, at the phenotypic, transcript and protein levels. The results are consistent with a function for the *Bma*EcR in regulating embryonic development in this parasite. Further studies will be needed to elucidate the role that this regulator plays in the other life cycle stages of the parasite as well. These data suggest that the *Bma*EcR may represent a fertile target for the development of drugs that would disrupt embryogenesis and bring about sterility in the human filarial parasites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- 20-hydroxyecdysone (20E) induces microfilarial release and immature abortion in *Brugia malayi.*
- 20E up-regulates 30 transcripts and 359 proteins in adult *B. malayi.*
- RNA interference (RNAi) phenotypes and their Gene Ontology (GO) terms predict involvement in embryogenic processes.

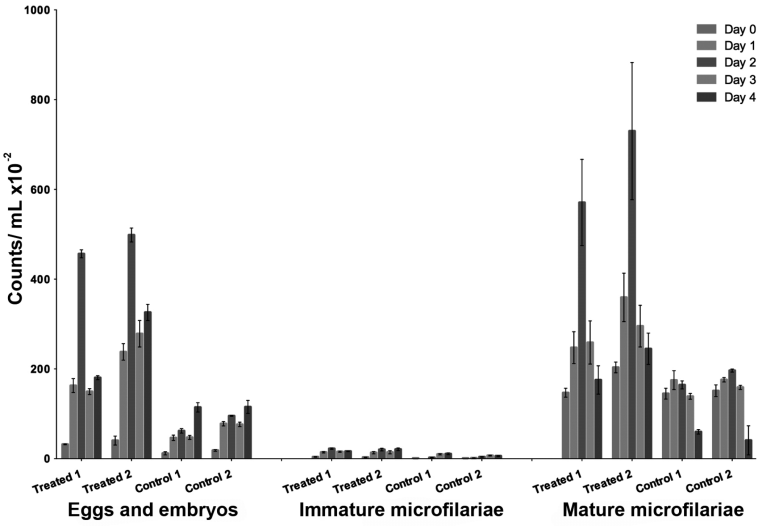

Fig. 1.

Effect of 20-hydroxyecdysone (20E) on the physiological activity of the adult female *Brugia malayi* worms in culture. The x-axis shows two biological replicates of the 20E-treated and control cultures. The bars depict counts observed. The error bars show the S.D. of the three technical replicates.

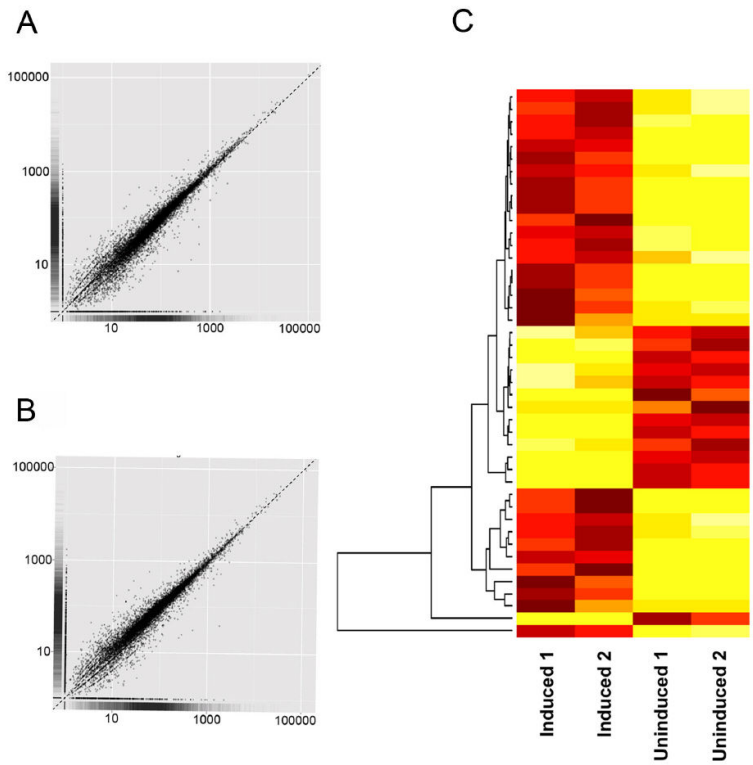

Fig. 2.

Transcriptomic analysis of the 20-hydroxyecdysone (20E)-treated and untreated *Brugia malayi* worms harvested after 48 h. (A) Comparison of expression scores of 20E-treated biological replicates. Expression scores were plotted on a $\log_{10}$ scale. (B) Comparison of expression scores of control biological replicates. Expression scores were plotted on a $\log_{10}$ scale. (C) Heat map of hierarchical clustering of differentially expressed genes of the adult female worms treated with 20E (induced) and controls in duplicate experiments ($n = 4$). Red, up-regulation; yellow, down-regulation. Intensity of color indicates the degree of expression.

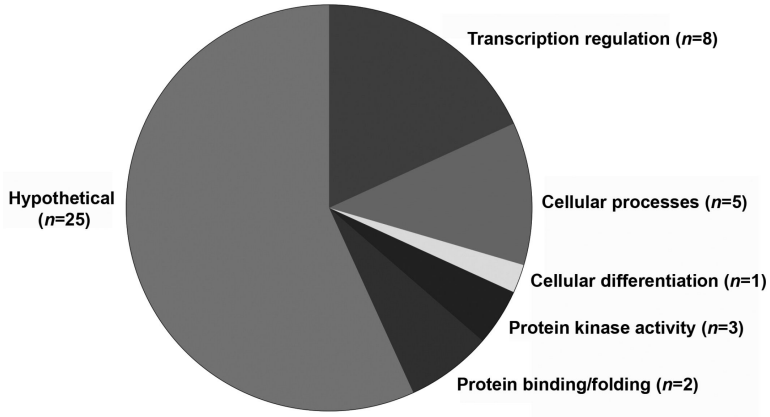

Fig. 3.

Pie chart of the functions of the differentially expressed *Brugia malayi* genes after treatment with 20-hydroxyecdysone (20E). Up-regulated genes were sorted based on their Gene Ontology (GO) classifications.

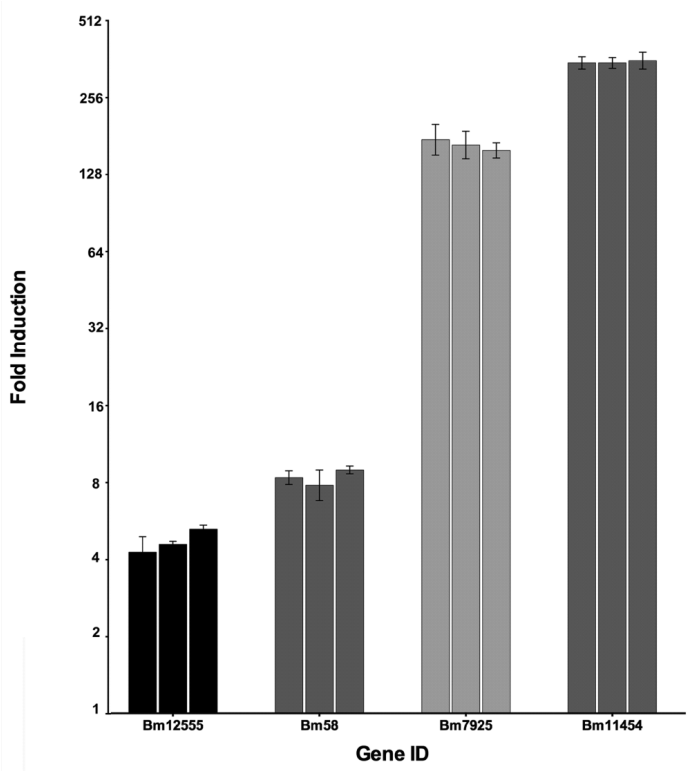

Fig. 4.

Real-time Reverse Transcriptase PCR **(**qRT-PCR) validation of the expression level demonstrated by the RNA-seq for four *Brugia malayi* genes (see Table 2). Columns represent fold inductions in three biological replicates and the error bars show the S.D. surrounding the estimates of three technical replicates per biological replicate.

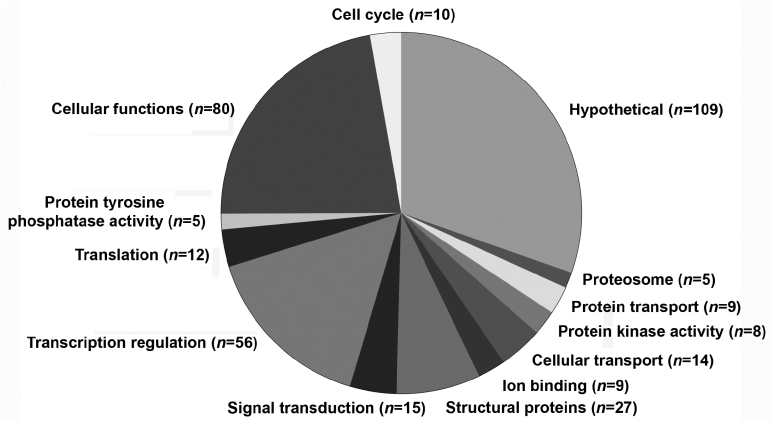

Fig. 5.

Pie chart of the Gene Ontology (GO) terms of the up-regulated *Brugia malayi* proteins. Three hundred and fifty-nine proteins with a greater than two peptide match, $P<0.01$ and greater than two-fold change were included in the analysis.

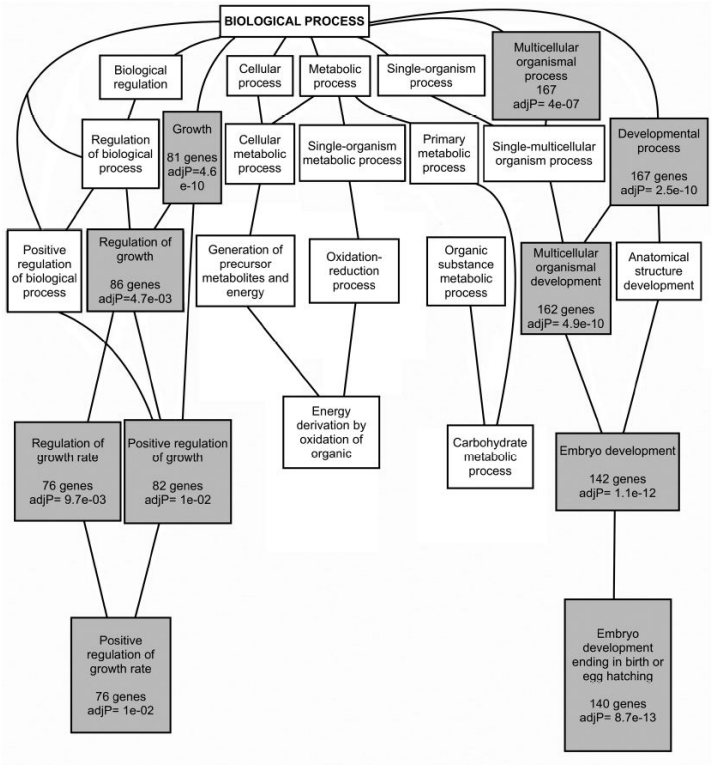

Fig. 6.

Gene Ontology (GO) Biological Process terms enrichment Directed Acyclic Graph. The terms highlighted in the graph were highly enriched (P <0.001).

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Table 1

RNA interference (RNAi) phenotype analysis of the *Brugia malayi* orthologs in the *Caenorhabditis elegans* genome.

B. malayi **Gene ID**	***C. elegans*** **Ortholog(s)**	**RNAi Phenotype(s)**
Bm5644	ANMT-1	Embryonic lethal, lethal, sterile
Bm14320	fem-2	Dauer constitutive, embryonic lethal, feminization of XX and XO animals, sterile, reduced brood size, no oocyte, variant, sperm absent
Bm2832	C07H6.4, F10E9.11	Embryonic lethal, spindle assembly defective early embryos
Bm3675	dpm-1, dpm-3	Embryonic lethal, cortical dynamics defective early embryogenesis, sterile progeny, small, sick
Bm5431	egl-15	Bag of worms, egg laying defective, embryonic lethal, L1 arrest, larval arrest, larval lethal
Bm9980	ZK265.7	Embryonic lethal, embryonic lethal, larval arrest
Bm8103	Y18D10A.23, F21D12.3	Reduced brood size, embryonic lethal
Bm58	cnt-2	Asymmetric cell division defective early embryogenesis, embryonic lethal, lethal, reduced brood size, sterile
Bm9280	MNM-2	Axon guidance variant, neuron morphology variant
Bm87	gck-4	Larval arrest, lethal, reduced brood size, slow growth
Bm7925	crh-1	Athermotactic, avoids bacterial lawn, cryophilic, isothermal tracking behavior variant, neuron calcium transient levels variant
Bm14681	dhps-1	Embryonic lethal
Bm11454	Y37E3.10	Pleiotropic defects severe early embryos, sterile, early embryonic lethal, sterile progeny, late embryonic arrest, L1 lethal, sick, lethal, larval arrest, embryonic lethal, nuclear morphology variation early embryos, reduced brood size
Bm432	mif-2	Embryonic lethal
Bm13025	F53E4.1	Maternal sterile
Bm12555	nhr-3, nhr-88	Transgene expression increased
Bm10799	B0336.3	Body wall muscle myosin organization defective, organism development variant, organism morphology variant, slow growth, transgene expression reduced, transgene induced co-suppression variant
Bm8538	fis-1	Egg retention, embryonic lethal

Table 2

Comparison of fold change between RNA-seq and quantitative reverse transcriptase PCR (qRT-PCR) for four *Brugia malayi* genes identified as transcriptional regulators in the Gene Ontology (GO) analysis.

B. malayi **Gene ID**	**Putative Functions**	**RNA-seq**	**qRT-PCR**
		I/UI	**I/UI**
Bm58	Signal transduction	8.3	8.4 ± 0.75
Bm7925	Regulation of transcription	159.9	169 ± 22.8
Bm12555	Nuclear receptor like gene	5.2	4.7 ± 0.38
Bm11454	Nematode larval development factor	392.0	356.5 ± 25.62