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Discovery of a Functional Ecdysone Response Element in *Brugia malayi*

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Discovery of a Functional Ecdysone Response Element in *Brugia malayi*

by

Tracy J. Enright

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science in Public Health
Department of Global Health
College of Public Health
University of South Florida

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Boo H. Kwa, Ph.D.

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Dedication

I would like to dedicate this thesis in remembrance of Chitra Chauhan, Ph.D.

It was a pleasure conducting my research among the talented and friendly past and present members of the TRU lab and the IDRB laboratory community. Thank you all for providing such a supportive work environment.

To my family and friends who were understanding and encouraging as I poured myself into my research and studies, I am forever grateful.
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Abstract

The aim of this study was to determine whether functional ecdysone response elements (EcREs) exist within the genome of Brugia malayi, a parasitic nematode that causes lymphatic filariasis. The hypothesis that EcREs exist in B. malayi stemmed from previous demonstration of a functional ecdysone response system in B. malayi (Tzertzinis et al., 2010). Real-time PCR (qPCR) experiments were conducted to measure gene expression levels for twelve genes proximal to five putative EcREs in 20-OH ecdysone treated and untreated B. malayi embryos. Seven genes showed consistent upregulation with 20-OH ecdysone treatment. Each of the five putative EcREs had at least one proximal gene consistently upregulated, suggesting that all five might be functional EcREs. One of the genes consistently upregulated in the qPCR experiments, Bm1_48650, codes for a MIZ zinc finger family protein, a likely transcription factor. Transgenic ecdysone induction assay experiments were conducted using embryos transiently transfected with a reporter construct driven by the EcRE-containing promoter of Bm1_48650. Significantly higher mean reporter gene activity (~3.5-fold) was seen in 20-OH ecdysone treated versus untreated embryos. In another set of transgenic ecdysone induction assays, the EcRE motif in the Bm1_48650 promoter was completely mutated, and this construct was tested in 20-OH ecdysone treated and untreated embryos. The mean reporter gene activity for
the treated and untreated embryos transfected with the mutant constructs did not differ significantly from the untreated embryos transfected with the native EcRE-containing promoter construct. These results showed that the EcRE in the promoter of Bm1_48650 is necessary for regulating gene expression in response to 20-OH ecdysone. This study substantiates previously discovered evidence of a functional ecdysone response system in B. malayi, which could potentially serve as a target for drug discovery for lymphatic filariasis.
Introduction

Lymphatic filariasis is a neglected tropical disease caused by infection with parasitic filarial worms *Brugia malayi*, *Brugia timori*, and *Wuchereria bancrofti*. The disease can be extremely debilitating and greatly impact the lives of those afflicted with it. It is estimated that 120 million people are infected with at least one of these filarial species, and more than a billion people are at risk worldwide. Currently, lymphatic filariasis is endemic in 81 countries. *W. bancrofti*, responsible for approximately 90% of lymphatic filariasis infections, exists throughout tropical Asia, Africa, the Americas, and the Pacific. *B. malayi* accounts for the majority of the remaining infections, and like *B. timori*, is transmitted in east and south Asia (World Health Organization [WHO], 2010).

Individuals are usually infected with lymphatic filarial parasites during childhood. As adults, many people never display clinical symptoms, but may experience lymphatic and kidney damage. Alternatively, individuals with long-term chronic infections may suffer from lymphoedema (elephantiasis) of the arm, leg, scrotum, penis, vulva or breast; hydrocele (fluid build-up around the testes); as well as lymphatic and kidney damage. Of the roughly 40 million people living with these clinical pathologies, 15 million suffer from lymphoedema, whereas 25 million men have urogenital swelling. Disease pathology is caused by blockage of the lymph system by the parasitic worms (WHO, 2000; WHO, 2010).
Often, there is a social stigma attached to those with outward manifestations of the disease. This can limit chances of marriage and security of disfigured individuals. Additionally, as a result of physical limitations brought on by the disease pathology, those affected may be unable to work. This loss of ability to make a living perpetuates poverty already apparent in the areas where lymphatic filariasis is endemic. The physical and societal impacts of this disease warrant its status as a public health problem (WHO, 2000; WHO, 2010).

The Global Programme to Eliminate Lymphatic Filariasis (GPELF) was established in 2000 by the World Health Organization (WHO) to facilitate elimination of lymphatic filariasis as a public health problem by 2020. The two main foci of the elimination strategy are transmission interruption and morbidity management. Mass drug administration (MDA) of two drugs to entire at-risk populations is the recommended approach for interrupting transmission. Currently, available drug treatments involve a single dose of albendazole with either ivermectin or diethylcarbamazine (DEC), administered once a year. The WHO (2010) recommends that this MDA regimen continue for at least five years. This is due to the fact adult worms, capable of producing millions of microfilariae, live for 4-6 years. Repeated treatments ensure microfilariae in patients' blood have been cleared and maintained at a low enough level to prevent further transmission. Fortunately, manufacturers of albendazole and ivermectin have pledged donations of these pharmaceuticals to aid in treatment plans (Molyneux, Bradley, Hoerauf, Kyelem, & Taylor, 2003; WHO, 2010). Huge strides have been made in eliminating the lymphatic filariasis as a public health problem in the first
ten years of the GPELF’s existence. China and the Republic of Korea have
officially been recognized for their achievement in eliminating their lymphatic
filariasis problem. Over 2.8 billion doses of pharmaceuticals have been dispersed
worldwide, covering 63% of the at-risk population (WHO, 2010).

Although MDA programs have exhibited a decrease in rates of infection
and disease, a number of problems and concerns exist with a limited selection of
pharmaceutical treatments available to combat lymphatic filariasis. Existing drugs
target either the larval stages or the parasites’ bacterial symbiont, *Wolbachia*,
however, treatment of adult worms is believed necessary to improve results.
Thus, discovery of targets in the adult stage is a priority for drug development
(Kumar et al., 2007; McNulty, Weil, Heinz, Crosby, & Fischer, 2008; Scott &
Ghedin, 2009). Without the ability to destroy or sterilize the adult worms,
subsequent and continued infection with new microfilariae ensues, perpetuating
the need for regular and repeated treatments (Scott & Ghedin, 2009). In addition,
treatment choices are further limited in areas where *Loa loa* is also endemic, as
people with high levels of microfilariae can experience adverse reactions with
ivermectin treatment (WHO, 2010). Furthermore, appraisal of potential drug
resistance is necessary to prevent a devastating setback due to possible
development of parasite resistance to treatment (Williams, 2004). Some
suggested filarial biological systems that may provide targets for drug therapies
include the nervous and reproductive systems, cuticle formation, and the molting
process. Interactions between *B. malayi* and its hosts rely on mechanisms
integral to survival of the worm, and thus provide attractive drug targets (Scott &
Ghedin, 2009). Increasing the field of viable treatments with novel, efficient, and cost-effective therapies for treatment and prevention is thus an important research priority.
Background

Research exploring the *B. malayi* genome began as part of the Filarial Genome Project (FGP), instituted by the WHO. The community of researchers that make up the Filarial Genome Project has made mapping the *B. malayi* genome, identifying genes, setting up data banks, and making these findings globally accessible the ultimate goal of their genomic investigations. Researchers chose *B. malayi* as the filarial model species based on a number of factors that facilitated working with these worms in laboratory studies. First, unlike *W. bancrofti*, *B. malayi* can be cultured in an animal host, and various life cycle stages are available for study. Furthermore, an added benefit exits in the availability of a *B. malayi* laboratory strain (Blaxter, Daub, Guiliano, Parkinson, & Whitton, 2002; Ghedin, Wang, Foster, & Slatko, 2004; Ghedin et al., 2007; Scott & Ghedin, 2009). Information garnered from research on *B. malayi* should be useful for study of other parasitic nematodes that may have homologous genes, regulatory systems, and biological pathways.

A notable accomplishment of the FGP research endeavors was the sequencing of the *B. malayi* genome. *B. malayi* was the first sequenced parasitic nematode genome. Additionally, the FGP utilized expressed sequence tags (ESTs) from all phases of the parasite life cycle to identify and analyze expressed genes (Ghedin et al., 2007; Scott & Ghedin, 2009; Williams, 1999).
Possession of this backbone of information with regard to a pathogenic species sets the foundation for investigation of gene expression patterns and functional genomic research (Kumar et al., 2007). The knowledge gathered through subsequent genetic research involving gene function and regulation should allow scientists to pinpoint the most promising targets for treatment of infection with *B. malayi*. These drug targets may also exist in other filarial worm species and similar treatments could be useful in combating those infections as well.

As a member of the superphylum Ecdysozoa, *B. malayi* go through the process of molting during their life cycle. Molting involves the shedding of the outer protective exoskeleton, or cuticle, after a new cuticle layer has formed to replace it. This process occurs four times in the development of the nematode worm (Kumar et al., 2007). Microfilariae living in the human bloodstream are taken up by a mosquito during a blood meal and molt twice into the L2 and L3 larval stages in the mosquito vector. The L3, or third stage larvae, are infective to humans, and in the human host these larvae further develop to L4, and subsequently adults that eventually produce microfilariae (Dieterich & Sommer, 2009). Each molt involves a complex set of events involving structural, metabolic, and behavioral changes (Craig, Isaac, & Brooks, 2007). Molting-associated genes and proteins make favorable targets as they are not found in humans or other vertebrates (Kumar et al., 2007).

Ecdysone is a steroid hormone that drives developmental processes such as molting, metamorphosis, oogenesis, and embryogenesis in insects. An ecdysone response system, such as that found in fruit flies, *Drosophila*, is also
hypothesized to regulate the process of molting in filarial parasites. The ecdysone response system involves two nuclear receptor proteins that form a heterodimer and act as a receptor for the ecdysone hormone. In *Drosophila*, these hormone receptor proteins are ecdysone receptor protein (EcR) and ultraspiracle protein (USP), a homolog of the retinoid X receptor (RXR) (Tzertzinis et al., 2010). Nuclear receptors, induced by ligands such as ecdysone, regulate transcription of target genes by binding to hormone response elements present in the promoters of those genes (Perera et al., 2005). A number of genes discovered to be induced by 20-OH ecdysone, as direct targets of ECR/USP, are transcription factors (Gauhar et al., 2009). The activation of the initial target genes triggers a cascade of gene expression, ultimately facilitating the necessary physiologic and morphologic changes in the organism (Tzertzinis et al., 2010).

Among the nuclear receptor family members identified in the *B. malayi* genome were a number of proteins similar to those that play a role in the *Drosophila melanogaster* ecdysone response system (Ghedin et al., 2007). Recently, both protein constituents of the ecdysone receptor were identified when *B. malayi* Ecr (*Bma-EcR*) and RXR (*Bma-rxr*) homologues were successfully cloned. Furthermore, the Bma-EcR and Bma-rxr proteins were shown to form a heterodimer capable of driving gene expression in response to an ecdysteroid hormone. In addition, Bma-EcR partnered with *Aedes aegypti* USP (AaUSP) displayed binding with a synthetic palindromic ecdysone response element (EcRE), PAL-1. Together, these findings reveal the presence of an
ecdysone receptor which has the ability to bind EcRE DNA sequences, and activate transcription in response to ecdysteroids. This ultimately indicates the existence of an ecdysone signaling system that functions in *B. malayi* (Tzertzinis et al., 2010). Research into manipulation of the ecdysone signaling system could provide mechanisms for halting procession of the filarial life cycle, and potential therapeutics.

Expression patterns of both *Bma-EcR* and *Bma-rxr* were studied for various life stages and *Bma-EcR* was found to be expressed in L1, L2, L3, and adult males and females, and *Bma-rxr* was expressed in L1, and adult males and females. The fact that both proteins were expressed in adult females provides evidence for the potential role that ecdysone plays in oogenesis and embryogenesis in *B. malayi*, as is does in insects (Tzertzinis et al., 2010; Wang, Miura, Miksicek, Segraves & Raikhel, 1998).

A transgenic hormone induction assay was successful in demonstrating increased gene expression attributed to 20-OH ecdysone treatment in *B. malayi*. This assay incorporated a luciferase reporter vector containing 5 tandem repeats of a synthetic EcRE (PAL-1), and helped bolster the evidence for the existence of an ecdysone response system in *B. malayi*. The reporter vector was introduced into *B. malayi* embryos using biolistic transient transfection techniques (Tzertzinis et al., 2010). Biolistic transient transfection has been optimized for *B. malayi* and has provided a useful mechanism for transfer of DNA into these parasites (Higazi, Merriweather, Shu, Davis, & Unnasch, 2002). Biolistic transfection involves the firing of DNA that is precipitated onto heavy metal bead particles into
an organism’s cells. Varying methods, such as helium discharge, and water vaporization caused by high-voltage spark, are capable of providing the high pressure needed for particle acceleration (Villemejane & Mir, 2009). Transient transfection allows for examination of gene regulation in a native environment, avoiding potential difficulties in conclusively determining promoter activity in non-native systems (Shu, Katholi, Higazi, & Unnasch, 2003).

While almost all components of the ecdysone response system have been discovered in *B. malayi*, it is necessary to determine the existence of functional ecdysone response elements (ECREs) within the *B. malayi* genome. The presence of functional EcREs would show that this system not only exists in *B. malayi*, but has the regulatory component within the genome to control expression of necessary molting genes. Whereas there is some degree of consensus, the sequences and structures that ECREs display in insect and nematode species have been found to vary. Naturally, EcREs have asymmetric configurations, occurring as imperfect palindromes or direct repeats. The first functional EcRE was discovered in the *Drosophila* hsp27 promoter. This EcRE has an imperfect palindrome arrangement, with one nucleotide separating its two half-sites (GGTCAATGC ACT). Other functional EcREs in *Drosophila* have configurations similar to the hsp27 EcRE, yet some are direct repeats containing variable numbers of spacer nucleotides. In general, steroid hormone receptors bind to inverted repeats of (Pu)G(G/A)(T/A)CA hexameric core motif. The motif (Pu)G(G/T)TCA is a common half-site pattern in natural ecdysone response elements. Receptor binding experiments have demonstrated that insect
EcR/USP heterodimers can also bind to symmetrical (perfect) palindromes (Perera et al., 2005; Vogtli, Elke, Imhof, & Lezzi, 1998; Wang, Miura, Miksicek, Segraves, & Raikhel, 1998).
Materials and Methods

Real-time PCR

Real-time PCR (qPCR) assays were conducted to determine expression levels of twelve *B. malayi* genes in embryos exposed to 20-OH ecdysone. These twelve target genes were selected due to their close proximity to putative EcREs.

Twenty adult female worms were dissected using a scalpel blade to isolate their embryos. Embryos from half of the worms were maintained in 10ml CF-RPMI embryo culture medium (RPMI tissue culture medium containing 25mM HEPES, 20% fetal calf serum, 20mM glucose, 24mM sodium bicarbonate, 2.5 mg/ml amphotericin B, 10 U/ml penicillin, 10 U/ml streptomycin, and 40 mg/ml gentamicin) supplemented with 5uM 20-OH ecdysone dissolved in 50% ethanol. The remaining embryos were cultured in 10ml CF-RPMI supplemented with ethanol solvent. The embryos were incubated for 48 hours at 37°C and 5% CO₂, after which they were harvested. The harvested embryos were combined with 1ml of Trizol reagent and were frozen at -80°C.

The embryo tissue in Trizol reagent was homogenized using a frozen mortar and pestle and transferred to a sterile microcentrifuge tube. For phase separation, 200 ul of chloroform was added, the mixture vortexed for 15 seconds, incubated at room temperature for 2-3 minutes, and centrifuged at 2-8°C and
14,000 rpm for 15 minutes. The upper aqueous phase was transferred to a fresh
tube and the RNA was precipitated by mixing with 500 ul isopropyl alcohol. The
samples were incubated at -20ºC for 10 minutes, and centrifuged at 2-4ºC,
14,000 rpm for 10 minutes. The RNA pellet was washed once with 1ml 70%
ethanol, vortexed, and centrifuged at 15,000 rpm for 5 minutes. The ethanol
wash procedure was repeated once again, and all ethanol removed. The RNA
pellet was air-dried for 5-10 minutes and resuspended in 50uL sterile water.

The BioRad IScript cDNA synthesis kit was used to synthesize cDNA from
the isolated B. malayi RNA. Real-time PCR reactions of the cDNA were set up
using iQ™ SYBR® Green supermix and were run in a Bio-Rad iCycler Thermal
Cycler. Primer sets were designed specifically for each of the twelve target
genes, as well as an endogenous control, ND4 NADH dehydrogenase. The
Comparative C_T Method (ΔΔ C_T) was used for quantitation of the amount of the
target. This quantitation method normalized the amount of target gene to the
endogenous control gene, and the ecdysone-exposed target was measured
relative to the unexposed target control.

**Ecdysteroid response reporter construct: BmRPS12-EcRE-rev**

An ecdysteroid response reporter construct, BmRPS12-EcRE-rev,
previously shown to be responsive to 20-OH ecdysone treatment, was used in
transient transfection control experiments run in parallel with the qPCR
experiments. This construct included the reverse PAL-1 EcRE, a double stranded
oligonucleotide that consisted of five tandem repeats of the EcRE (GGACAAAGTCAGTGACCTCCTTGTTC), in a reverse orientation, inserted into the BmRPS12 promoter from which the endogenous repeat enhancer had been deleted, driving the expression of firefly luciferase in the promoterless vector pGL3 Basic. The control experiments tested promoter activity of the constructs in transiently transfected B. malayi embryos treated with 20-OH ecdysone. These control experiments were used to verify whether each set of embryos were responsive to the 5uM 20-OH ecdysone treatment (Tzertzinis et al. 2010).

Construction of EcRE promoter reporter plasmid: Bm1_48650-EcRE

First, PCR amplification with Taq polymerase high fidelity was performed using primers (5’-AAGCTTGTGTTATTTTAGGCGTTATCAAGTTG-3’ and 5’-AAGCTTAGGAGACGTATAAGGATCTAAGACAAC-3’) that included HindIII restriction sites. The primers were designed to isolate the upstream promoter region of gene Bm1_48650 from B. malayi DNA. The PCR product was purified using the QIAquick PCR purification system. Following purification, dA-tailing of the PCR products was performed in order to ensure successful ligation into pCR®2.1 TA vectors. Following ligation of the promoter fragments into the TA vectors, XL10-Gold competent E. coli cells were transformed with the TA constructs, and spread on LB plates containing carbenicillin. TA constructs were isolated for sequencing with a PureYield™ Plasmid Miniprep kit. Qiagen Maxiprep protocol was used to for increased yield of TA constructs.
Restriction digestion reactions with HindIII were performed on 10ug of the TA vector constructs as well as pGL3-Basic firefly luciferase reporter vectors. SuperSAP™ Shrimp Alkaline Phosphatase was included in the pGL3-Basic vector digestion reaction to enable dephosphorylation, and was subsequently heat inactivated at 65°C for 15 minutes. Phenol extraction and ethanol precipitation of the pGL3-Basic vector digestion product followed. The TA vector clone digestion products were run on a 1% agarose gel, and the EcRE promoter insert bands were removed and purified with the GeneCleanII gel purification kit.

Ligation of the pGL3-Basic vector and the EcRE promoter insert using T4 DNA ligase was successfully performed with 75 ng insert DNA and 35 ng vector DNA (2:1 insert-to-vector ratio), as well as with a 4:1 insert-to-vector ratio. The ligation product was then transformed into XL10 Gold cells and plated on LB agar containing carbenicillin. PureYield™ Plasmid Miniprep and Qiagen Maxiprep kits were used to isolate the pGL3-Basic constructs containing the EcRE promoter.

**Mutagenesis**

The GeneTailor™ site-directed mutagenesis system was used to mutate the EcRE portion of the promoter within the pGL3-Basic construct. The EcRE (AGGTCATTGACCT) was completely mutated using primers designed according to protocol specifications (5’-GTTTATTTAATAAGGAACATCTCAGAGTACGTCGACTTGAATGT -3’ and 5’-TTCCTTTATTTAAAATACATTTTCTGCTA -3’).
Transgenic hormone induction assay

A transgenic hormone induction assay was used to determine promoter activity with 20-OH ecdysone treatment. As described, gravid adult female *B. malayi* worms were dissected in order to isolate embryos. The embryos were biolistically transfected with pGL3-Basic luciferase reporter constructs. Following transfection, embryos were incubated for 48 hours in CF-RPMI media, with a subset of embryo samples treated with 5uM 20-OH ecdysone dissolved in 50% ethanol and the control subset treated with solely 50% ethanol solvent. Finally, promoter activity was measured with a Dual Luciferase assay. For each construct analyzed, multiple independent experiments were conducted, with triplicate transfections performed during each experiment (Shu, Katholi, Higazi, & Unnasch, 2003; Tzertzinis et al. 2010).

Statistical analysis

A Dunnett’s test was performed on the luciferase assay results to determine whether any differences in mean activity were statistically significant. Mean activity of each experimental group (ecdysone-exposed embryos with EcRE promoter, unexposed embryos with mutant promoter, ecdysone-exposed embryos with mutant promoter) was compared with mean activity of the control group (unexposed embryos with EcRE promoter).
Results

Based on previous research findings that revealed a functional ecdysone response system in *B. malayi*, it was believed that functional ecdysone response elements (EcREs) would be present within the *B. malayi* genome (Tzertzinis et al., 2010). The aim of this study was to locate EcREs within the *B. malayi* genome and demonstrate their functionality as gene expression regulators in an ecdysone response system. In order to locate putative EcREs within the *B. malayi* genome, contig DNA segments ≥200 kilobases were searched with strict criteria that corresponded to naturally occurring EcRE sequences. These pattern criteria specified either a perfect or imperfect palindrome containing a half site (R)GGTCA, with a 0-1 nucleotide spacer between half-sites. Thirty-one different sequences were found, with 6 of them existing twice. In total, thirty-seven motifs were located that fit the search criteria. Of these, 10 motifs were not considered for further analysis because they lacked a spacer nucleotide between the palindrome half-sites. Five of the remaining putative EcREs were selected randomly for further study (motif sequences GGGTCAATGACTC, AGGTCATTGAACT, AGGTCATTGACCT, GGGTCAGTGACCT, AGGTCAGTGACAT).

The first step to determine whether these sequences might serve as functional EcREs in *B. malayi* was to quantify expression levels of genes
proximal to the putative EcREs. This was accomplished by performing real-time quantitative PCR (qPCR) on cDNA synthesized from embryo RNA, and calculating fold-differences in gene expression of 20-OH ecdysone exposed embryos relative to untreated embryos. If the EcREs were a functional part of an ecdysone response system, nearby genes should be differentially expressed in embryos treated with 20-OH ecdysone as compared to untreated embryos. In total, twelve target genes located near the five EcREs, were studied. The locations of these genes varied relative to the five EcREs, with five upstream genes (one per EcRE), five downstream genes (one per EcRE), and two genes containing an EcRE within the gene. The open reading frames of the genes also varied in their distance from putative EcREs, ranging from zero to 8165 base pairs away.

Transfection experiments were conducted in parallel with the qPCR experiments to verify that each set of embryos was responsive to the 5uM 20-OH ecdysone treatment. Embryos were transfected with BmRPS12-EcRE-rev, a luciferase reporter vector containing 5 tandem repeats of the PAL-1 EcRE in reverse orientation within an enhancer-less BmRPS12 promoter. This reporter construct had previously been used by Tzertzinis et al. (2010) to examine activity of an EcRE-containing promoter in response to 20-OH ecdysone treatment. This construct had exhibited significantly higher reporter gene expression in embryos treated with 20-OH ecdysone versus unexposed embryos (Tzertzinis et al., 2010). Because this construct reliably produced differential activity levels in 20-OH exposed versus unexposed embryos, it was used in the current study as an
indicator of 20-OH ecdysone responsiveness in the embryo population.

Throughout the control experiments, the mean luciferase activity levels in the 20-OH ecdysone treated embryos were significantly higher (approximately 2.4-fold) than those of the uninduced embryos (Figure 1). A Dunnett’s statistical test showed that the difference in mean activities seen between the treated and untreated embryos was statistically significant (p-value <0.0001). The differences in mean activity observed in these control experiments were similar to those obtained by Tzertzinis et al. (2010) with the same construct, and 1μM 20-OH ecdysone treatment. The 20-OH ecdysone triggered reporter gene activity seen in this subset of embryos indicated that the embryos used in the qPCR experiments should have been responsive to 20-OH ecdysone treatment.

Figure 1. Mean activity of uninduced BmRPS12-EcRE-rev promoter and 20-OH ecdysone treated BmRPS12-EcRE-rev promoter, showing a significant increase in activity with 20-OH treatment. Bars represent mean activity relative to the uninduced BmRPS12-EcRE-rev promoter. Error bars are the standard deviations resulting from multiple independent transfections. The asterisk indicates statistically significant difference in activity levels of denoted promoter compared with the uninduced BmRPS12-EcRE-rev promoter (p ≤ 0.05, Dunnett’s test).
Seven of the twelve target genes were consistently upregulated when embryos were exposed to 20-OH ecdysone in all three repeats of the qPCR experiments. The remaining target genes showed inconsistency in differential expression across the three repeat experiments (Table 1). Neither relative location nor distance from an EcRE appeared to have an effect on the induction of the genes. All five of the EcREs had at least one nearby gene consistently upregulated. These results suggest that all five of these EcREs perform a functional role in ecdysone response and gene regulation. However, they do not appear to regulate all flanking genes. One of the consistently upregulated genes codes for a MIZ zinc finger family protein (Bm1_48650), and is located approximately 2 kilobase pairs downstream of an EcRE (AGGTCATTGACCT) (Figure 2). Zinc finger proteins have the ability to bind DNA and act as transcription factors. Because this gene biologically makes sense as a target for increased gene expression in the primary stage of an ecdysone cascade, it was chosen as the focus for further study.
Table 1. Mean fold-change in gene expression levels of 20-OH ecdysone treated embryos relative to untreated embryos, for twelve genes proximal to putative EcREs in the \textit{B. malayi} genome. Standard deviations result from three independent qPCR experiments. Genes in shaded cells showed consistent upregulation throughout the repeat experiments. The MIZ zinc finger family protein, chosen for further analysis, is highlighted.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mean fold-change</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>major facilitator superfamily protein</td>
<td>Bm1_12830</td>
<td>4.66</td>
</tr>
<tr>
<td>protein kinase domain containing protein</td>
<td>Bm1_12835</td>
<td>2.14</td>
</tr>
<tr>
<td>hypothetical protein</td>
<td>Bm1_12840</td>
<td>1.34</td>
</tr>
<tr>
<td>6 phosphogluconolactonase family protein</td>
<td>Bm1_33900</td>
<td>3.04</td>
</tr>
<tr>
<td>probable beta arrestin</td>
<td>Bm1_33905</td>
<td>2.83</td>
</tr>
<tr>
<td>probable NADH-ubiquinone oxidoreductase 20 kDa subunit</td>
<td>Bm1_48645</td>
<td>1.13</td>
</tr>
<tr>
<td>MIZ zinc family protein</td>
<td>Bm1_48650</td>
<td>3.15</td>
</tr>
<tr>
<td>hypothetical protein</td>
<td>Bm1_52900</td>
<td>1.00</td>
</tr>
<tr>
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<tr>
<td>filarial antigen Av33 precursor</td>
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The qPCR results indicate that the five EcREs are functional, but to determine that the increased level of gene expression is actually being driven by the EcRE requires proof that the EcRE sequence alone is necessary to upregulate gene expression. A transgenic 20-OH ecdysone induction assay was used to examine activity of the EcRE-containing promoter of the Bm1_48650 gene. Transfections with this promoter cloned into a luciferase reporter vector showed a significantly higher level of expression of the luciferase reporter gene (roughly 3.5-fold) in embryos treated with 20-OH ecdysone relative to uninduced
embryos (Figure 3). A statistical analysis comparing mean luciferase activities of the 20-OH ecdysone treated versus untreated embryos showed that the observed difference was statistically significant (p-value <0.0001, Dunnett’s). These results confirmed the qPCR results with regard to the EcRE in the Bm1_48650 promoter, by showing that this EcRE-containing promoter was capable of driving increased expression under 20-OH ecdysone treatment conditions. Conversely, this experiment revealed that 20-OH ecdysone was able to trigger an increase in transcription above the background transcriptional activity of this EcRE-containing construct. These results demonstrate that this promoter was able to upregulate gene expression in an ecdysone-responsive manner, and is therefore likely a viable part of a B. malayi ecdysone response system. However, it was necessary to remove the EcRE from the promoter in order to show that the EcRE alone is responsible for the observed gene regulation. The EcRE portion of the Bm1_48650 promoter within the luciferase reporter constructs was completely mutated (Figure 4), and the activity of the mutated promoter determined using the transgenic 20-OH ecdysone induction assay. Statistical analysis of the difference in mean activities between the uninduced native EcRE-containing construct and the uninduced mutant showed no statistically significant difference (p-value = 0.9946). Most importantly, there was no statistically significant difference between mean activities of the uninduced native EcRE-containing construct and the 20-OH ecdysone treated mutant (p-value = 1.0000). These results confirm that this EcRE sequence was in
fact necessary for the increase in gene expression displayed when embryos were treated with 20-OH ecdysone (Figure 5).
Figure 2. Map showing locations of genes, including Bm1_48650 MIZ zinc finger family protein, relative to an EcRE.
Figure 3. Mean activity of uninduced native Bm1_48650 EcRE-containing promoter and 20-OH ecdysone treated Bm1_48650 EcRE promoter, indicating a significant increase in activity occurring with 20-OH ecdysone treatment in an EcRE-containing promoter. Bars represent mean activity relative to the uninduced native Bm1_48650 EcRE promoter. Error bars are the standard deviations resulting from multiple independent transfections. The asterisk indicates statistically significant difference in activity levels of denoted promoter compared with the uninduced native Bm1_48650 EcRE promoter ($p \leq 0.05$, Dunnett’s test).

native EcRE sequence: AGGTCAATTGACCT
mutant replacement sequence: CATCTCAGAGGTAC

Figure 4. Sequence of Bm1_48650 promoter EcRE, and corresponding mutated sequence.
**Figure 5.** Mean activity of uninduced mutant promoter and 20-OH ecdysone treated mutant promoter. Bars represent mean activity relative to the uninduced native Bm1_48650 EcRE promoter. Error bars are the standard deviations resulting from multiple independent transfections. There was no significant difference in activity levels of the treated and uninduced mutant promoter compared with the uninduced native Bm1_48650 EcRE promoter.
Discussion

The finding by Tzertzinis et al. (2010) describing the existence of a viable ecdysone response system in *B. malayi* led to the hypothesis that *B. malayi* has functional EcREs within its genome that regulate gene expression as part of this system. The results from this study conclusively demonstrated the existence of a *B. malayi* EcRE that was necessary for regulating gene expression in response to 20-OH ecdysone. In addition, qPCR results indicated that additional viable EcREs may exist in *B. malayi*. These findings provided further evidence to support the existence of a functional 20-OH ecdysone-inducible gene expression system in *B. malayi*.

The EcRE located within the promoter of gene Bm1_48650 is functional and necessary for regulating gene expression with 20-OH ecdysone treatment. Furthermore, there is enough evidence to consider Bm1_48650 an “early” gene participant in a *B. malayi* ecdysone response cascade. The Bm1_48650 gene codes for a MIZ zinc finger family protein, and zinc finger proteins have the ability to bind DNA and act as transcription factors. As one of the initially activated proteins, it would have the capability of regulating another group of genes, through repression or activation, thus expanding the field of proteins involved in the response cascade.
The results of the qPCR showed variability in expression levels and fold-differences between 20-OH ecdysone treated and untreated embryos across independent experiments. However, the results indicated functionality of each of the five putative EcREs examined, as at least one gene proximal to each of the five EcREs displayed consistent upregulation across the repeat experiments. Furthermore, the results from the transgenic ecdysone induction assay experiments verified the functionality of one of these EcREs, thus confirming the qPCR results for one of the five EcREs. The established transgenic hormone induction assay is recommended for verification of functionality of additional putative EcREs.

It is feasible that a much larger number of possible EcREs exist within the B. malayi genome than were identified in this study. First, only a selection of potential EcREs that were initially located were included in the qPCR experiments. Furthermore, the search criteria to identify putative EcREs in B. malayi were fairly strict. By including only perfect and imperfect palindromic sequences, other naturally occurring motifs - direct repeat arrangements - were not considered. Moreover, with only a single mismatched nucleotide allowed, sequences having greater nucleotide variation would not have been included. Widening the search parameters and performing qPCR with genes nearby to additional putative EcREs could reveal a larger collection of functional motifs.

Once the ecdysone response system’s initial targets have been identified, there are methods available that would facilitate the identification and description of an ecdysone response cascade hierarchy and pathways within B. malayi. In
insects, ecdysone is responsible for regulating critical processes throughout the life cycle, including oogenesis, embryogenesis, molting, and metamorphosis (Gauhar et al., 2009; Tzertzinis et al., 2010; Wang, Miura, Miksicek, Segraves & Raikhel, 1998). Based on evidence of a functional ecdysone response system, and expression of ecdysone receptor proteins in various life stages, including adult females, it can be hypothesized that ecdysone also controls aspects of reproduction and molting in *B. malayi*. Determination of which genes are induced by ecdysone, and the function of their encoded proteins, is needed to elucidate which aspects of the *B. malayi* life cycle ecdysone regulates. RNA interference (RNAi) experiments could target *B. malayi* genes homologous to those necessary for successful oogenesis, embryogenesis, and molting in organisms such as *C. elegans* and *D. melanogaster*. The results would indicate whether these genes were critical players in these processes and ultimately, survival of the *B. malayi* parasite (Ghedin et al., 2007). Real-time PCR (qPCR) could be used to measure expression of these critical genes in 20-OH ecdysone treated and untreated worms or embryos. If these genes were upregulated in the treated worms, this would suggest that 20-OH ecdysone regulates the processes these genes are essential to. Microarrays could be used to compare gene expression profiles for 20-OH ecdysone treated versus untreated worms. The results would reveal genes that are affected by 20-OH ecdysone, providing an overall picture of what genes are participating in the cascade. However, the results would lack an indication of the response hierarchy of the cascade. Further analysis of the upregulated genes using Gene Ontology (GO) terms and biological pathways
that are described for other organisms would aid in determining potential pathways regulated by ecdysone in *B. malayi*. Furthermore, a comparison of the upregulated genes with ESTs for each stage of the *B. malayi* life cycle would aid in determining which processes the ecdysone response system regulates, based on the affected stages of the parasite’s life.

The existence of an ecdysone response system in *B. malayi* provides a desirable avenue for drug targets (Kumar et al., 2007; Tzertzinis et al., 2010). Because this system is fundamentally controlled by an ecdysteroid, it could also be manipulated by ecdysteroid agonists or antagonists. Transactivation experiments by Tzertzinis et al. (2010) showed that both Ponasterone-A (an ecdysteroid) and RSL1 (an ecdysteroid agonist) were able to activate the ligand binding domain of Bma_EcR, leading to reporter gene expression. This verifies that different ecdysteroid compounds, as well as an ecdysteroid agonist have the ability to activate the ecdysone response system in *B. malayi* (Tzertzinis et al., 2010). By interfering with the ecdysone response cascade, molting, oogenesis, or embryogenesis processes could be arrested, interrupted, or advanced inappropriately, leading to death. In fact, there are currently compounds being used as insecticides that target ecdysone receptors in larval insect stages (Nakagawa & Henrich, 2009; Wheelock et al., 2006).

A rapid and reliable screening system capable of detecting ecdysteroid agonists and antagonists was created using transformed silk moth (*Bombyx mori*) cells. It is a high-throughput reporter assay system that can be used for initial appraisal of natural plant extracts as well as synthetic compounds for
ecdysone agonistic or antagonistic properties. In addition, preferred chemical structural characteristics of agonist and antagonist binding can be determined and predicted through modeling methods such as Quantitative structure-activity relationship (QSAR) and Comparative Molecular Field Analysis (CoMFA). (Swevers et al., 2004; Wheelock et al., 2006). Active agonist and antagonist compounds identified by the high-throughput assay can be tested for activity in a native B. malayi environment using the transgenic induction assay initially described and recommended in Tzertzinis et al. (2010). In experiments with putative agonists, treatment of the embryos with 20-OH ecdysone would be replaced by treatment of the embryos with the agonist compound. Whereas, testing of antagonistic compounds would involve concurrent treatment of the embryos with both the putative antagonist and 20-OH ecdysone.

In conclusion, this study’s findings show that B. malayi has a functional EcRE within its genome. The EcRE was considered necessary for increased transcriptional activity seen with 20-OH ecdysone treatment. This functional EcRE sits within the promoter region of the Bm1_48650 gene, suggesting that this gene, which codes for a transcription factor, may act as a regulator as part of a signaling cascade. Furthermore, the findings from qPCR experiments indicate that B. malayi may have additional functional EcREs. The results from this study provide further validation of the existence of an ecdysone response system in B. malayi. Future work is necessary to determine whether the ecdysone response system controls any vital processes within this parasite. If so, the ecdysone response system would make for a promising target for drug development. The
lack of ecdysone receptors in humans substantiates this system's practicality as a drug target (Wheelock et al., 2006). Moreover, availability of mechanisms for screening potential compounds for their ability to disrupt the ecdysone response system may allow for expeditious discovery of drugs capable of combating lymphatic filariasis.
List of References


