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Potential involvement of *Brugia malayi* cysteine proteases in the maintenance of the endosymbiotic relationship with *Wolbachia*

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ABSTRACT

Brugia malayi, a parasitic nematode that causes lymphatic filariasis, harbors endosymbiotic intracellular bacteria, *Wolbachia*, that are required for the development and reproduction of the worm. The essential nature of this endosymbiosis led to the development of anti-*Wolbachia* chemotherapeutic approaches for the treatment of human filarial infections. Our study is aimed at identifying specific proteins that play a critical role in this endosymbiotic relationship leading to the identification of potential targets in the adult worms. Filarial cysteine proteases are known to be involved in molting and embryogenesis, processes shown to also be *Wolbachia* dependent. Based on the observation that cysteine protease transcripts are differentially regulated in response to tetracycline treatment, we focused on defining their role in symbiosis. We observe a bimodal regulation pattern of transcripts encoding cysteine proteases when *in vitro* tetracycline treated worms were examined. Using tetracycline-treated infertile female worms and purified embryos we established that the first peak of the bimodal pattern corresponds to embryonic transcripts while the second takes place within the hypodermis of the adult worms. Localization studies of the native proteins corresponding to *Bm-cpl-3* and *Bm-cpl-6* indicate that they are present in the area surrounding *Wolbachia*, and, in some cases, the proteins appear localized within the bacteria. Both proteins were also found in the inner bodies of microfilariae. The possible role of these cysteine proteases during development and endosymbiosis was further characterized using RNAi. Reduction in *Bm-cpl-3* and *Bm-cpl-6* transcript levels was accompanied by hindered microfilarial development and release, and reduced *Wolbachia* DNA levels, making these enzymes strong drug target candidates.
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1. Introduction

Filarial parasites are responsible each year for millions of human infections worldwide, causing chronic diseases that include lymphatic filariasis (elephantiasis) due to *Brugia malayi* or *Wuchereria bancrofti* infection, and onchocerciasis (river blindness) as a result of infection by *Onchocerca volvulus* (Lustigman et al., 2012). Present international control programs are focused on the reduction of transmission with the ultimate goal of eliminating these diseases. They are, however, almost universally based on a single strategy: the mass administration of microfilaricidal drugs (Molyneux and Taylor, 2001; Molyneux et al., 2003; Chu et al., 2010; Taylor et al., 2010; Hoerauf et al., 2011). Importantly, treatment of filarial infections in humans is still suboptimal due to a lack of macrofilaricidal drugs (i.e. drugs that can kill adult worms), and no vaccines are yet available to prevent new infections (Hoerauf et al., 2011; Lustigman et al., 2012; Prichard et al., 2012). Additional research is critically needed to discover novel drug targets and to develop a new generation of drugs with macrofilaricidal effects.

Many filarial species harbor an endosymbiotic bacterium of the genus *Wolbachia*. A recent investigation of *Wolbachia* distribution

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in 35 filarial species including 28 species and 7 genera and/or subgenera have shown that 37% of them harbor *Wolbachia* (Ferri et al., 2011). In filarial nematodes, *Wolbachia* appear to have evolved towards a mutualistic symbiosis. Genomic information for *B. malayi* and its endosymbiont *Wolbachia* have enabled us to better understand this co-dependency (Foster et al., 2005a; Ghedin et al., 2007; Slatko et al., 2010). As the endosymbiont has limited biosynthetic capabilities, it is highly plausible that the host supplements *Wolbachia* with amino acids required for growth (Foster et al., 2005a,b). Conversely, *Wolbachia* appears to supply the filarial host with riboflavin, flavin adenine dinucleotide, nucleotides and possibly also heme, although the recent genome study of *Loa loa*, which does not harbor *Wolbachia* indicates that this might be overly inferred (Desjardins et al., 2013). Notably, the endosymbiont appears to be essential for optimal filarial development in the definitive human host, including development of L3 to L4 and reproduction; as such, it has become a target for the development of novel chemotherapeutic drugs (Foster et al., 2005a; Slatko et al., 2010; Johnston et al., 2014; Taylor et al., 2014). *Wolbachia* appears to be less important for L1 to L3 development in the intermediate host (Arumugam et al., 2008). Treatment of humans with antibiotics was shown to have a strong anti-filarial effect, confirming the essential role *Wolbachia* plays in survival and reproduction of the worm. For example, antibiotic treatment of *W. bancrofti* or *O. volvulus*-infected patients with doxycycline resulted in a long-term embryostatic effect, sterility of adult female worms with a sustained reduction of microfilarial loads (Taylor et al., 2010; Hoerauf et al., 2011). Notably, this treatment resulted also in slow death for the adult worms, with the majority of the worms (70% for onchocerciasis and 90% for LF) dying 2 years after treatment, with subsequent improvement in the pathological manifestations of both diseases (Debrah et al., 2006, 2007, 2011; Hoerauf et al., 2008; Specht et al., 2008, 2009).

To better understand endosymbiosis at the molecular level and the dependency of *B. malayi* on its endosymbiont, we analyzed *B. malayi* gene expression patterns in response to depletion of *Wolbachia* by tetracycline treatment *in vivo* (Ghedin et al., 2009). This study highlighted *B. malayi* metabolic pathways—including proteolysis, translation, energy metabolism, and signal transduction—as being affected by *Wolbachia* depletion, thus indirectly implicating them in the endosymbiotic relationship (Ghedin et al., 2009). Some of the most up-regulated genes encoded proteins known to be involved in regulating degradation of intracellular proteins, including the cathepsin L-like cysteine proteases *Bm-cpl-3* (Wormbase gene ID WBGene00233004) and *Bm-cpl-6* (WBGene00233058). *Bm-cpl-3* was up-regulated at day 7 post-treatment while *Bm-cpl-6* was up-regulated at day 14 post-treatment. In comparison, *Bm-cpl-4* (WBGene00227937) was down-regulated 7 and 14 days after depletion of the endosymbiont with tetracycline (Ghedin et al., 2009). The regulation of the cathepsin L-like cysteine proteases by tetracycline treatment was of interest, as it identified a potential connection between the essential dependency of the filarial parasite on *Wolbachia* and the known functions of these proteins in the filarial host (Ghedin et al., 2009).

Remarkably, the role of *Wolbachia* during filarial development, molting and embryogenesis is similar to the roles attributed to these proteases in filarial development (Lustigman et al., 1992, 1996, 2004; Tort et al., 1999; Guiliano et al., 2004; Ford et al., 2009). In *B. malayi*, two clade I subfamilies of the cathepsin L- like cysteine proteases (*Bm*-CPL) were identified (Guiliano et al., 2004): clade group Ia includes *Bm*-CPL-1, -4 and -5, and clade Ic includes *Bm*-CPL-2, -3, -6, -7 and -8. The cathepsin-L like proteases of group Ia have been studied extensively (Britton and Murray, 2004, 2006; Guiliano et al., 2004; Ford et al., 2009), and were shown by RNA interference (RNAi) to be essential for molting of *O. volvulus* larvae (Lustigman et al., 2004) and *B. malayi* larvae (Song et al., 2010), as well as for embryogenesis in *B. malayi* female worms (Lustigman et al., 2004; Ford et al., 2009). Electron microscopy analysis of adult female worms treated with double-strand RNA corresponding to *Bm-cpl-5* indicated that the number of *Wolbachia* in the hypodermis of the adult worms as well as in microfilariae were much reduced in the RNAi treated worms, as compared to the untreated controls. In comparison, the number of *Wolbachia* in the oocytes and embryos was similar to those of normal worms (Ghedin et al., 2008). To further understand the possible role of the filarial cysteine proteases during symbiosis we focused in the present study on the group Ic cathepsin-L like proteases, since expression of *Bm-cpl-3* and *Bm-cpl-6* is modulated by *Wolbachia* depletion (Ghedin et al., 2009) and very little is known about these proteins.

2. Materials and methods

2.1. Treatment with tetracycline of young and adult B. malayi worms

Young (42 days post-infection) and adult female worms (120 days post-infection) were treated with tetracycline *in vitro* in short-term cultures. The parasites were obtained from the NIH/NIAID Filariasis Research Reagent Resource Center (www.filariasiscenter.org). Worms were washed several times with incomplete RPMI-1640 media (Gibco, Grand Island, New York) containing 100 U/ml streptomycin, 100 μg/ml penicillin, and 0.25 mg/ml of amphotericin-B (Sigma). The young or adult female worms were cultured in groups of 4 in 2 ml of complete media (CM; incomplete RPMI 1640 supplemented with 25 mM HEPES buffer, 2 mM glutamine, and 10% heat-inactivated fetal calf serum) or with 40 μg/ml tetracycline (Sigma) in CM in 24-well culture plates (Costar, Cambridge, Massachusetts). The cultured worms were incubated at 37 °C in an atmosphere of 5% CO_2 in air. The culture medium was replaced daily with CM or CM + tetracycline. Worms were harvested on day 6 or earlier based on the experiment. Each experiment was carried out in triplicate.

The worms and embryos were treated with 40 μg/ml tetracycline as previous studies had demonstrated that this was the minimum concentration capable of reducing microfilariae release *in vitro* by close to 100% (Rao and Well, 2002), and in our previous study (Ghedin et al., 2009) caused 100% degradation of *Wolbachia* without affecting the worms' motility. Moreover, the concentration of 40 μg/ml is equivalent to a concentration of 83 μM, which is well within the range of drug concentrations reported in other published studies (Rao and Well, 2002).

2.2. Treatment with tetracycline of B. malayi embryos

B. malayi adult female worms (500 worms, 120 days post-infection) were cut into small pieces using a scalpel in approximately 10 ml of complete media (CM; incomplete RPMI 1640 supplemented with 25 mM HEPES buffer, 2 mM glutamine, and 10% heat-inactivated fetal calf serum). One hour of gentle rocking at room temperature followed by overnight incubation at 37 °C resulted in the release of all embryonic stages. The embryos were then separated from the cuticles by passing the suspension twice through a filter (BD Falcon, 70 μm Nylon, 35-2350). The embryos were centrifuged for 10 min at 2000 rpm at room temperature and their number quantified using the improved Neubauer haemocytometer. Treatment of embryos with tetracycline (40 μg/ml) was carried out *in vitro* in 24-well culture plates (Costar, Cambridge, Massachusetts) for 6 days at 37 °C under 5% CO_2. Each well contained 7×10^5 embryos and the treatment was carried out in triplicate for each time point.

2.3. qRT-PCR

B. malayi worms or embryos treated with 40 μg/ml tetracycline along with their respective controls were snap frozen and crushed in liquid nitrogen using a mortar and pestle. Crushed samples were re-suspended in TRIzol reagent (Invitrogen, Carlsbad, CA) and total RNA was prepared according to the manufacturer's protocol. RNA samples were treated with amplification grade DNase I (Gibco BRL, Gaithersburg, MD) to eliminate genomic DNA contamination. Quantitation of RNA was performed using a NanoDrop 2000 (Thermo Scientific).

Synthesis of first strand complementary DNA (cDNA) was prepared from 1 μg total RNA using the SuperScript™ III First Strand Synthesis System and random primers followed by treatment with RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA). The qRT-PCR reaction included 5 μl cDNA template mixed with 12.5 μl SYBR Green/Rox PCR master mix (SABiosciences), 1 μl each of 5 μM forward and reverse gene-specific primers in a 25 μl total reaction volume. The gene-specific primers used are listed in Supplementary Table 1. The PCR reaction conditions were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles for 15 seconds at 95 °C, 30 seconds at 60 °C and 30 seconds at 72 °C (ABI 7300 Real-time PCR system). Threshold cycle values were normalized against *B. malayi* NADH dehydrogenase subunit 1 (ND1) as the internal control gene.

For qRT-PCR of the related *Acanthocheilonema viteae cpl* genes, the specific primers listed in Supplementary Table 2 were used. The cDNA samples used for the analyses were from female *A. viteae* adult worms recovered from infected *Meriones unguiculatus* that were not treated, or treated orally for six weeks with 0.5% (w/v) tetracycline in drinking water (Strubing et al., 2010). The PCR conditions were as described above for *B. malayi*, however the threshold cycle values were normalized against the *A. viteae* actin as the internal control gene.

To measure the relative expression level of each transcript in the treated worms or embryos versus their corresponding control groups, the ΔΔCt method was implemented: $\Delta\Delta Ct = \Delta Ct_{treated} - \Delta Ct_{control}$. The ΔΔCt was obtained from subtracting the ΔCt value of the control group from that of the tetracycline treated samples. Finally, gene expression fold change in the tetracycline treated over control *B. malayi* worms was determined using the formula $2^{-\Delta\Delta Ct}$.

2.4. Expression and purification of B. malayi Bm-CPL-3 and Bm-CPL-6 proteins

cDNAs corresponding to *Bm*-CPL-3 and *Bm*-CPL-6 were amplified by PCR from female *B. malayi* total RNA using the following forward and reverse primer sets: 5′-CGGGATCCAATCCACTTAATGAACTGG-3′ and 5′-CCGCTCGAGTTTGAAGATTGGTACGTTCG-3′; 5′-CGGGATCCCTAATTTATCAGCAATTC-3′ and 5′-CCGCTCGAGCATTGCAGCAAGTGATTCAAG-3′, respectively. The 1074bp *Bm-cpl-3* and 973bp *Bm-cpl-6* products were cloned directly into TOPO TA plasmid vector (Invitrogen), followed by the subcloning of the specific inserts into the pET43 expression vector (Novogen) according to the manufacturers' instructions. The fidelity of the cloned PCR amplicons and their orientation were confirmed by DNA sequencing. Expression of *Bm*-CPL-3 and *Bm*-CPL-6 recombinant proteins was done through the transformation of the pET43_*Bm-cpl-3* and pET43_*Bm-cpl-6* plasmids into BL21 (DE3) *Escherichia coli.* This cloning strategy resulted in His-*Bm*-CPL-3 and His-*Bm*-CPL-6 fusion proteins, which were purified, respectively, under natural and 6 M Urea denaturing conditions using the His Bind Columns (Novagen) according to the manufacturer's instructions. Recombinant His-Bm-CPL-6 protein purified under denaturing condition was dialyzed (50 mM Tris–HCl, 18 mM NaCl, 1 mM EDTA, pH 7.6) after purification. Both purified recombinant proteins were analyzed by SDS–PAGE. Protein concentration was determined using NanoDrop 2000 (Thermo Scientific).

2.5. Production of mouse antibodies against Bm-CPL-3 and Bm-CPL-6 recombinant proteins

A group of five female BALB/c mice were immunized subcutaneously (s.c.) with 30 μg of recombinant His-*Bm*-CPL-3 or His-*Bm*-CPL-6 proteins formulated with the Sigma Adjuvant System®, as recommended by the manufacturer (Sigma–Aldrich, St. Louis, MO, USA) and under an approved protocol (IACUC protocol #224, New York Blood Center). Boost immunizations were given on days 14 and 28 post primary immunizations. Blood was collected pre-immunization and day 7 after last immunization. Pooled serum was analyzed by western blot. The protein bands of both recombinant proteins, as well as their corresponding native proteins in *B. malayi* crude extract, were detected with each of the protein specific polyclonal antibodies. There was no recognition of the recombinant proteins when pre-immunization serum was used (data not shown).

2.6. Localization of Bm-CPL-3 and Bm-CPL-6 in B. malayi worms by immunoelectron microscopy

B. malayi female worms were fixed in 0.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 containing 1% sucrose for 60 min at room temperature and processed for immunoelectron microscopy, as described previously (Guiliano et al., 2004). Thin sections of embedded worms were blocked and probed with mouse antibodies raised against recombinant His-*Bm*-CPL-3 or His-*Bm*-CPL-6 (1:10 dilution) followed by 15 nm or 18 nm gold labeled goat anti-mouse IgG (H + L) (Amersham Biosciences, UK). Pre-immunization serum was used as the control.

2.7. Phylogenetic analysis of the filarial CPL family

Orthologous filarial genes were identified by BlastP searches using curated *B. malayi* gene models against the GenBank nr database limited to filarial nematodes. Scaffolds encoding *A. viteae* CPL family members were downloaded from the 959 nematodes project page (http://nematodes.org/genomes/acanthocheilonema_viteae/), and manual gene predictions were generated for orthologous CPLs. A multiple peptide alignment of filarial CPLs was generated using Muscle (v. 3.8.3) (Edgar, 2004). The alignment was then inspected manually, and peptide sequences with partial or missing mature regions, or proregions were removed from the analysis. A character set for phylogenetic analysis was generated using Gblocks (Talavera and Castresana, 2007), then manually adjusted.

A phylogenetic tree was generated using PhyML (v3.0) (Guindon and Gascuel, 2003) from 197 characters, using the LG substitution model, and rates across sites modeled on a discrete gamma model approximated with four rate categories; 1000 bootstrap replicates were performed. Only nodes with bootstrap support values above 50% (500) are shown on the tree (Fig. 3). Clades were determined based on membership of annotated Brugia CPLs. The tree was rooted on the CPZ clade.

2.8. RNA interference (RNAi) treatment of B. malayi adult females

RNAi treatment of *B. malayi* adult females was carried out by soaking with siRNA as previously described (Ford et al., 2009) with few modifications. For RNA transcription, purified plasmid DNA containing *Bm-cpl-3* (bp 119-1128) or *Bm-cpl-6* (bp 83-1035) cDNA sequence was first amplified with M13 forward and M13 reverse primers (Invitrogen). Purified PCR products were then used for

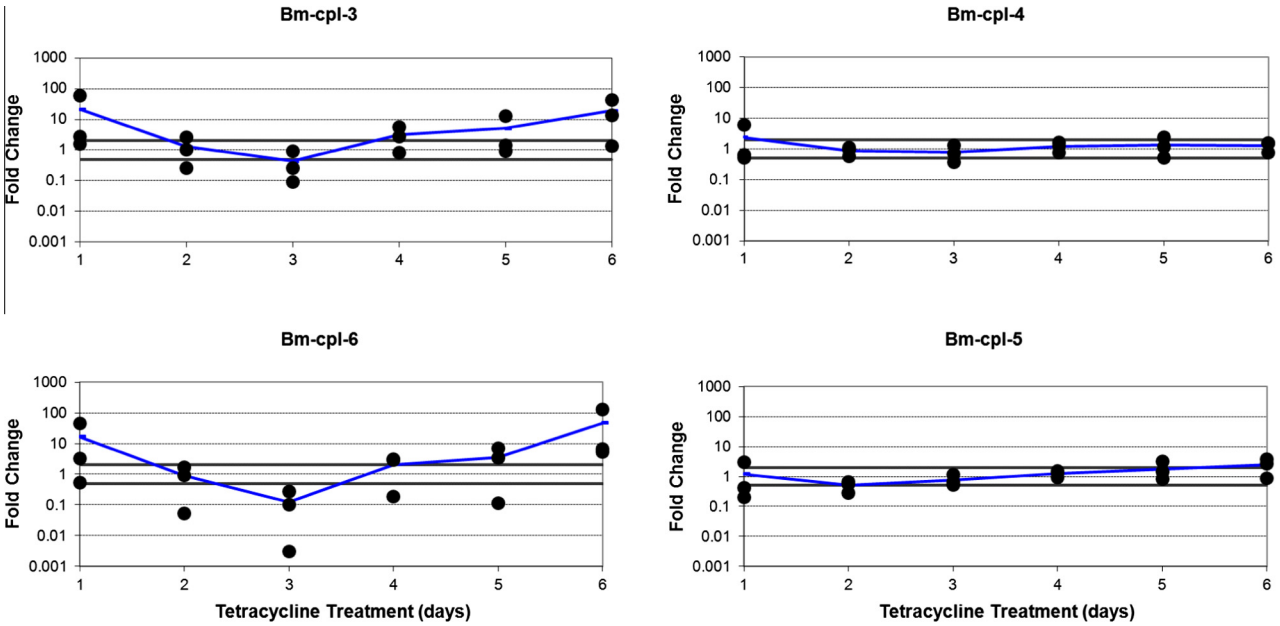

Fig. 1. Fold changes in mRNA expression of the cathepsin L-like Ia and Ic family (*Bm-cpl*) members over six days of tetracycline treatment. Adult female *B. malayi* worms (120 days post infection) were treated with 40 μg/ml tetracycline for up to six days; experiments were performed in triplicate. Levels of *cpl* mRNA were compared each day between the treated and the untreated worms. Fold changes are presented in log scale. Each data point (bullet) represents one independent experiment. The blue line represents average values of three independent experiments. The cutoff of 2- or 0.5-fold increase or decrease, respectively, are marked by a solid black line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

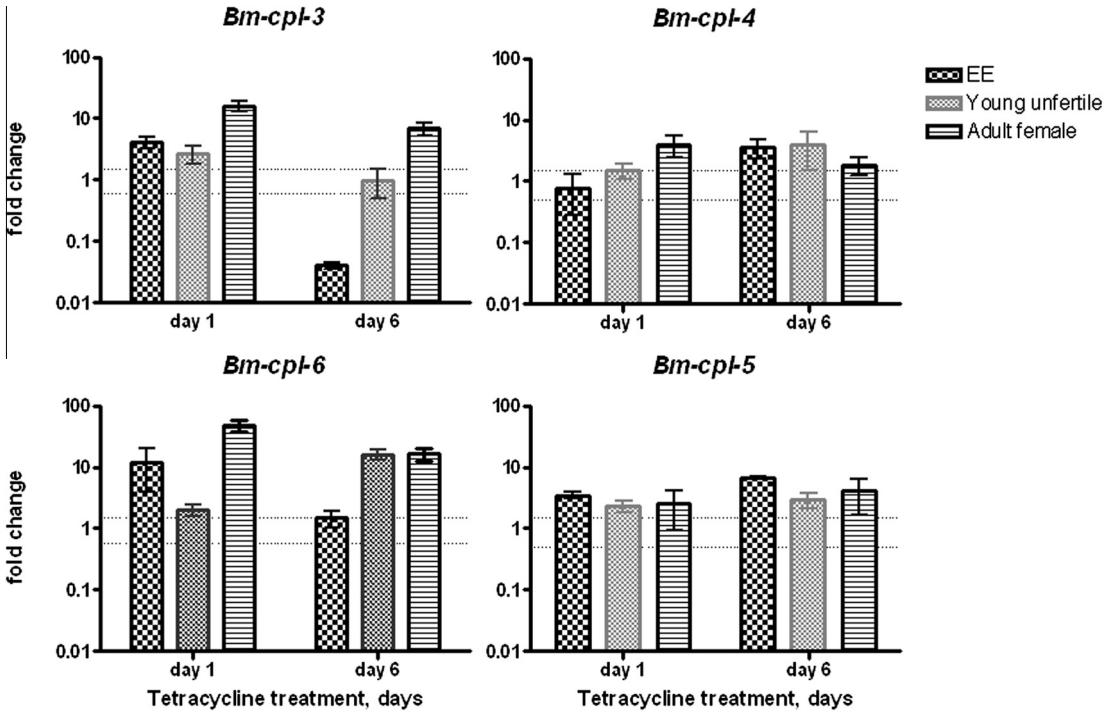

Fig. 2. Fold changes in mRNA levels of *Bm-cpl* genes after tetracycline treatment in egg and embryos (EE), young unfertile female *B. malayi* worms (42 days post infection) or fertile adult worms (90 days post infection). Each stage was treated with 40 μg/ml of tetracycline for up to six days. The *Bm-cpl* genes analyzed belong to the Ic (*Bm-cpl-3* and *Bm-cpl-6*) and the Ia (*Bm-cpl-4* and *Bm-cpl-5*) cathepsin L-like families. The mRNA levels of the treated embryos or young and adult worms were compared to untreated control groups cultured for one and six days with or without tetracycline, respectively. Each column represents the mean fold change per treatment group based on three biological replicas and the error bar is the SD. The cut offs of 2- fold increase and decrease are marked by solid black lines.

single-stranded sense or antisense RNA synthesis using T3 or T7 RNA polymerase and the MEGAscript high yield transcription kit (Ambion Inc., Austin, TX). Integrity of dsRNA was confirmed by standard agarose gel electrophoresis. The siRNA corresponding to the specific target was produced by digesting transcribed dsRNA with RNase III (Ambion) according to the manufacturer's instructions. Undigested and partially RNase III digested material was removed using a siRNA purification unit (Ambion). The siRNA was quantified by measuring absorbance and the concentration calculated according to the manufacturer's instructions.

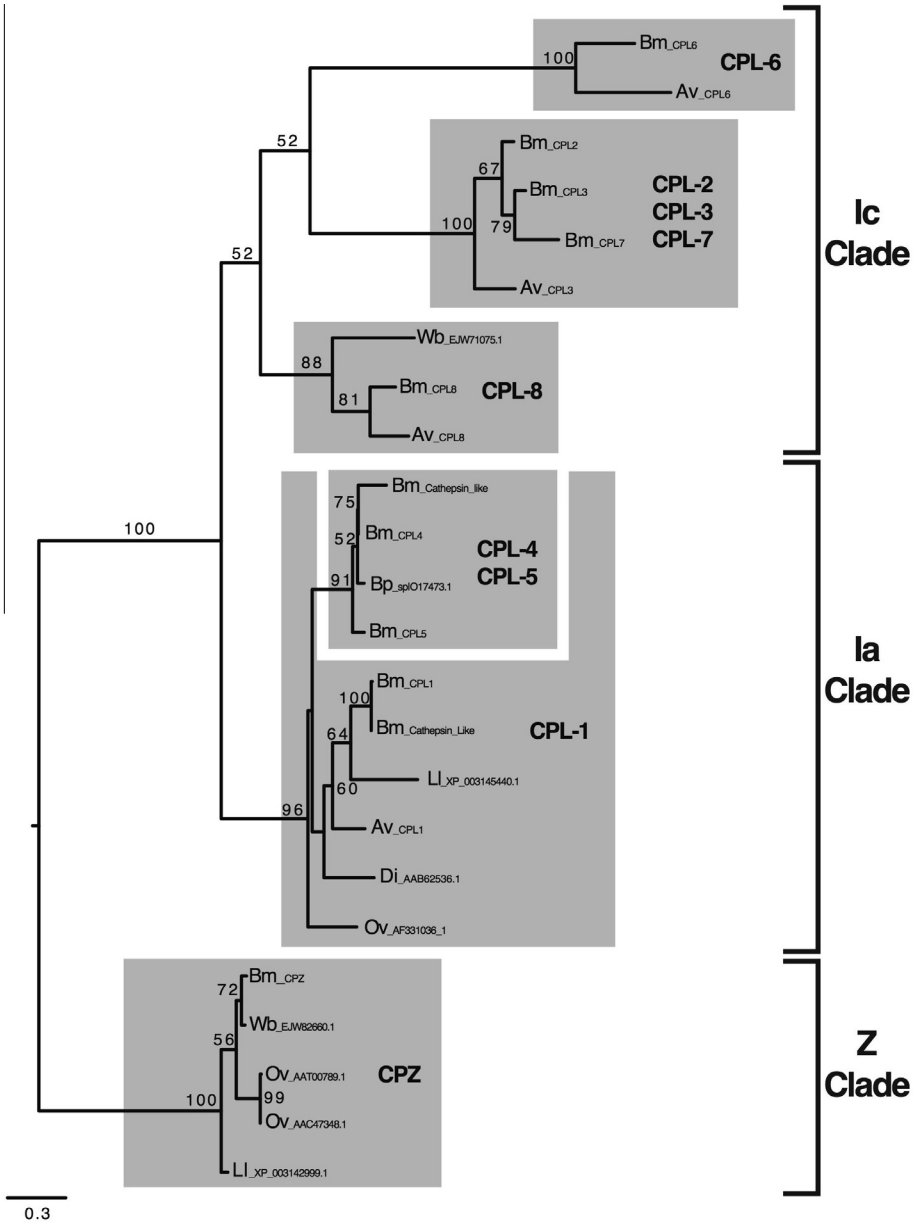

Fig. 3. PhyML maximum likelihood tree of filarial CPLs and CPZ. Bootstrap support values are shown at nodes. Taxon names are abbreviated to simplify the figure. Full names are as follows: *Acanthocheilonema viteae* (Av), *Brugia malayi* (Bm), *Brugia pahangi* (Bp), *Dirofilaria immitis* (Di), *Loa loa* (Ll), *Onchocerca volvulus* (Ov), *Wuchereria bancrofti* (Wb). Grey boxes surrounding clades indicate CPL membership within each of these clades, brackets on the right edge of the figure show CPL clade nomenclature described in Guiliano et al. (2004). All sequences with the exception of *A. viteae* sequences were downloaded from Genbank, and corresponding Genbank accessions are shown with abbreviated names. *Brugia* malayi CPLs are labeled according to their predicted clade. Genbank accessions for these are as follows: Bm_CPL1 (AAT07060), Bm_CPL2 (BAC24764), Bm_CPL3 (BAC24765), Bm_CPL4 (AAT07057), Bm_CPL5 (AAT07058), Bm_CPL6 (AAT07055.1), Bm_CPL7 (AAT07056), Bm_CPL8 (AAT07054), Bm_cathepsinlikeA (AAK16513.1), Bm_cathepsinlikeB (XP_001896823.1). *A. viteae* coding sequences were predicted based on the *A. viteae* assembly (May 2012). Contigs on which these were predicted are as follows: Av_CPL1 (nAv.1.0.scaf01660), Av_CPL3 (nAv.1.0.scaf04999), Av_CPL6 (nAv.1.0.scaf03681), Av_CPL8 (nAV00022).

Following 24 h culture of female worms recovered 120 days post infection in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) plus 1% glucose and 1X antibiotic-antimycotic solution (Sigma–Aldrich, St Louis, MO, USA) at 37 °C in a 5% CO_2 incubator, and the fitness of individual worms and their microfilaria release was scored. The highly viable and motile individual worms were then transferred individually into 48-well plates containing culture medium with 10% heat-inactivated fetal calf serum and 5 μM *Bm*-cpl-3 or *Bm*-cpl-6 siRNA. The negative controls included: culture medium containing RNA storage buffer (medium control) and siRNA corresponding to an *O. volvulus* intronic sequence, *Ov-cpz-int2* (negative control).

The following phenotypic and molecular outcomes were measured after 48 h and 96 h in culture: (1) release of microfilariae from individual female worms after siRNAi treatment in comparison to medium and RNAi negative control groups (9-10 worms per treatment); (2) embryogram (2–3 worms per treatment group), analysis of the stages of uterine embryonic development (eggs, embryos, pre-microfilariae, stretched microfilariae); (3) *Bm-cpl-3* and *Bm-cpl-6* transcript levels as determined by qRT-PCR (4 worms per treatment group); and (4) quantification of *Wolbachia* using a sensitive quantitative-PCR (qPCR) (Fenn and Blaxter, 2004; McGarry et al., 2004), genomic DNA extracted from individual worms (3 worms per treatment group), and the primers described in Supplementary Table 3.

For the embryogram analyses, the numbers for various embryonic stages (eggs, embryos, pre-microfilariae, stretched microfilariae) were counted following the method of Lok et al. (1988) with small modifications. In brief, individual worms were transferred into Eppendorf tubes containing 200 µl of PBS, and gently homogenized for 5–10 s using a disposable sterile pestle. The pestle was washed with 300 µl of PBS and the final volume of the homogenate was then adjusted to 500 µl. The numbers for various embryonic stages in the resulting suspension were determined microscopically (Nikon Eclipse E600) using a hemacytometer. More than 100 events were counted in each of two samples per worm. The segmental data were added to get the total numbers per worm for the different culturing conditions. The embryonic stages were classified into 4 categories and the relative proportions of progeny at different stages of development were then calculated.

2.9. Statistical analysis

Comparisons between microfilariae release in the RNAi treated groups were done using the two-tailed non-parametric Mann–Whitney *U*-test. A *P* value of <0.05 was considered statistically significant.

Comparisons between the phenotypic outcomes in the RNAi treated groups were done using the chi-square test for association with a *P* value of <0.05 considered as statistically significant for the family of comparisons. *P*-values are provided for Bonferroni multiple comparison adjustment. We utilized all available data, such that there are two-worm and three-worm sets of analyses for the 48- and 96-hour experiments.

3. Results

3.1. Depletion of Wolbachia by tetracycline treatment in vitro also regulates the transcription of B. malayi cysteine proteases

The effect of *Wolbachia* depletion on the filarial *Bm-cpl* mRNA expression was analyzed using qRT-PCR. The RNA was prepared from groups of 4 adult female *B. malayi* worms cultured *in vitro* in the presence or absence of tetracycline. *Bm-cpl* mRNA expression was determined over the six day tetracycline treatment. Notably, a bimodal expression pattern was observed. *Bm-cpl-3* and *Bm-cpl-6* expression levels were significantly higher on day 1 with expression levels 16- and 47-fold higher, respectively, as compared to the untreated worms (Fig. 1). By day 3, however, *Bm-cpl-3* and *Bm-cpl-6* gene expression was down-regulated in the treated worms as compared to the similarly cultured control worms. The second expression peak was observed on day 6 of the treatment: *Bm-cpl-3* expression was 7-fold and *Bm-cpl-6* 17-fold higher than controls (Fig. 1). In contrast, *Bm-cpl-4* and *Bm-cpl-5* expression was not affected and remained unchanged over the six days of treatment (Fig. 1).

In a previous study we found a similar gene regulation pattern for four other genes belonging to the signal transduction pathway: kinesin light chain protein 2 (WBGene00228531), phosphatase 2A regulatory A subunit (*Bm-paa-1* WBGene00233942), a protein kinase (*Bm-kin-3* WBGene00221966), and secretion associated Ras-related COPII vesicle coat protein (*Bma-sar-1* WBGene00233918) (Ghedin et al., 2009). We hypothesized that this bimodal pattern of regulation is possibly associated with an immediate effect (first peak) of *Wolbachia* depletion on the *B. malayi* pre-embryonic and embryonic stages, which appear to be more sensitive to the death of the endosymbiont. While the second peak is possibly associated with a delayed response to the elimination of the endosymbiont within the hypodermal tissues of the adult worms (Ghedin et al., 2009). Consequently, we decided to investigate the dependency of gene expression regulation in different *B. malayi* life stages: young unfertile female worms (42 days post infection), and embryonic stages within the gonads, described as eggs and embryos (EE). Both stages were treated with tetracycline for six days *in vitro*. The mRNA isolated from samples cultured for one or six days was transcribed into cDNA using random primers and the expression profiles of the *Bm-cpl* genes were recorded using qRT-PCR (Fig. 2).

As expected, tetracycline treatment of eggs and embryos (EE) resulted in the up-regulation on day 1 of *Bm-cpl-3* and *Bm-cpl-6* transcript expressions, 4-fold and 12-fold respectively. However, both were down-regulated on day 6 when compared to their levels on day 1—more than 103-fold for *Bm-cpl-3* and 8 fold for *Bm-cpl-6*—which probably reflected the consequent death of the EE due to treatment, as previously hypothesized (Ghedin et al., 2009). In the young unfertile female worms the expression of the *Bm-cpl-3* was not affected on day 1 or day 6 (2.6 and 0.99 fold increase, respectively), while *Bm-cpl-6* was only up-regulated at day 6 (16 fold increase). In comparison, in fertile female adult worms both transcripts were highly up-regulated at both time points after treatment. In contrast, the expression profile of *Bm-cpl-4* and *Bm-cpl-5* did not show differential regulation in the different *B. malayi* life stages. These data support the hypothesis that the bimodal regulation of *Bm-cpl-3* and *-6* cysteine proteases of subgroup Ic is related to the differential effect of tetracycline treatment on *Wolbachia* in different tissues.

To confirm that the regulation of these two cysteine proteases has to do with the fitness of *Wolbachia*, we analyzed the regulation of the orthologous transcripts in the *Wolbachia* free rodent filarial parasite *A. viteae*. The orthologous *A. viteae* cathepsin L- like proteins were predicted based on the recently publically available genome (http://nematodes.org/genomes/acanthocheilonema_viteae/). As a first step in this process, the *A. viteae* genes were assigned to the various clades by phylogenetic analyses (Fig. 3). This analysis recapitulates the topology of the tree presented in Guiliano et al. (2004) in terms of CPL clades with filarial membership, and relationships between CPL clades. The newly identified *A. viteae* CPL-1, CPL-3, CPL-6 and CPL-8 cysteine proteases branch with their corresponding orthologues in *B. malayi*, confirming their assignment to these clades.

Using primers designed to the coding regions of the *A. viteae* transcripts (Supplementary Table 2), the regulation of *Av-cpl-3*, *Av-cpl-6* and *Av-cpl-8* was analyzed (we could not identify homologues of *Bm-cpl-4* or *Bm-cpl-5*). The cDNA samples used for the analyses were generously provided by Dr. Kenneth M. Pfarr, University Clinic Bonn, Germany. They were prepared from female *A. viteae* adult worms recovered from infected *Meriones unguiculatus* (Mongolian jird) that were treated orally for six weeks with 0.5% (w/v) tetracycline in drinking water or from untreated infected jirds (Strubing et al., 2010). As seen in Fig. 4, *Av-cpl-3* and *Av-cpl-6* transcript levels were unaffected by tetracycline treatment.

3.2. Profiles of cathepsin L-like proteases life stage-specific expression over the B. malayi lifecycle

To further understand the potential interrelationship between *Bm-cpl-3* and *Bm-cpl-6* with *Wolbachia*, we compared their transcriptional regulation over the different life stages of the *B. malayi* lifecycle in comparison to that of the Ia family members of CPLs. As shown in Fig. 5, the relative expression of the cysteine protease genes varied considerably among the four developmental stages analyzed: adult male, adult female, microfilaria and L3. Transcripts from the adult male stage were used as the reference point in the pair wise comparisons of the mRNA levels because embryo and microfilaria transcripts are inherently a subset of the adult female transcriptome. The expression profiles differed between the family

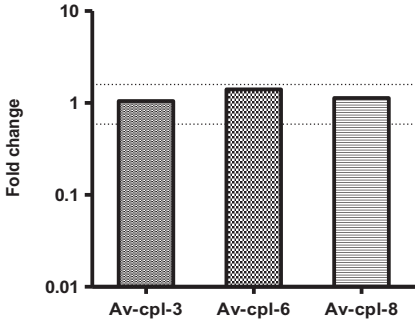

Fig. 4. Analysis of mRNA levels of the *A. viteae* cathepsin L-like lc gene family after tetracycline treatment *in vivo*. The mRNA levels of *Av-cpl-3*, *Av-cpl-6* and *Av-cpl-8* were compared between treated worms collected 6 weeks after tetracycline treatment and untreated age-match control worms; three biological replicates were analyzed. The Ct values of the *A. viteae* cysteine protease genes were normalized using geometrical median of the housekeeping *Av*-actin gene. The cut offs of 2-fold increase and decrease are marked by solid black lines.

members of the group 1c (*Bm-cpl-3*, *Bm-cpl-6*) and 1a (*Bm-cpl-4*, *Bm-cpl-5*) of the cathepsin L-like cysteine proteases, in particular in the L3s; higher levels expression of *Bm-cpl-3* and *Bm-cpl-6* had in adult female and microfilaria and very little in L3s, while *Bm-cpl-4* and *Bm-cpl-5* were highly expressed in all stages tested—adult female, microfilaria and L3s—with highest expression in L3s.

3.3. Subcellular immunolocalization of the B. malayi native Bm-CPL-3 and Bm-CPL-6 proteins by immunoelectron microscopy

Two specific polyclonal antibodies generated against the *Bm*-CPL-3 and *Bm*-CPL-6 recombinant proteins were used to identify the corresponding native CPL proteins in *B. malayi*. The endogenous native proteins were localized by immunoelectron microscopy on sections prepared from the different life-cycle stages of *B. malayi*. The anti-*Bm*-CPL-3 antibodies reacted specifically with *Wolbachia* and the area within the hypodermis in the adult female worm surrounding the *Wolbachia* (Fig. 6a). Notably, there was no labeling of the region of the hypodermis below the cuticle, where anti-*Bm*-CPL-4 and -5 are present (Guiliano et al., 2004). In sections of microfilaria, the *Bm*-CPL-3 was localized within the inner bodies (Fig. 6c). The antibodies raised against *Bm*-CPL-6 reacted similarly with *Wolbachia* and the area within the hypodermis in the adult female worm surrounding the *Wolbachia* (Fig. 6b). In microfilaria these antibodies labeled the protein in the cuticle and inner bodies (Fig. 6d). Pre-immune sera did not cross-react with any proteins in the sections of the life-cycle stages of *B. malayi*. An example of a region containing *Wolbachia* within the hypodermis of adult female worms is shown in Fig. 6e.

3.4. RNAi-mediated silencing of B. malayi Bm-cpl-3 and Bm-cpl-6 resulted in the reduction of microfilariae production in vitro and within the uterus, as well as in the reduction of Wolbachia density within each worm

To further determine the possible function(s) of the *B. malayi Bm-cpl-3* and *Bm-cpl-6* cathepsin-like cysteine proteases during embryogenesis, RNAi that selectively interferes with their expression was carried out. Release of microfilaria per female worm was determined prior to siRNA treatment to provide a baseline reading, showing that in the 4 worm groups the counts were evenly distributed (Fig. 7a). Release of microfilariae from individual worms cultured for 48 h or 96 h in control conditions or treated with 5 μM *Bm-cpl-3* and *Bm-cpl-6* siRNA was also determined. In comparison to the media control and *Ov-cpz-int2* siRNA control, a significant reduction in the number of microfilariae released into

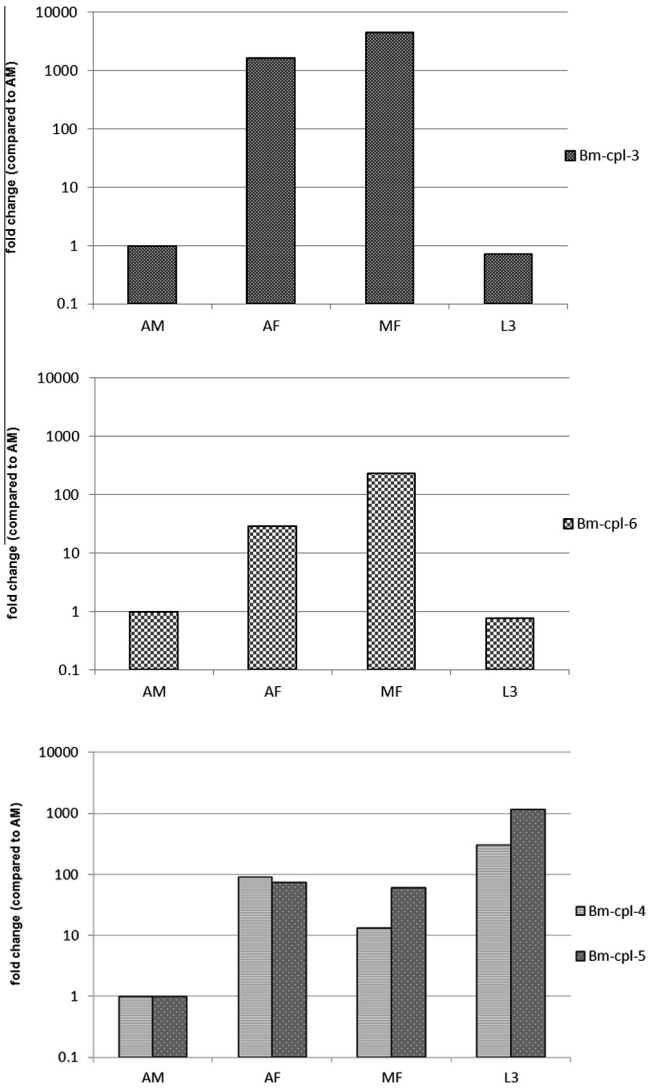

Fig. 5. Stage-specific *B. malayi cpl* gene expression. The Ct values of cysteine protease genes were normalized using the geometric median of housekeeping genes (tubulin and *gst*). The *cpl* mRNA levels in the different life stages of the parasite were compared to adult male worms (AM) using the ΔΔCt method. Abbreviations: AM, adult male; AF, adult female; MF, microfilaria; and L3 – third-stage larvae.

the culture medium was observed in the *Bm-cpl-3* and *Bm-cpl-6* siRNA treated worms: 32.5% (*Bm-cpl-3*) and 39.7% (*Bm-cpl-6*) reduction after 48 h RNAi treatment, and 55.7% (*Bm-cpl*-3) and 41.6% (*Bm-cpl*-6) reduction after 96 h RNAi treatment (Fig. 7b and c).

Subsequently, we also analyzed the effect of the siRNA treatment on embryogenesis. RNAi treatment with *Bm-cpl-3* and *Bm-cpl-6* siRNA had dramatic effects on intrauterine embryogram profiles (Table 1). Intrauterine progeny were examined 48 and 96 hours after RNAi treatment and expressed as the relative proportions of progeny at different stages of development: eggs, developing embryos, pre-microfilariae (pre-Mf) and Mf (Fig. 8). In comparison to the percentage of intrauterine Mf in the medium control (27.1% and 23.6% at 48 h and 96 h, respectively), the adult female worms treated with *Bm-cpl-3* and *Bm-cpl-6* siRNA showed significant reductions in the number of intrauterine Mf in the *Bm-cpl-3* and *Bm-cpl-6* siRNA treated worms: 67.5% and 45% reduction after 48 h, and 41.1% and 59.7% reduction 96 h after RNAi treatment, respectively. When the percentage of intrauterine Mf

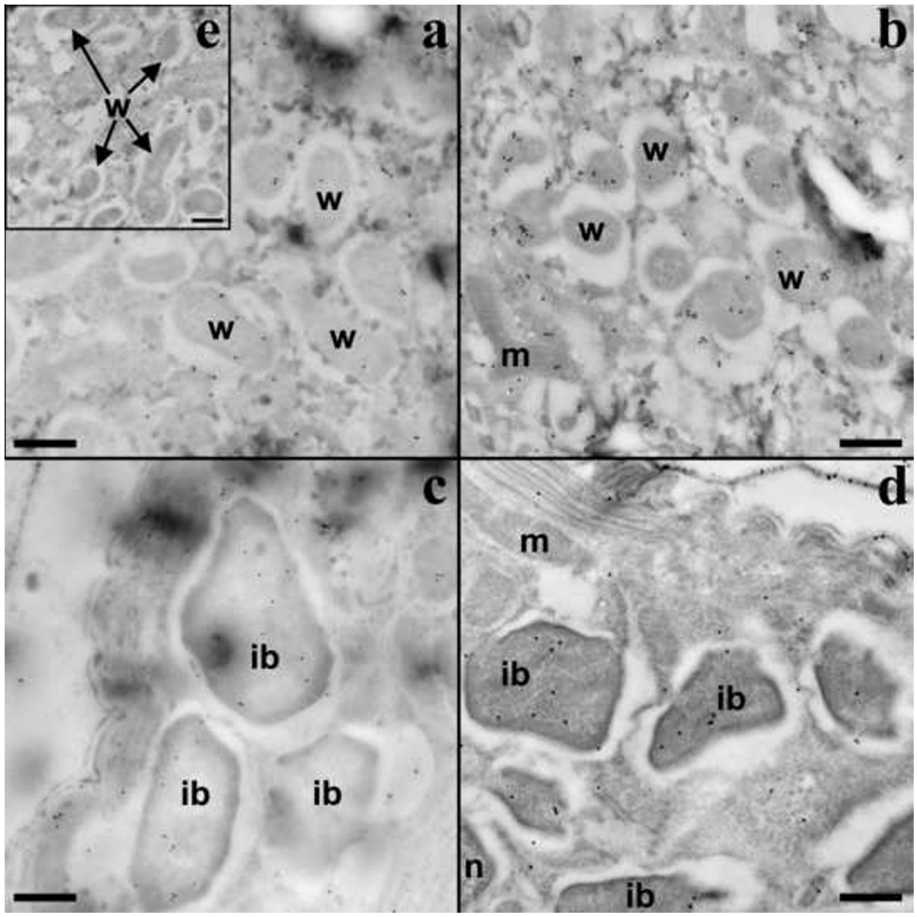

Fig. 6. Localization of the Ic members of the *B. malayi* cathepsin L-like cysteine proteases. (a) *Bm*-CPL-3 labeling in adult female worms. (b) *Bm*-CPL-6 labeling in adult female worms. (c) *Bm*-CPL-3 labeling in purified microfilariae. (d) *Bm*-CPL-6 labeling in purified microfilariae. (e) NRS control. w = *Wolbachia*; n = nucleus; m = mitochondria; ib = inner body. The bar is 500 nm.

was compared to the negative control, *Ov-cpz-int2* (23.2% and 31.5% at 48 h and 96 h, respectively), significant reductions were also observed in the *Bm-cpl-3* and *Bm-cpl-6* siRNA treated worms: 57.7% and 35.3% reduction after 48 h, and 63.8% and 61.5% reduction 96 h after RNAi treatment, respectively. While the numbers of Mf were reduced, the percentage of pre-Mf was significantly increased 48 h after RNAi in comparison to medium control (8.4%): 251.1% in the *Bm-cpl-3* and 515.5% in the *Bm-cpl-6* siRNA treated worms. This increase, however, was less apparent when the percentage of the pre-Mf in the *Bm-cpl-3* and *Bm-cpl-6* siRNA treated worms was compared to *Ov-cpz-int2* (22.7%); 30.3% and 125.1% increase, respectively. The significant increase in the percentage of pre-Mf was also observed 96 h post siRNA treatment when compared to medium control (14.8%); 216.2% in the *Bm-cpl*-3 and 260.8% in the *Bm-cpl-6* RNAi treated worms. Similarly, a significant increase in the percentage of pre-Mf was observed when it was compared to the RNAi negative control, *Ov-cpz-int2* (10.2%); 346% in the *Bm-cpl*-3 and 380.4% in the *Bm-cpl-6* RNAi treated worms. The percentages of embryos and eggs were also significantly decreased in the *Bm-cpl-3* and *Bm-cpl-6* siRNA treated worms 48 h or 96 h after RNAi treatment.

Importantly, when the *Wolbachia* levels were measured by DNA qPCR in the *Bm-cpl-3* and *Bm-cpl-6* siRNA treated worms, it appeared that in comparison to worms cultured in medium alone or in the presence of negative control siRNA (*Ov-cpz-int2*), the density of *Wolbachia* was moderately reduced 48 h after *Bm-cpl-3* and *Bm-cpl-6* siRNA treatment: 20.2% and 20.8%, respectively, in comparison to media control, or 31.0% and 31.6%, respectively, when compared with negative siRNA control (data not shown). The reduction was much more significant after 96 h of *Bm-cpl-3* and *Bm-cpl-6* siRNA treatment: 92.6% and 89.5%, respectively, in comparison to media control, and 82.4% and 75%, respectively, when compared with the negative siRNA control (Fig. 9). The *Bm-cpl-3* and *Bm-cpl-6* levels were reduced by 90% and 45%, respectively, 96 h after treatment when compared to the siRNA negative control as determined by qRT-PCR (data not shown).

4. Discussion

In this study we characterized in more detail the interrelationship between the expression levels of the *B. malayi Bm-cpl-3* and *Bm-cpl-6* cysteine proteases and the fitness of *Wolbachia*, and consequently that of the parasite. Our present *in vitro* data support our previous *in vivo* studies showing that killing *Wolbachia* using tetracycline treatment of *B. malayi* worms resulted in differential expression of these two enzymes (Ghedin et al., 2009). In contrast to *B. malayi*, tetracycline treatment of *A. viteae*, which lacks a *Wolbachia* endosymbiont, did not affect the expression of these enzymes. That being said, the comparison of *cpl* expression in *B. malayi* worms cultured for 6 days *in vitro* in the presence of tetracycline with *A. viteae*, which were recovered from jirds after six weeks of treatment with 0.5% (w/v) tetracycline in drinking water (Strubing et al., 2010) was not optimal. However, six weeks of similar oral tetracycline treatment of *Litomosoides sigmodontis*-infected jirds has been shown to lead to a persistent depletion of *Wolbachia*, demonstrating sufficient tetracycline delivery with this treatment (Arumugam et al., 2008). In contrast, similar tetracycline

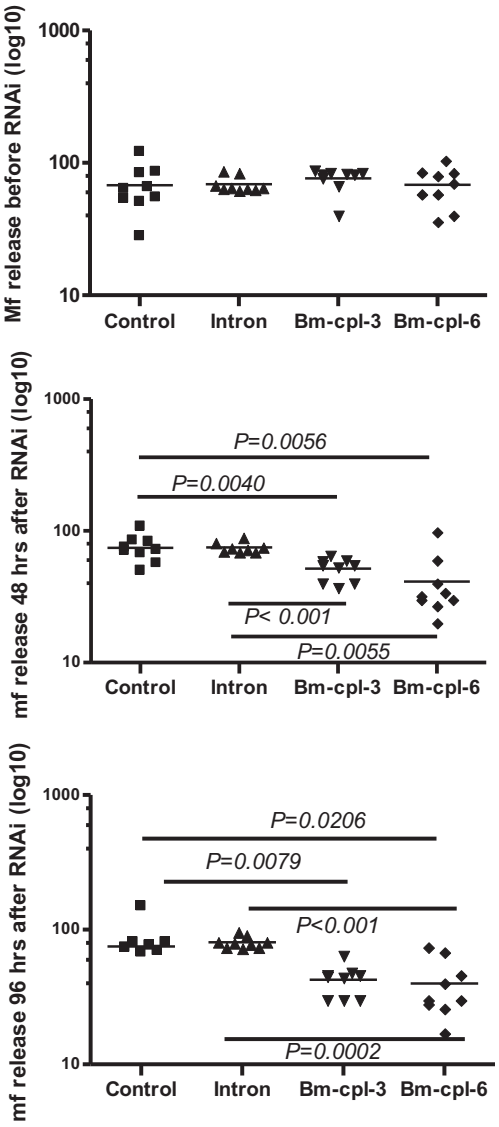

Fig. 7. RNAi treatment of adult female *B. malayi* with *Bm-cpl-3* and *Bm-cpl-6* siRNA leads to a reduction in microfilaria release from *B. malayi in vitro*. Following 24 h culture in normal culture medium *B. malayi* female worms (one worm per well, nine worms per group) were treated for 48 h and 96 h with 5 μM siRNA corresponding to *Bm-cpl-3*, *Bm-cpl-6* or negative control (*Ov-cpz-Int2*), or cultured in medium alone (Control). Released microfilariae were collected and counted. Results are expressed as microfilaria (mf) release before siRNA treatment (A), 48 h (B) and 96 h (C) after treatment. Each graph represents one experiment, which is representative of at least 3 separate experiments. *P* values denote a significant difference between dsRNA-treated worms and either untreated medium controls or negative control (*Ov-cpz-Int2*) (Mann–Whitney *U*-test).

treatment of *A. viteae*, a *Wolbachia*-free filarial nematode did not show any phenotypic effects on the worm (Hoerauf et al., 1999). In our previous studies we have shown that treatment of *B. malayi in vitro* with 40 μg/ml tetracycline caused 100% degradation of *Wolbachia* by day 5 without affecting the worms' motility (Ghedin et al., 2009).

The *in vitro* studies support our previous observations of a bimodal expression pattern of *B. malayi* transcripts after tetracycline treatment, including the two enzymes we studied here. We hypothesize that this bimodal expression may be due to stage-specific responses of the host in response to *Wolbachia* death; the first effect is in the pre-embryonic and embryonic stages of the worms and the later effect is due to reduced fitness of the worms. The differential expression patterns in female worms at various stages of fertility were not surprising because adult female worms (>70 days after infection) can produce eggs and contain developing embryonic stages, which would account for the differential expression profiles in comparison to those in unfertile females. Clinical studies in humans have also shown that in filaria-infected patients treated with antibiotics the first phenotype is reduction in the levels of microfilariae followed by a long-term sterility and eventual death of adult worms (Taylor et al., 2010).

However, it is possible that some of the responses observed upon tetracycline treatment may also be unrelated to its antibacterial effects. Tetracycline is known to have pleiotropic effects that are unrelated to its antibiotic activity. For example, bacteriologically inactive analogs of tetracycline have been shown to inhibit molting in *B. malayi* larvae, suggesting that the effect on molting observed in tetracycline treated larvae is not related to its anti-*Wolbachia* activity (Rajan, 2004). Similarly, tetracycline has been shown to exhibit anti-collagenase activity, an enzymatic activity that is likely important in molting, as collagens are an important component of the parasite cuticle (Griffin et al., 2010). Dissection of the exact mechanism of the effect of tetracycline on adult worms will require additional studies utilizing bacteriologically inactive analogs in addition to tetracycline, perhaps coupled with RNAi studies that specifically target the enzymes in question in parallel of both *B. malayi* and *A. viteae* worms.

To better understand the possible functions of the Ic cysteine proteases in *B. malayi*, we analyzed the localization of these proteins. Contrary to what was observed for cathepsin L-like cysteine proteases in the Ia group (Guiliano et al., 2004), the proteins in the Ic group were not localized to the hypodermal lamellae or the eggshell surrounding all stages of the developing microfilariae. They were instead found in the inner bodies of microfilariae and co-localized with *Wolbachia*. In some cases, they were found within the *Wolbachia* cells. As for our qRT-PCR analysis, RNAseq transcriptome analysis of *B. malayi* stages (Choi et al., 2011) indicate that *Bm-cpl-3* is specifically up-regulated in immature microfilariae while *Bm-cpl-6* is up-regulated in both immature and mature microfilariae. However, *Bm- cpl-1*, *Bm-cpl-4* and *Bm-cpl-5* are up-regulated in the L3 stage, further indicating their potential differing roles during development of the parasite. Our work supports the hypothesis that there is tissue-specific differential regulation of CPL gene expression in *B. malayi*.

To better understand *Wolbachia*'s dependence on these enzymes, RNAi experiments targeting these transcripts will be required to examine the consequences of their down-regulation on *Wolbachia* in the different tissues of the parasite. Initial studies have shown that decreases in *Bm-cpl-3* and *Bm-cpl-6* transcript levels after 96hr of siRNA treatment resulted in a significant reduction of *Wolbachia* in the *Bm-cpl-3* and *Bm-cpl-6* siRNA treated worms. It was also accompanied by a significant reduction in the number of microfilariae produced within the uteri of the treated female worms and in the number of microfilaria released into the culture medium. This supports our hypothesis that there is a definite link between the *Bm*-CPL-3 and *Bm*-CPL-6 enzymes and *Wolbachia*. This link might be directly or, more likely, indirectly involved in the maintenance of the symbiotic relationship and, consequently, proper embryonic development leading to the release of stretch microfilaria. The mechanism mediating this effect is still unknown. One possible indirect biochemical link may be secondary: for example, these enzymes may be used by *Wolbachia* for amino acid provisioning as genome analysis of *Wolbachia* has shown that they lack the capability for amino acid biosynthesis (Foster et al., 2005a).

Further characterization of the *Bm*-CPL-3 and -6 function(s) may lead to an improved understanding of filarial nematode biology and identify these proteases as anti-filarial drug targets.

Table 1
Effects on embryogenesis after siRNA treatment of adult female *B. malayi*.

Intrauterine progeny*	% Progeny 48 h after siRNA treatment ($n = 3$ per group)			% Progeny 96 h after siRNA treatment ($n = 2$ per group)		
	Control	*Bm-cpl-3*	*Bm-cpl-6*	Control	*Bm-cpl-3*	*Bm-cpl-6*
mf	27.1	8.8	14.9	23.6	13.9	9.5
pmf	8.4	29.5	51.7	14.8	46.8	53.4
emb	36.7	45.6	24.2	39.8	32.2	26.3
eggs	27.9	16.0	9.2	21.8	7.1	10.8
Intrauterine progeny*	% Progeny 48 h after siRNA treatment ($n = 2$ per group)			% Progeny 96 h after siRNA treatment ($n = 3$ per group)		
	Ov-cpz-Int2	*Bm-cpl-3*	*Bm-cpl-6*	Ov-cpz-Int2	*Bm-cpl-3*	*Bm-cpl-6*
mf	23.2	9.8	15.0	31.5	11.4	12.1
pmf	22.7	29.6	51.1	10.2	45.5	49.0
emb	43.9	43.8	24.6	43.3	34.8	27.8
eggs	10.3	16.9	9.3	15.1	8.3	11.1

Chi-square *P*-value of <0.001 were obtained for the treated and control groups and for the treated and *Ov-cpz-int2* siRNA control groups at both 48 h and 96 h.
* Expressed as the relative proportions of progeny at different stages of development (see Fig. 8).

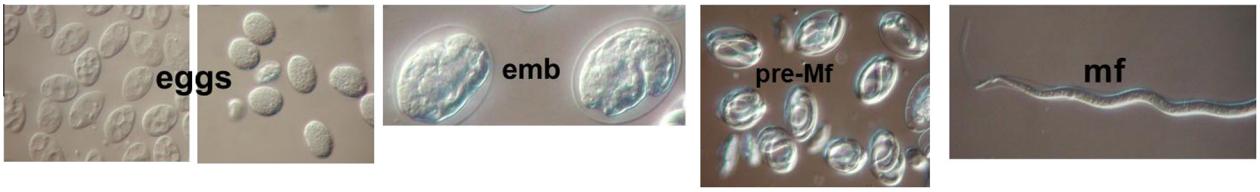

Fig. 8. Embryonic stages of development within a normal uterine of *B. malayi* female worm. Images of eggs, embryos, pre-microfilaria (pre-Mf) and stretched microfilaria found in a normal uterine of *B. malayi* female worm are presented. These stages correspond to the stages counted when an embryogram is performed and as presented in Table 1, where the effects of siRNA treatment on embryogenesis are reported. The images of eggs, pre-microfilaria (pre-Mf) and stretched microfilaria were taken using a 20 X magnification, while that of the embryos was taken using a 40 X magnification.

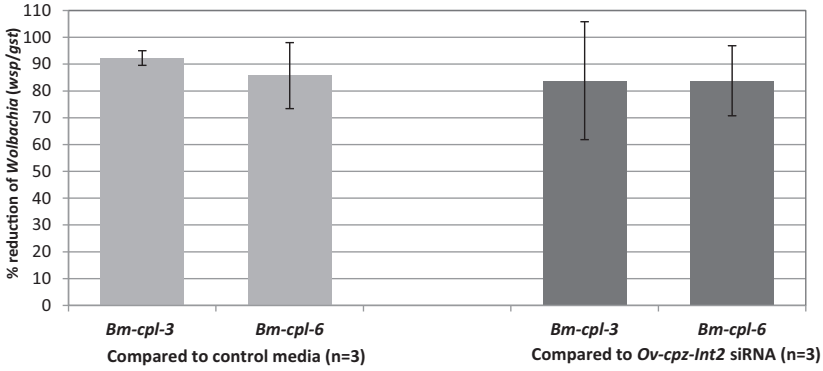

Fig. 9. RNAi treatment of *B. malayi* adult female worms with *Bm-cpl-3* and *Bm-cpl-6* siRNA results in significant reduction of *Wolbachia* density. At the end of the experiment (96 h after treatment), groups of three adult female worms were removed from each of the culture conditions. Total genomic DNA was extracted from individual worms, and single copy genes from the *Wolbachia* genome (*wsp*) and the *B. malayi* (*gst*) nuclear genome were amplified. The PCR products were quantified in real time through incorporation of SYBR green dye using an ABI 7300 Real-time PCR system calibrated with standard dilutions of control DNA targets. The ratio of *Wolbachia* gene copy number to *B. malayi* gene copy number (*wsp*/*gst* ratio) per sample was then calculated. The percent reduction (mean ± SD) in the number of *Wolbachia* genomes per worm ($n = 3$) were determined based on the *wsp*/*gst* ratio found in the worms that were cultured with media control or in worms that were treated with *Ov-cpz-Int2* siRNA.

Cysteine proteases are known to have essential functions in a variety of parasitic systems, including other nematodes, and are being developed as potential targets for drugs and vaccines (McKerrow, 1999; Newton and Meeusen, 2003; Sajid et al., 2011; Marco and Coteron, 2012; Vermeire et al., 2012).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpddr.2014.08.001.

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