# Silencing of essential genes by RNA interference in *Haemonchus contortus*

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SUMMARY

In this study we assessed three technologies for silencing gene expression by RNA interference (RNAi) in the sheep parasitic nematode *Haemonchus contortus*. We chose as targets five genes that are essential in *Caenorhabditis elegans* (*mitr-1, pat-12, vha-19, glf-1* and *noah-1*), orthologues of which are present and expressed in *H. contortus*, plus four genes previously tested by RNAi in *H. contortus* (ubiquitin, tubulin, paramyosin, tropomyosin). To introduce double-stranded RNA (dsRNA) into the nematodes we tested (1) feeding free-living stages of *H. contortus* with *Escherichia coli* that express dsRNA targetting the test genes; (2) electroporation of dsRNA into *H. contortus* eggs or larvae; and (3) soaking adult *H. contortus* in dsRNA. For each gene tested we observed reduced levels of mRNA in the treated nematodes, except for some electroporation conditions. We did not observe any phenotypic changes in the worms in the electroporation or dsRNA soaking experiments. The feeding method, however, elicited observable changes in the development and viability of larvae for five of the eight genes tested, including the 'essential' genes, *Hc-pat-12, Hc-vha-19* and *Hc-glf-1*. We recommend the *E. coli* feeding method for RNAi in *H. contortus* and provide recommendations for future research directions for RNAi in this species.

Key Words: *Haemonchus contortus*, RNA interference, RNAi, dsRNA, electroporation, *Caenorhabditis elegans*, gene knockdown.

## INTRODUCTION

In this review we describe our investigation of a set of potential anti-nematode drug targets (hereafter abbreviated 'targets') using RNA interference (RNAi) in *Caenorhabditis elegans* and *Haemonchus contortus*. We discuss the available methodology for delivering double-stranded RNA (dsRNA) to nematodes, briefly describe how the targets were identified, and then describe and discuss the methods and results of three different technologies for delivering dsRNA to *H. contortus*. Our experiences with these technologies were rather mixed and we discuss the factors that may have contributed to this.

### *RNAi technologies available*

RNAi has become a routine tool for studying gene function in *C. elegans*. The technique is readily applied to this organism using a variety of dsRNA delivery methods (for reviews and methods see (Kamath *et al.* 2001; Timmons *et al.* 2001; Timmons, 2006; Johnson *et al.* 2008) – injection of dsRNA (Fire *et al.* 1998), feeding *Escherichia coli* expressing gene-specific dsRNA (Timmons and Fire, 1998; Kamath and Ahringer, 2003), soaking nematodes in dsRNA (Tabara *et al.* 1998; Maeda *et al.* 2001), employing RNAi-hypersensitive strains such as *rrf-3(pk1426)* (Simmer *et al.* 2002), *eri-1(mg366)* (Kennedy *et al.* 2004), *eri-1(mg366);lin-15B(n744)* (Wang *et al.* 2005), and engineering nematodes to express dsRNA internally to allow dsRNA expression in specific tissues, particularly neurons (Tavernarakis *et al.* 2000; Johnson *et al.* 2005; Briese *et al.* 2006; Calixto *et al.* 2010).

While gene knock-down by these methods has been very effective in *C. elegans*, their direct application to parasitic nematodes has not been straightforward. RNAi studies in the gastrointestinal nematodes *Trichostrongylus colubriformis* (Issa *et al.* 2005), *H. contortus* (Geldhof *et al.* 2006; Kotze and Bagnall, 2006; Samarasinghe *et al.* 2011) and *Ostertagia ostertagi* (Visser *et al.* 2006) have tested a

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variety of methods for the delivery of dsRNA, specifically feeding, soaking and electroporation. The achievements of these studies will be briefly discussed below.

### *Delivery of dsRNA by electroporation*

Electroporation has been used successfully to deliver dsRNA and small interfering RNA (siRNA) to adult and schistosomula life stages of *Schistosoma mansoni* (Correnti *et al*. 2005; Krautz-Peterson *et al*. 2007), but its application in nematodes has met with mixed success. Issa *et al*. (2005) reported that electroporation of *T. colubriformis* L1 stage larvae with dsRNA or siRNA targetting the (essential) ubiquitin gene resulted in death or developmental delays in >90% of the larvae. Successful RNAi was assessed by phenotype alone and the authors were able to show an RNAi effect under some, but not all, of the conditions examined and for some, but not all, of the genes tested. This variability has also been reported by all subsequent studies. Geldhof *et al*. (2006) found significant decreases in expression levels for two out of four targeted genes following electroporation of long dsRNA with *H. contortus* L1 larvae, but any phenotypic effects could not be examined due to the high mortality of the treated larvae. Visser *et al*. (2006) found that electroporation of *Ostertagia ostertagi* larvae (L1) caused transcript level reductions in only two out of eight genes tested, and that these reductions were difficult to reproduce. They also noted that in control assays the electroporation conditions caused the death or failure to develop to the L2 stage in a variable percentage of larvae. Finally, electroporation of *Heligomosomoides polygyrus* L1 larvae failed to transport Cy3-labeled siRNA through the cuticle (Lendner *et al*. 2008). These workers reported that the electroporation conditions used in their experiments had significant adverse effects on survival of the larvae, with a mortality of 70–90%.

### *Delivery of dsRNA by soaking*

Issa *et al*. (2005) reported successful inhibition of development induced by soaking L1 stage *T. colubriformis* in a solution containing siRNA targetting ubiquitin; in contrast, long dsRNA targetting ubiquitin was not effective. Using *H. contortus* exsheathed-L3s (xL3), Kotze and Bagnall (2006) observed significant decreases in transcript levels of two tubulin genes; development to L4 was reduced in each case, while motility was reduced for one of the targetted genes. Transcript levels for both genes were also reduced when L4s or adult worms were soaked in dsRNA, though no effects on motility were observed. Geldhof *et al*. (2006), also using *H. contortus*, tested whether soaking in long dsRNA induced effective RNAi in L1 or xL3 stages. They measured transcript levels and provided evidence for transcript down-regulation in two (*Hc-ben-1* and *Hc-sec-23*) of the 11 genes tested. No phenotypic effects were observed for any of these genes. The authors also tested the effects of soaking with siRNA targetting either *Hc-sec-23* or *Hc-elt-2*, but did not observe any transcript down-regulation. Visser *et al*. (2006) tested RNAi by soaking in exsheathed-L3s of *O. ostertagi*; they observed reduced transcript levels for five of the eight genes tested, but the effects were quite variable. Transcript levels for the remaining three genes were consistently unchanged in these experiments.

More recently, Samarasinghe *et al*. (2011) investigated transcript down-regulation of a number of *H. contortus* genes by soaking exsheathed-L3s for 72 h and determining relative expression levels of the targetted genes. The results were mixed: they observed consistently reduced transcript levels for six genes, including the *β*-tubulin *Hc-iso-1*, inconsistent knock-down for two genes, increased transcript levels for one gene, and no change for the remaining three genes. No phenotypic changes were observed in the treated larvae. The effects of larval RNAi on the adult *in vivo* phenotype were tested for one gene, the intestinal aminopeptidase H11. Sheep infected with H11 RNAi-treated larvae exhibited reduced faecal egg output and a lower adult worm burden. The authors hypothesised that, using the soaking method, RNAi is more effective against genes expressed in 'accessible' tissues such as the intestine, excretory cell and amphids; this was generally supported by their results, although transcript levels remained unchanged for one gene that (in *C. elegans*) is expressed in the excretory cell.

### *Delivery of dsRNA by feeding with* E. coli *expressing dsRNA*

RNAi experiments using the feeding method successfully inhibited development of *T. colubriformis* L1s to L3 when tropomyosin, but not ubiquitin, was targetted (Issa *et al*. 2005). For *H. contortus* L1s, no transcript reduction was evident for any of the four genes tested in RNA extracted from worms after 48 h of feeding on dsRNA-expressing *E. coli* (Geldhof *et al*. 2006).

### *Our approach*

Taken together, the studies outlined above have shown that gene silencing by RNAi is possible in most of the parasitic nematodes tested, but they also illustrate the inconsistency and highlight a number of difficulties encountered with the application of the different RNAi methodologies. It is likely that a combination of approaches, potentially different for

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each species and life cycle stage, may be required for successful gene silencing.

Apart from the expense associated with the production of *in vitro*-transcribed dsRNA, an apparent problem with the application of the soaking method to parasitic nematodes is the requirement for their maintenance in a small volume of concentrated dsRNA for an extended period of time, which tends to have a negative effect on subsequent development. Electroporation, while effective for the delivery of dsRNA, also requires *in vitro*-transcribed dsRNA. A benefit of this method, however, is the ability to reduce the amount of time spent in concentrated dsRNA, thereby reducing the impact on subsequent development. Unfortunately, as noted above, electroporation is a harsh treatment that was reported to cause considerable nematode death in several of the previous studies; those nematodes that survive do, however, tend to continue with development. With carefully optimised methods of electroporation it may be possible to reduce the impact on nematode viability.

The feeding method has the advantage that it does not require *in vitro*-transcribed dsRNA, so it is less expensive, less laborious and can be easily applied to large numbers of nematodes. In this method, *E. coli* are engineered and induced to produce dsRNA, then fed to the nematodes, releasing dsRNA into the intestine following digestion. The dsRNA is then absorbed and may be distributed to all or some of the tissues of the nematode. This approach is dependent on the ability of the nematode to ingest and digest bacteria, thereby limiting the application to specific life cycle stages of appropriate species. In the studies outlined above, changes to gene expression or phenotype were, with one exception, not observed when the feeding method was used, which may indicate that initial exposure to the dsRNA is slow or that the silencing is short-lived. It may be possible to overcome some of these problems with optimised methods.

For RNAi to become a reliable tool for the analysis of gene function in parasitic nematodes, it has become clear that considerable effort must be given to the optimisation of specific techniques for each parasite and possibly for each life cycle stage. We describe below our efforts to optimise procedures for RNAi in *H. contortus*. First we describe our assessment of the feeding method for delivery of dsRNA to the free-living stages. Although Geldhof and colleagues did not have success with this method in *H. contortus* (Geldhof *et al*. 2006), Issa and colleagues (2005) observed a developmental phenotype with the feeding method in *T. colubriformis* for tropomyosin, but not ubiquitin. Successful application of this procedure would be highly beneficial to the research community as it would enable continuous exposure to gene-specific dsRNA without compromising the *in vitro* culture conditions required by the larvae, thus allowing observers to monitor for an induced effect throughout development. Second, we describe our exploration of methods for delivery of dsRNA by electroporation of the free-living stages of *H. contortus*. Our approach to this method focused initially on defining optimal electrical conditions for dsRNA uptake (voltage, pulse length) without compromising the health of the larvae. In this way we aimed to avoid the control mortality issues that had clouded previous attempts to apply this method to nematode larvae. Finally, we investigate delivery of dsRNA to adult *H. contortus* using the soaking method. Although, as noted above, this method has the disadvantage of requiring a significant amount of *in vitro*-transcribed dsRNA, it was considered to be most applicable to this life stage due to likely technical difficulties associated with utilising the bacterial feeding method with adult worms contained in a nutrient medium.

### *Selection of the target genes*

In the first stage of this work we used *C. elegans* resources to generate a set of essential and potentially nematode-specific target genes to test. We searched the databases of RNAi phenotypes reported in the whole-genome RNAi screens in *C. elegans* published by Fraser *et al*. (2000), Kamath *et al*. (2003) and Sönnichsen *et al*. (2005) and selected candidate genes using the following general criteria: (1) Strong RNAi phenotype showing compromised viability, fertility or locomotion; (2) Silenced gene conserved in nematodes; translated product of the gene having no or low ($e < 10^{-5}$) homology to translated products of any known mammalian genes; and (3) Evidence of a (predicted) protein sequence homologue in a relevant parasitic nematode(s).

The resulting set of candidates was further refined by examining available expression information for each gene in *C. elegans* (including EST expression, microarray experiments, reporter gene expression, compiled in Wormbase (www.wormbase.org)) and, where available, for homologues in relevant parasitic nematodes using, principally, the EST databases (www.nematode.net; www.nematodes.org) and any relevant published information. We gave some priority to genes apparently expressed in late larval or adult stages of parasitic nematodes, given that these are the stages normally targetted in nematode infections. This criterion was not applied absolutely, however, because different proteins can have greatly different turnover half-lives and/or post-translational modifications, such that some genes may be expressed as protein in a later developmental stage than that at which high levels of mRNA are expressed. At this stage of the project we did not formally apply 'druggability' criteria (see McCarter, 2004; Behm *et al*. 2005; Grant and Behm, 2007; Kumar *et al*.

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2007) to the targets because we were seeking strong RNAi phenotypes to maximise the probability of observing a phenotype in the next stage of the project, the *H. contortus* RNAi experiments.

We then verified and examined in detail the reported *C. elegans* RNAi phenotypes for each candidate gene by RNAi in *C. elegans*, using the *E. coli* feeding method (Timmons and Fire, 1998) for delivery of dsRNA. RNAi experiments were carried out in two ways, (1) by continuous exposure to dsRNA from the 1st larval stage (L1 of P0 generation) and following the development and behaviour of the nematodes through the F1 generation; and (2) by exposure to dsRNA from the 4th larval stage (L4 of P0 generation) and examining the phenotype of the P0 adults and the subsequent F1 generation.

Verified candidates that satisfied the selection criteria were then taken to the next stage, where gene-specific sequence information from *C. elegans*, and other free-living and parasitic nematodes (where available) was used to design degenerate PCR primers to probe cDNA preparations from *H. contortus*, as described below. Amplified products were sequenced and positive cDNAs were selected and used for RNAi experiments in *H. contortus*.

### *Target genes tested*

*Ce-mitr-1*. The *C. elegans* protein, *Ce*-MITR-1 (**mit**ochondrial **r**espiration gene 1; F36A2.7), is expressed in mitochondria and plays an essential role in mitochondrial respiration and energy metabolism (Johnson *et al*. unpublished data). Silencing of *Ce-mitr-1* by RNAi leads to extended longevity, small body size, 'clear' worms, and slow growth. In addition, *Ce-mitr-1(RNAi)* adults produce few or no progeny. This fecundity phenotype, if reproduced in the parasitic stages of *H. contortus*, would lead to a decrease in transmission.

*Ce-pat-12*. The gene *Ce-pat-12* (***p***aralysed ***a***rrest at ***t***wo-fold; T17H7·4), also known as *Ce-gei-16* (*ge*x *i*nteracting), encodes a protein with 27 predicted isoforms (www.wormbase.org), many of which have been confirmed (Hetherington *et al*. 2011). In *C. elegans* many of the isoforms of PAT-12 are expressed in the epidermis and also in the apical and basal plasma membranes of the pharynx. PAT-12 was initially chosen as a target as it has a high degree of homology to the B20 antigen from the filarial parasite *Onchocerca volvulus* (Abdel-Wahab *et al*. 1996). In addition, post-embryonic RNAi of *Ce-pat-12* leads to paralysis and death, often associated with moulting defects and muscle detachment (Hetherington *et al*. 2011). Further, *Ce-pat-12 (RNAi)* embryos fail to undergo correct morphogenesis and arrest at the two-fold stage. PAT-12 was subsequently shown to be a novel component of the hemidesmosome, which is essential to maintain the attachment of the body wall muscles to the cuticle (Hetherington *et al*. 2011). If similar phenotypes are induced by silencing the expression of *pat-12* in the parasitic stages of *H. contortus*, the consequence should be expulsion of paralysed parasites from the host, plus a reduction in transmission.

*Ce-vha-19*. Ce-VHA-19 (***v***acuolar ***H*** ***A***TPase 19; Y55H10A.1) is predicted to encode a V-ATPase accessory protein (Knight *et al*. unpublished data). In *C. elegans* VHA-19 is expressed in the excretory canal cell, excretory cell, apical membrane of the intestine and in the epidermis. The initial bioinformatic screen carried out for this study suggested VHA-19 to be nematode-specific. With availability of increased genome sequencing data, however, VHA-19 was later found to share a highly-conserved 13 amino acid motif with the mammalian protein Ac45, which is an accessory protein of the vertebrate V-ATPase (Xu *et al*. 2007). VHA-19 was chosen as a target because postembryonic RNAi knockdown of *Ce-vha-19* led to developmental arrest and death. In addition, *Ce-vha-19(RNAi)* worms produced fewer viable embryos, resulting in a greatly reduced brood size when compared with controls (Knight *et al*. in preparation). Thus, targeting *Hc-vha-19* could result in death of *H. contortus* within the host and a decrease in transmission.

*Ce-glf-1*. GLF-1 encodes UDP-galactopyranose mutase (UPM), an enzyme that catalyses the interconversion of UDP-galactopyranose (UDP-Gal*p*) and UDP-galactofuranose (UDP-Gal*f*) (Beverley *et al*. 2005; Novelli *et al*. 2009). In *C. elegans*, GLF-1 is expressed in the epidermal syncytium and seam cells at all post-embryonic stages. *Ce*-GLF-1 plays an essential role in formation of the nematode surface coat, in motility and embryogenesis (Novelli *et al*. 2009); Fritz *et al*. unpublished data). The nematode surface coat is an essential interface and protective barrier between the worm and its environment, particularly between a parasite and its host. The dynamic properties of the surface coat have been implicated in host immune evasion (reviewed by Blaxter *et al*. (1992)). Targetting *Hc*-GLF-1 could result in expulsion of immobile parasites from the gut, increased susceptibility to the immune response of the host, plus potentially increased uptake of anti-nematode drugs, which could lead to increased sensitivity to drug treatments.

*Ce-noah-1, Ce-noah-2*. NOAH-1 and NOAH-2 are PAN- and ZP-domain-containing proteins that have homology to the *Drosophila* extracellular matrix component, NompA. In *C. elegans noah-1* and *noah-2* mRNA is expressed in the epidermis in all embryonic and postembryonic larval stages (Wise, Biswas, Behm *et al*. unpublished data; Hill *et al*.

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2000). RNAi of either *Ce-noah-1* (C34G6.6) or *Ce-noah-2* (F52B11.3) leads to developmental arrest, with worms unable to complete the moulting process (Frand *et al*. 2005). This phenotype, if reproduced in the parasitic stages of *H. contortus*, would result in termination of the life cycle and a reduction in transmission rates.

### *Preparation of* H. contortus *RNAi clones for the target and control genes*

Partial cDNAs for *Hc-pat-12, Hc-vha-19, Hc-noah-1, Hc-glf-1* and *Hc-mitr-1* were amplified using oligonucleotides designed using publicly available sequences from the *H. contortus* genome project (www.sanger.ac.uk). Oligonucleotides used to amplify the fragments were as follows:

*Hc-pat-12:* *Hc-pat-12*F2 5′-CCAGTGCGATTCTCGTACCT-3′ and *Hc-pat-12*R1 5′-TGTGTACGTGCGACCGCTTG-3′; *Hc-vha-19:* *Hc-vha-19*F3 5′-CCTGGCCTTTTGCTGGCCTT-3′ and *Hc-vha-19*R1 5′-TTTCAGGCTGCGCAACTGTT-3′; *Hc-noah-1:* *Hc-noah-1*F2 5′-TCGCATGACTCGTATACGGC-3′ and *Hc-noah-1*R5 5′-TGTCATGATATACAGTACAC-3′; *Hc-glf-1:* *Hc-glf-1*F2 5′-TGCAGGAATCCTATGCTGGTGGT-3′ and *Hc-glf-1*R3 5′-CCTGGATAAAGCAGTGATCCTGAA-3′; *Hc-mitr-1:* *Hc-mitr-1*F1 5′-CGTGCTGTCAGTTGCCTGAA-3′ and *Hc-mitr-1*R1 5′-TCGTGTTCCTCTCTTCTTTG-3′. PCR fragments were cloned into pGEM®-Teasy using standard techniques before being sub-cloned into the RNAi vector, pL4440, via NotI restriction enzyme digestion and ligation.

A fragment of the *Arabidopsis thaliana lhcb4.3* gene (GenBank AF134128) was used as a dsRNA negative control gene (Fritz and Behm, 2009) as the sequence did not match any known *H. contortus* DNA. From previous studies (see above), the three genes that have shown the greatest potential for silencing by RNAi in the trichostrongylids were ubiquitin, beta-tubulin and tropomyosin. These genes were included in this study for comparison with the literature. Partial cDNAs for *Hc-ubiquitin, Hc-beta-tubulin* and *Hc-tropomyosin* were amplified using oligonucleotides designed using publicly available sequences from the *H. contortus* genome project (www.sanger.ac.uk). Oligonucleotides used to amplify the fragments were as follows:

*Hc-ubiquitin:* *Hc-ubq-1*F 5′-CGCACACTTTCGGATTACAA-3′ and *Hc-ubq-1*R 5′- AAAATGAGCCTTTGCTGGTC-3′; *Hc-beta-tubulin:* *Hc-ben-1*F3 5′-CGTAATTATCTGGGCGGAAA-3′ and *Hc-ben-1*R1 5′-GATCCCCCTGTGAATCAAGA-3′; *Hc-tropomyosin:* *Hc-tmy-1*F2 5′-ATCGAGAAGGACAACGCTCT-3′ and *Hc-tmy-1*R5 5′-TTCAGTGGCGATCTTCAAAC-3′. PCR fragments were cloned into pL4440 via NotI restriction enzyme digestion and ligation.

Positive clones were confirmed by sequence analysis. For the RNAi by feeding experiments the clones in pL4440 were transformed into the RNaseIII-deficient *E. coli* strain, HT115 (DE3), which has isopropyl-*β*-D-thiogalactopyranoside (IPTG)-inducible T7 polymerase activity, for dsRNA expression (Timmons and Fire, 1998).

## *H. CONTORTUS* RNAI BY FEEDING OF FREE-LIVING LARVAL STAGES

### *Expression of dsRNA for* H. contortus *target genes*

To induce the expression of dsRNA, a single *E. coli* colony was grown overnight at 37 °C in Luria Broth (LB) supplemented with ampicillin (100 $\mu$g/ml) and tetracycline (12·5 $\mu$g/ml) and then diluted 1/100 into fresh LB with supplements and grown to an $OD_{600}$ of 0·6. The culture was then induced by addition of 0·4 mM IPTG and grown for a further three hours. The cells were pelleted by centrifugation at 2,000 × *g* for 10 min, resuspended in sterile Physiological Saline (sPS) (0·85% NaCl) and adjusted to an $OD_{600}$ of 0·23–0·24. *E. coli* preparations expressing dsRNA were either used fresh or stored at 4 °C for subsequent use in the RNAi feeding assay, as described below.

### *Purification of* H. contortus *eggs*

A benzimidazole-resistant strain of *H. contortus* (Haecon-5, obtained from Novartis Animal Health, Australia) was maintained by serial passage in 6–12 month old Merino weaner sheep. These donor sheep were treated orally with abamectin and a combination drench to remove any existing nematode infections, then maintained off-pasture to prevent any further parasite infection. After one month each animal was inoculated with 5,000–7,500 *H. contortus* infective L3 and patent infection established.

Faeces from infected donor sheep were collected and *H. contortus* eggs isolated by sequential filtration and collected on a 20 $\mu$m sieve. Eggs were separated from faecal material by flotation in saturated sucrose (SG 1·26) and centrifugation at 400 × *g* for 5 min to pellet the faecal debris. The upper filtrate was passed through sequential nylon screens to remove fine debris and the eggs collected on a 37 $\mu$m nylon screen. Recovered eggs were washed to remove fine debris and reduce commensal bacteria and fungi. Eggs were sterilised twice in an antibiotic-antimycotic solution (70 mM NaCl, 2·5 mM KCl, 5 mM glucose, 10 mM $Na_2HPO_4$, 2·5 mM $NaH_2PO_4$, 100 U/ml Penicillin, 100 U/ml Streptomycin, 80 $\mu$g/ml Ciprofloxacin, 10 $\mu$g/ml Amphotericin B, 40 $\mu$g/ml Gentamicin, 100 $\mu$g/ml Ampicillin) for 5 min to kill remaining commensals, washed thoroughly to remove residual antibiotics and concentrated by centrifugation at 1,000 × *g* for 5 min. Aliquots were taken to estimate

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recovery. Eggs were used in the RNAi feeding assay as described below.

### *RNAi feeding assay*

The bacterial feeding method was used to introduce dsRNA to the larvae in a larval development assay. L1 were fed on the recombinant *E. coli*, which were ingested and digested, releasing dsRNA which was then available for uptake by the nematode intestine.

The Culture Medium (CM) used for the assay had the following composition: Nutrient Medium (Earle's Balanced Salt Solution and 1% yeast extract in sPS)(20%), eggs (17%), gene-specific recombinant *E. coli* (17%), carbenicillin (100 $\mu$g/ml), amphotericin B (2 $\mu$g/ml) and 5-fluorocytosine (5 $\mu$g/ml) in sPS (46%). 2·5 ml of the CM was added to each culture flask (12·5 $cm^2$; 4 replicates), sealed and incubated at 27 °C with 80% relative humidity for 6–10 days. Controls were included in each experiment: a negative control with no added bacteria (to ensure the absence of commensals during development) as well as a fragment of the *A. thaliana Lhcb4.3* gene in pL4440 and HT115(DE3) (to capture any effect due to the presence of irrelevant dsRNA). The success of egg hatch and development of the L1 through to L3 was monitored at three times during development, on days 1, 3/4 and 7–9. At each of the times, the replicate cultures were examined by microscopy to ensure reproducibility and a single culture was harvested for further phenotypic and molecular analysis. From this culture, three sub-samples were taken and larvae photographed. The images were used to differentiate between developmental stages and to compare culture success of larvae fed the control or target genes. This enabled a detailed examination of any phenotypic changes induced by the RNAi treatments. The remaining culture (2–4,000 larvae) was processed to provide cDNA for molecular analyses of transcript levels by quantitative RT-PCR in duplicate experiments to ensure reproducibility.

For an experiment to be considered successful, the following criteria had to be met. First, an egg hatch of 50% or greater was required when the culture was harvested on day 1, between 24 and 28 h after initial incubation. Any significant delay in hatch can allow the bacteria to overgrow, which has a negative impact on subsequent egg hatch and development. The bacterial lawn should not have progressed beyond light to moderate at this time and there should be no bacterial growth in the negative control. Second, by days 3/4, the L2 in the control gene cultures should have completed their moult and be active and growing. Finally, at days 7–9, the majority of larvae in the control gene cultures should be active L3, with few L2 still in lethargus. It is important to note that there are differences in developmental rates between experiments so it is crucial always to compare target gene phenotypes with the control gene cultures within each specific experiment and not between experiments.

For analysis of transcript levels, cDNA was produced from DNase-treated total RNA using OligodT primers (Promega) and Superscript III Reverse Transcriptase (Invitrogen) for each sample harvested. Quantitative PCR was performed using the Roche Light Cycler system. The $MgCl_2$ concentration, annealing temperature and PCR efficiency were optimised for each target gene primer set and the integrity of each reaction determined by dissociation curve analysis. Gene-specific quantitative PCRs were carried out in a 20 $\mu$l final volume containing 5 pmol forward primer; 5 pmol reverse primer; 2–5 mM $MgCl_2$; 1 x FastStart™ DNA master SYBR Green I mix; and 2 $\mu$L template cDNA. The PCR program consisted of a denaturation cycle of 95 °C for 10 mins, followed by amplification for 40 cycles at 95 °C for 10 sec; 60–62 °C for 5 sec; and 72 °C for 12–16 sec. This was followed by a dissociation curve analysis component of 95 °C for 0 sec, 65 °C for 15 sec, then increasing to 95 °C at 0·1 °C/sec. The primers for these analyses used are presented in Table 1. The levels of expression were determined by the generation of a normalised ratio for each specific cDNA using *Hc-ama-1* as a reference. This normalised ratio was then used to calculate relative expression for each target gene compared with expression in the dsRNA control samples, using the Light Cycler Relative Quantification Software version 4.05 (Roche).

### *Results*

The feeding RNAi assays were performed in the free-living stages as described above. While all assays were repeated to ensure reproducibility, the developmental results presented below (Fig. 1) are for a representative experiment:

For *Hc-tropomyosin, Hc-noah-1* and *Hc-mitr-1*, no phenotypic effect was apparent during development, while gene expression profiles showed an increase during early development; this was followed by a decrease at Day 8.

For *Hc-ubiquitin*, there was an increased proportion of sick L2 present on days 4 and 8 (24%) and sick L3 at day 8 (23%) compared with the Control A, which is indicative of a larval lethality phenotype. Gene expression had also decreased at all three time points. For *Hc-tubulin*, there was a proportion of sick L2 present on day 8 (25%), indicative of a slight developmental arrest at L2. Gene expression had decreased at the first two times but had increased by the third. Down-regulation of *Hc-vha-19* gene expression correlated with a subtle larval lethality phenotype, although by day 8 gene expression had been up-regulated similar to the pattern observed for *Hc-tubulin*.

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Table 1. RNAi of free-living stages of *H. contortus* using the feeding method: PCR primers used for quantitative RT-PCR analyses.

| Gene | Primer sequence (5′→3′) |
|---|---|
| *Hc-ubiquitin* | F: GAGGCACTTCGTCTTCGAG<br>R: TGACCGGGAAGACAATCAC |
| *Hc-tubulin* | F: CCAATTGTTACCCGCTCCT<br>R: TGAGGTCCACCCTGAATTTC |
| *Hc-tropomysin* | F: ATCGAGAAGGACAACGCTCT<br>R: GAGCGGTTTTTCCATCACTTT |
| *Hc-vha-19* | F: CTTGCCTTGTCCCAATACAC<br>R: ATCCCGATTTCACTTTAGGG |
| *Hc-noah-1* | F: GGACTTTCCCTCCAAGCATA<br>R: GGAGGTGGCAAATTCAAACT |
| *Hc-glf-1* | F(SL1):<br>GGTTTAATTACCCAAGTTTGAG<br>R: TGGCTTTATTTCCCTGATCC |
| *Hc-pat-12* | F(SL1):<br>GGTTTAATTACCCAAGTTTGAG<br>R: AGAAGGACAACCACCTGGAC |
| *Hc-mitr-1* | F: GGCCTCCAACATTTCATCTT<br>R: GTTCCTTGCTGGATTCTGGT |
| *Hc-ama-1* | F: GCTCAAAGAAAATGGCATCA<br>R: CCAGGTTGAGCAATAGCTTG |

Phenotypic observations for *Hc-glf-1* and *Hc-pat-12* were the most notable, with a dramatic increase in sick L2 observed at day 3 and sick L3 at day 9 for both genes. For *Hc-pat-12* the RNAi effect at day 9 was more notable, with a combination of sick L1, L2 and L3 and only 35% healthy L3 present. In addition, for *Hc-glf-1*, the L3 were active, but less inclined to super-coil and movement was also slower than observed in the control (data not shown). Interestingly, these phenotypes were not supported by the transcript data, where gene expression levels increased substantially at days 1 and 3 before a decrease by day 8. This is similar to the transcript profiles obtained for *Hc-tropomyosin, Hc-noah-1* and *Hc-mitr-1*.

## RNAI IN FREE-LIVING STAGES OF *H. CONTORTUS* USING ELECTROPORATION

### *Experimental approaches*

The reports outlined above showed that while delivery of dsRNA or siRNA via electroporation is possible with at least some species of nematode larvae, it was not applicable to all genes, and that some effort was still required to optimise the voltage conditions in order to reduce control mortality.

As part of our efforts to optimise RNAi in *H. contortus* we undertook to further assess this method for delivery of dsRNA to various free-living life stages. Our initial requirement was to optimise the electroporation conditions in order to maximise the electrical voltage while maintaining larval viability. Electroporation was applied using a Bio-Rad GenePulser® II to three different life stages of the parasite (eggs, L1 larvae and L3 larvae) held in 2 mm cuvettes. The *H. contortus* used for these experiments were from the Kirby isolate (Albers and Burgess, 1988). Eggs were isolated from faeces using filtration and sucrose gradient centrifugation (Gill *et al*. 1995) and either electroporated immediately or allowed to hatch overnight, then collected as L1s for electroporation. L3 larvae were collected as they migrated from faecal cultures and were stored at 12 °C for no longer than 2 weeks before being used in electroporation experiments.

The consequences of the electroporation were assessed in the three life stages as follows: (1) Eggs: measuring their ability to hatch; (2) L1 larvae: measuring the ability of the larvae to develop to the L3 stage in 96-well-based larval development assays (adapted from Gill *et al*. 1995); and (3) L3 larvae: measuring the ability of the larvae to migrate though an agar/mesh system 3, 6, 10 and 16 days after the treatment (as described by Kotze *et al*. 2006).

The experiments were conducted using eggs and larvae held in siPORT™ siRNA electroporation buffer (Ambion) containing a control dsRNA at 0·5 $\mu$g/$\mu$l. This control dsRNA was produced by *in vitro* transcription (MEGAscript® RNAi Kit, Ambion) from an approximately 625 nt section of the rubisco gene of wheat, *Triticum aestivum* (GenBank EU492898.1, nucleotides 110–733). This material was considered an appropriate dsRNA control as there were no known matches to the sequence in the DNA of *H. contortus*.

### *Optimising the electroporation conditions*

Fig. 2 shows the electroporation conditions around the point at which effects on worm viability were first detected. The arrow above each graph indicates the parameters chosen for subsequent dsRNA delivery experiments. The choice of optimal parameters was based initially around maximising the voltage, then on increasing pulse length (the pulse length increased as the resistance setting on the electroporator was increased), as well as assessing 1, 3 or 5 pulses (only 3-pulse results are shown in Fig. 2). The use of these experimental conditions provided some confidence that we were imposing the highest possible voltage conditions on the eggs or worms without compromising their viability, thus allowing any effects of worm-specific dsRNA on worm viability to be examined clearly.

The conditions chosen for subsequent RNAi experiments were as follows: (1) Eggs: 250 volts, 3 ohm, 3 pulses (pulse length approx. 0·4 ms) (4,000 eggs in 100 $\mu$l in cuvette); (2) L1 larvae: 500 volts, 25 ohm, 3 pulses (pulse length approx. 3·3 ms) (4,000 L1 larvae in 100 $\mu$l in cuvette) and (3) L3 larvae:

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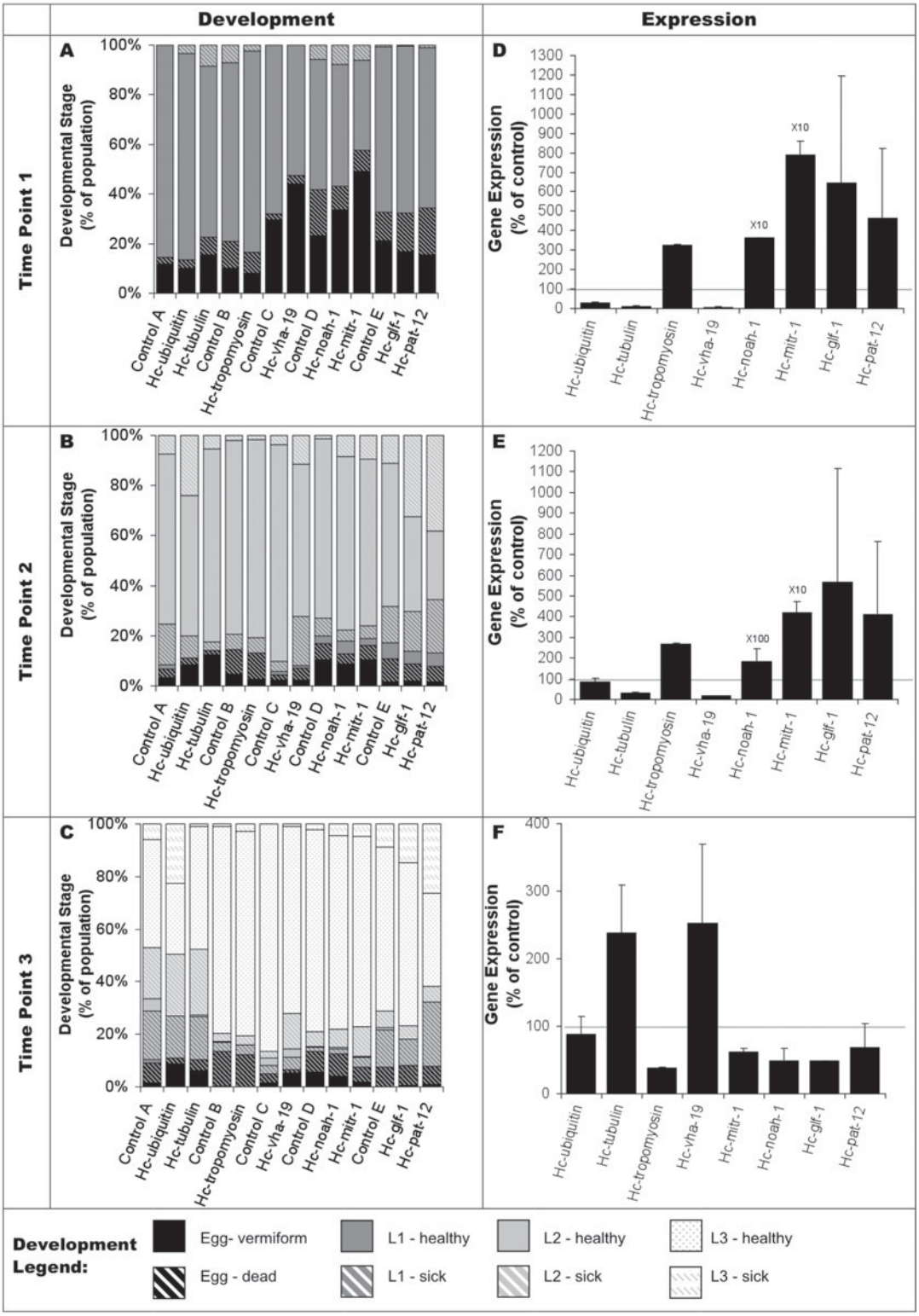


Fig. 1. RNAi in the free living stages of *H. contortus* with dsRNA introduced by the feeding method. Target genes were analysed by RNAi in two separate experiments, of which a representative experiment is shown. Panels A, B and C: Percentage of each developmental stage in treated and control larvae at 24–28 h (Time Point 1), 3–4 days (Time Point 2) and 7–9 days (Time Point 3), respectively. Each column represents mean larval counts (80–120 larvae) from three sub-samples for each gene. Data for each gene should be compared with a specific control experiment as follows: Control A, *Hc-ubiquitin* and *Hc-tubulin*; Control B, *Hc-tropomyosin*; Control C, *Hc-vha-19*; Control D, *Hc-noah-1* and *Hc-mitr-1*; Control E, *Hc-glf-1* and *Hc-pat-12*. Panels D, E and F: Transcript expression in treated larvae relative to control larvae at 24–28 h, 3–4 days and 7–9 days, respectively. Each column represents mean ± SE, n = 2 separate quantitative PCR experiments, each from a single sample (2–4,000 nematodes) for each gene compared with a control sample. Up-regulation of transcripts for *Hc-noah-1* and *Hc-mitr-1* in the early time points was considerably higher than for the other target genes; to allow graphical representation factors of x10 or x100 have been used.

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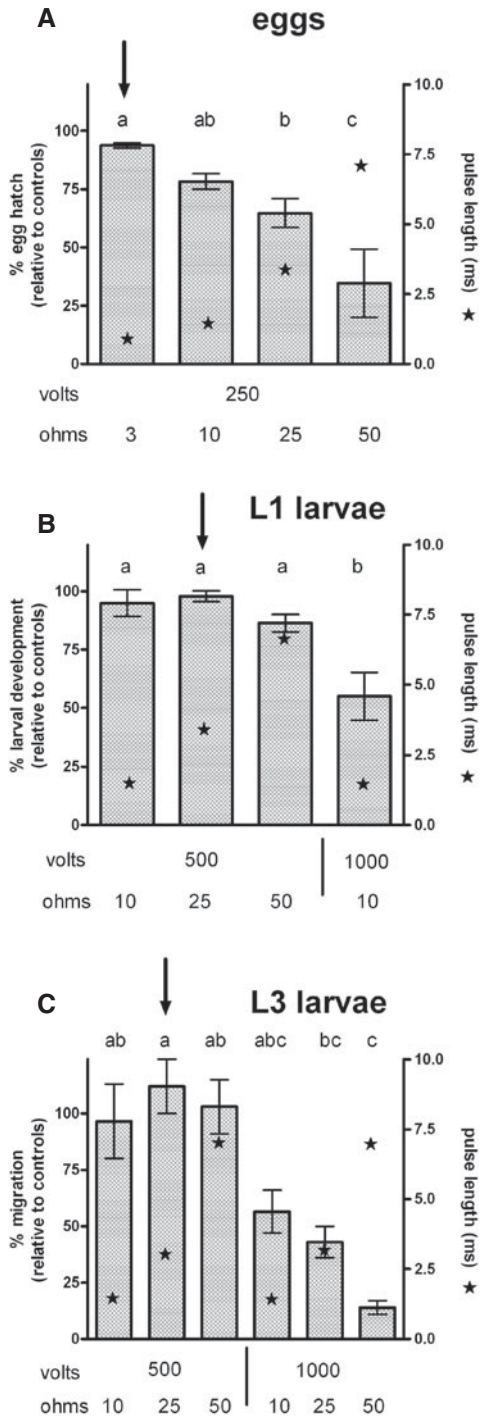


Fig. 2. Effect of electroporation conditions on the viability of free-living life stages of *H. contortus*. Electroporation was applied to eggs **(A)**, L1 larvae **(B)** or L3 larvae (**C**) and the effects assessed by examining egg hatch, larval development to the L3 stage, or larval migration, respectively. Each column represents mean ± SE, n = 2–3 separate experiments (**A**), 5 separate experiments (**B**), or 2 separate experiments (**C**). Each separate experiment consisted of a single electroporated sample at each set of electrical conditions, assessed in 3–5 separate assay wells. Within each life stage, columns labelled with the same letter were not significantly different (ANOVA, $P = 0{\cdot}05$). Arrows above columns indicate electroporation conditions subsequently used for RNAi experiments.

500 volts, 25 ohms, 3 pulses (pulse length approx. 3·3) (10,000 L3 in 100 $\mu$l in cuvette).

As our primary aim was to maximise voltage while maintaining larval viability, these parameters represented significantly higher voltages, alongside shorter pulse lengths, than reported previously in the literature. For example, Issa *et al*. (2005) and Geldhof *et al*. (2006) electroporated *T. colubriformis* and *H. contortus* L1 larvae, respectively with a single pulse of 100 volts (pulse lengths were not reported). Visser *et al*. (2006) used a pulse length of 25 ms, alongside the same voltage conditions as the two previous groups, with *O. ostertagi* L1 larvae. As noted above, however, both Geldhof *et al*. (2006) and Visser *et al*. (2006) described significant levels of death under these conditions in control electroporation samples. Electroporation of schistosomes is generally performed with a single 20 ms pulse at 125 V (Correnti *et al*. 2005; Krautz-Peterson *et al*. 2007).

We then used these electroporation conditions in RNAi experiments with each life stage, using long dsRNA produced by *in vitro* transcription of 300–600 nt lengths of the target genes of interest. The target genes were ubiquitin (Nembase 4 sequence ID HCC00644), tropomyosin (Nembase 4 sequence ID HCC00209), paramyosin (Nembase 4 sequence ID HCC00644), *Hc-vha-19, Hc-noah-1, Hc-glf-1, Hc-pat-12/Hc-gei-16*, and *Hc-mitr-1*. Samples of eggs or larvae were electroporated and placed into 6- or 12-well plates at 27 °C. After 48 h the majority of each sample (approximately 90% of the larvae) was removed and snap frozen in liquid nitrogen for gene expression analysis, while the remaining 10% of the larvae were diluted and transferred to 96-well plates and incubated further. For eggs and L1 experiments, these larvae were allowed to develop to the L3 stage over a 5–6 day period feeding on *E. coli* (XL1-Blue® Stratagene), and then numbers of L3 larvae were scored. For L3 experiments, samples of the remaining larvae were collected at days 3, 6, 10 and 16 after dsRNA treatment and placed onto agar overlaying 20 micron mesh for assessment of migration ability (Kotze *et al*. 2006). For molecular analyses, cDNA was prepared and quantitative PCR performed as described previously (Kotze and Bagnall, 2006). Gene expression was analysed using REST (Relative Expression Software Tool) (Pfaffl *et al*. 2002) software, and expression of each target gene was compared in gene-specific dsRNA treatments versus rubisco dsRNA controls, using three reference gene PCRs (GAPDH, $\beta$-actin and $\beta$-tubulin) to normalise the data.

## *Results*

The electroporation gene expression and phenotype results are shown in Table 2. Gene expression and

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Table 2. Summary of gene expression and phenotypic data for RNAi experiments with *H. contortus* eggs and larvae against various genes using delivery of long dsRNA by electroporation.

| Gene | Life stage[1] | Gene expression relative to control worms[2,3] | Phenotype effects[2,3,4,5] |
|---|---|---|---|
| ubiquitin | Egg | 1·06 ± 0·21 (2) | 110 ± 13 (3) |
| | L1 | 0·42 (1)** | 100 (1) |
| | L3 | 0·15 ± 0·04 (2)** | ns (2) |
| paramyosin | Egg | 1·134 (1) | 74 ± 34 (3) |
| | L1 | 0·91 (1) | 87 ± 5 (3) |
| | L3 | 0·77 ± 0·01 (2) | ns (2) |
| tropomyosin | Egg | 0·94 ± 0·04 (2) | 86 ± 15 (3) |
| | L1 | 0·81 ± 0·01 (2) | 93 ± 4 (2) |
| | L3 | 0·34 ± 0·07 (2)** | ns (2) |
| *Hc-vha-19* | Egg | 0·97 ± 0·08 (2) | 97 ± 11 (3) |
| | L1 | 0·50 ± 0·07 (3)** | 93 ± 8 (5) |
| | L3 | 0·67 ± 0·07 (2)* | ns (2) |
| *Hc-noah-1* | Egg | 0·82 ± 0·22 (2) | 86 ± 12 (3) |
| | L1 | 0·46 ± 0·12 (3)** | 89 ± 3 (3) |
| | L3 | 0·81 ± 0·03 (2) | ns (2) |
| *Hc-glf-1* | Egg | 1·18 ± 0·21 (2) | 105 ± 6 (3) |
| | L1 | 0·53 (1)** | 73 (1) |
| | L3 | 0·68 ± 0·15 (2)* | ns (2) |
| *Hc-pat-12/Hc-gei-16* | Egg | 0·90 ± 0·08 (2) | 121 ± 11 (3) |
| | L1 | 1·03 (1) | 106 (1) |
| | L3 | 0·71 ± 0·05 (2) | ns (2) |
| *Hc-mitr-1* | Egg | 0·95 (1) | 124 ± 4 (2) |
| | L1 | 1·02 ± 0·33 (2) | 82 ± 6 (2) |
| | L3 | 0·66 ± 0·11 (2)** | ns (2) |

[1] L1, 1st stage larvae; L3, 3rd stage larvae.
[2] Relative to controls, which were treated with dsRNA targetting rubisco
[3] Values are mean ± SE; values in parentheses are the number of separate experiments, each with single electroporated worm samples assessed in triplicate or quadruplicate larval development or migration assays;
[4] For eggs and L1, values are % development to L3;
[5] For L3 larvae, ns indicates migration of treated worms was not significantly different from control worms at days 3, 6, 10 and 16 after treatment.
* values were significantly different ($P \leqslant 0{\cdot}05$) from controls in at least one of the separate experiments;
** values were significantly different ($P \leqslant 0{\cdot}05$) from controls in each separate experiment or, in the single experiment for ubiquitin and *Hc-glf-1*, with L1s.

phenotype were unaffected in any larvae that had been treated with dsRNA by electroporation at the egg stage. On the other hand, electroporation of L1 stage larvae caused a significant decrease in expression of ubiquitin, *Hc-vha-19, Hc-noah-1* and *Hc-glf-1*. The greatest decrease was approximately two-fold with ubiquitin. These gene expression changes at 48 h did not, however, interfere with the ability of the larvae to develop to the L3 stage over the 5–6 day incubation period. Electroporation of L3 stage larvae had more significant effects on gene expression, with ubiquitin reduced approximately 7-fold, tropomyosin 3-fold, and several other genes 1·5-fold. Again, however, this did not interfere with the ability of the worms to migrate at time points 3, 6, 10 and 16 days after the dsRNA treatment.

Overall, these results showed that while reductions in gene expression could be achieved using electroporation with *H. contortus* free-living life stages, the reductions were minor (with ubiquitin showing the greatest effects) and no gross phenotypic effects were evident using our assessment methods. The inability to induce significant development or motility defects in larvae developing from the L1 to the L3 stage following electroporation of eggs or L1s contrasts with the development defects observed in the dsRNA bacterial feeding experiments. This suggests that electroporation is not a very useful technique for RNAi with these stages of *H. contortus*. However, as feeding techniques are not applicable to exsheathed L3 larvae (as they have no functioning mouthparts) the use of soaking or electroporation methods remains the only option with this life stage. Although our electroporation experiments with L3 larvae did not produce observable phenotypes, they did lead to reduced gene expression in a number of cases. This method may

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therefore be useful for studying some genes with this life stage.

### RNAI IN ADULT *H. CONTORTUS* USING SOAKING IN LONG DSRNA

#### *Experimental approach*

As part of our assessment of different methods to elicit RNAi in *H. contortus* we also examined the effects of soaking adult worms in long dsRNA *in vitro*. Experiments with adult *H. contortus* are generally compromised by the inability to maintain the worms in a 'fit' state for lengthy periods. Indeed, even after 2–3 days the degree and form of movement shown by adult worms *in vitro* is significantly reduced compared with their state when first removed from the abomasum (Kotze, unpublished data). Therefore, it is questionable whether any mild general effects on worm viability that result from RNAi-mediated gene silencing would even be distinguishable from changes due to reduced worm fitness throughout the *in vitro* incubation period. Nonetheless, we reasoned that gene expression changes should be measurable following dsRNA treatment in the time scales possible with such experiments even in the presence of compromised worm fitness in both control and treated groups. Indeed, Kotze and Bagnall (2006) demonstrated reduced beta-tubulin expression in adult *H. contortus* bathed in a solution of dsRNA. Hence, we examined the effects of targeting the same set of genes examined in the larval electroporation experiments using adult worms incubated in solutions containing dsRNA (also at 0·5 $\mu$g/$\mu$l).

Adult Kirby isolate *H. contortus* worms were recovered from sheep and placed into RPMI 1640-based culture medium as described previously (Kotze and McClure, 2001). Groups of five worms were held in 200 $\mu$l of medium in the presence of 0·5 $\mu$g/$\mu$l dsRNA for 24 h, then 0·8 ml of culture medium was added, and the incubation continued. After a further 24 h, worms were snap frozen for molecular analysis, while parallel assays were continued for measurement of phenotypic effects. On day 3 (72 h) FITC-labelled dextran (Sigma Chemical Co.) (molecular weight approximately 40,000 Da) was added as a marker of feeding levels. After a further 24 h, worms were examined under an illuminated magnifying lens, and each individual was scored as motile or non-motile (no sign of movement). The worms were then homogenised and the levels of ingested dextran were measured using a Tecan Spectrafluor Plus at excitation and emission wavelengths of 485 and 535 nm, respectively. For each separate experiment, each targeted gene was examined using a single group of 5 worms, alongside either two groups of 5 worms treated with rubisco dsRNA for molecular analyses, or 2–4 groups of 5 worms treated with rubsico dsRNA for feeding and motility assessments.

#### *Results*

Significant reductions in gene expression were measured for each of the targeted genes (compared with rubisco dsRNA-treated controls) at 48 h after initial exposure to the dsRNA (Fig. 3A). The reductions were greatest for ubiquitin (50-fold) and *Hc-vha-19* (30-fold). However, worm survival at day 4 was not affected (Fig. 3B). In addition, while feeding (i.e. dextran uptake) appeared to be reduced over the 24 h period from 72–96 h after initial exposure to the dsRNA in some treated groups compared with controls (Fig. 3C), the standard errors were quite large, and none of the treatments was significantly different from the controls (ANOVA, $P = 0{\cdot}05$).

### 'DIFFERENTIAL RNAI' IN *C. ELEGANS* TO ASSESS FUNCTIONS OF ONE ORTHOLOGOUS PROTEIN FROM *H. CONTORTUS*

The five nematode-specific genes tested in this project – *Hc-vha-19, Hc-noah-1, Hc-mitr-1, Hc-glf-1* and *Hc-pat-12* – were chosen for this study because they are essential in *C. elegans* and their silencing by RNAi in this species elicits severely compromised phenotypes. Silencing the orthologues of three of these genes in *H. contortus* elicited observable phenotypes in the larval feeding experiments (Fig. 1), while the remaining two genes – *Hc-noah-1* and *Hc-mitr-1* – did not, even though there was evidence of reduced transcript levels for these genes. There could be a number of reasons for this (see Discussion below), one of which could be that the physiological functions of the orthologous protein in *H. contortus* are different and are not critical under the conditions of our experiments. To examine this we tested the relative functions of one of these genes, *Hc-mitr-1*, in *C. elegans* using a 'differential RNAi' approach, in which transgenic *C. elegans* expressing both *Ce-mitr-1* and *Hc-mitr-1* were subjected to RNAi targetting *Ce-mitr-1*, in order to assess whether *Hc-mitr-1* could replace the function of *Ce-mitr-1* and thus 'rescue' the RNAi phenotype.

#### *Preparation of transgenic strains and RNAi methods*

A fragment of the ORF of *Hc-mitr-1* was amplified by PCR from mixed-stage *H. contortus* cDNA using the following primer pair, which was designed from EST clone Hc_d11_12A05 (Genbank BF060164): 5′-AGAAACCGAATGAGGAAGAGGG-3′ and 5′-CCAGAATCCAGCAAGGAACAGAAG. 3′- and 5′-RACE techniques (Marathon™ cDNA Amplification kit) were then used to obtain the full-length ORF (Genbank EF065614), which was ligated into pGEM-T easy. This was then employed as the PCR template to amplify the complete ORF of

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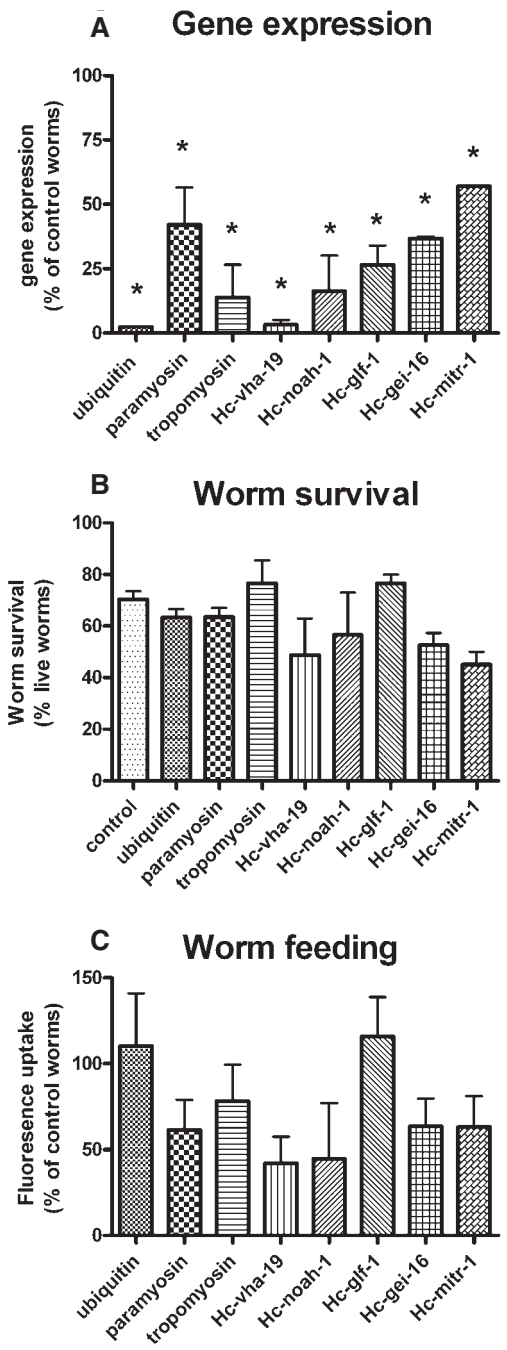


Fig. 3. RNAi with adult *H. contortus* incubated in the presence of dsRNA. **A**, gene expression in treated worms relative to control (rubisco dsRNA) worms after 48 hrs. **B**, percentage of motile worms in treated and control worms after 96 h; **C**, worm feeding levels during the 72–96 h period after dsRNA exposure in treated worms compared with control worms. Each column represents mean ± SE, n = 2 separate experiments in **A**, each with a single worm sample (5 individuals) for each gene compared separately with two rubisco-treated control worm samples (except for *Hc-mitr-1* where n = 1 experiment only); and n = 2–4 experiments for **B** and **C**, each with a single worm sample (5 individuals) alongside the mean value from 2–4 control assays (**B**), or expressed as a percentage of the mean value from 2–4 control assays (**C**). Asterisks above columns in **A** indicate significantly different (at $P = 0{\cdot}05$) from expression in control worms as assessed using REST software.

*Hc-mitr-1*, excluding the stop codon, using the following primers: 5′-B1.1-ATGTCCATTA-CTCGTGCTGTC-3′ and 5′-B2.1-TCGTGT-TCCTCTCTTCTTTGCGGAC-3′. B1.1 and B2.1 correspond to the Gateway® vector recombination sites GGGGACAACTTTGTACAAAAAAGT-TGGC and GGGGACAACTTTGTACAAGAA-AGTTGGGCA, respectively. The plasmid containing the ORF of *Ce-mitr-1* was kindly provided by Dr Denis Dupuy, Harvard Medical School, Boston, USA from the ORFeome collection (see worfdb.dfci.harvard.edu) and used as PCR template with the primers 5′-B1.1-ATGTCTCTCTCACT-CTTGAC-3′ and 5′-B2.1-TCTGGTTCCTCTC-TCCTTGG to amplify the complete ORF minus the stop codon.

The resulting PCR fragments were Gateway®-cloned into pDONR223 to generate pCB102 (*Hc-mitr-1* ORF) and pCB103 (*Ce-mitr-1* ORF). To generate the final destination vectors, pCB104 and pCB105, that were used to create WT257, WT258 and WT259, the *C. elegans* strains used in the differential RNAi experiments, the *C. elegans mitr-1* Promoterome clone (pF36A2.7; kindly provided by Dr Denis Dupuy, Harvard Medical School, Boston, USA; see worfdb.dfci.harvard.edu/promoteromedb) was incubated with either pCB102 (*Hc-mitr-1* ORF), or pCB103 (*Ce-mitr-1* ORF), and the destination vector pDEST-MB14 (kindly provided by Dr Mike Boxem, Harvard Medical School, Boston, USA) in the presence of LR clonase™ (Invitrogen). This generated pCB104 (*Ce-mitr-1*p::*Hc-mitr-1*::gfp) and pCB105 (*Ce-mitr-1*p::*Ce-mitr-1*::gfp), respectively. Micro-particle bombardment of *C. elegans unc-119 (ed3)* hermaphrodites (Praitis *et al*. 2001; Berezikov *et al*. 2004) with pCB104 and pCB105 was carried out to create the transgenic strains WT257 and WT258/259, respectively

Each transgenic strain was fed on *E. coli* HT115 (DE3) expressing dsRNA corresponding to either a fragment of the *A. thaliana lhcb4.3* gene (pCB19, a negative control) or a fragment of *Ce-mitr-1* (F36A2.7; Fraser *et al*. (2000)), using standard procedures (Johnson *et al*. 2008).

## *Results*

The treated worms were scored for the ‘clear’ phenotype (Fig. 4A) and for fecundity, as assessed by the total number of progeny produced (Fig. 4B). While the ‘clear’ phenotype was effectively rescued by expression of the *Hc-mitr-1* transgene, and also partially by overexpression of *Ce*-MITR-1 from the *Ce-mitr-1* transgene, there was no apparent rescue of the ‘low fecundity’ phenotype. Lack of rescue of the latter phenotype is most likely due to germline silencing of the *Hc*-MITR-1 transgene, a common phenomenon with transgenes in *C. elegans* (Merritt

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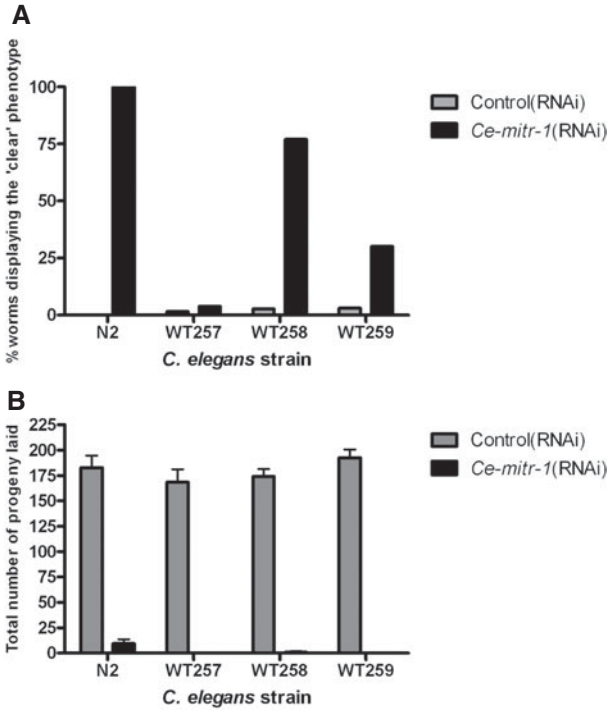


Fig. 4. 'Differential RNAi' experiment using transgenic *C. elegans*. Panel A: rescue of the *Ce-mitr-1* RNAi 'clear' phenotype by expression of *Hc-mitr-1*. Panel B: lack of rescue of the *Ce-mitr-1* RNAi reduced progeny phenotype by expression of *Hc-mitr-1*. RNAi was carried out against *C. elegans mitr-1* in the following strains: N2 (wild-type), WT257 (*Ce-mitr-1*p::*Hc*-MITR-1::GFP) and WT258 and WT259 (*Ce-mitr-1*p::*Ce*-MITR-1::GFP). Each strain was fed on dsRNA corresponding to either a control sequence (pCB19) or *Ce-mitr-1* from the L1 stage. After three days at 20 °C, worms were scored for the 'clear' phenotype (Panel A). Strains fed on control dsRNA displayed the clear phenotype at a penetrance of 0–3%. Strains containing wild-type *Ce-mitr-1* (N2) or a *Ce-mitr-1* transgene (WT258 and WT259) and fed on *Ce-mitr-1* dsRNA, displayed a 'clear' phenotype with a penetrance of 100% (n = 132), 77% (n = 130) and 30% (n = 50), respectively. In contrast, WT257, which expresses the *Hc-mitr-1* transgene, showed a 'clear' phenotype with a penetrance of 4% (n = 104) when fed on *Ce-mitr-1* dsRNA. The total number of progeny laid by up to 15 individual adults was also counted for each strain (Panel B). Adults from each strain fed on control dsRNA (pCB19) produced greater than 150 progeny. All strains, regardless of whether they contained the wild-type *Ce-mitr-1* (N2), a *Ce-mitr-1* transgene (WT258 and WT259) or a *Hc-mitr-1* transgene (WT257) did not produce more than an average of 10 progeny when fed on *Ce-mitr-1* dsRNA. Error bars are SEM.

and Seydoux, 2010). In addition, *Hc*-MITR-1 is only 49% identical to *Ce*-MITR-1 at the amino acid level, so it is also possible that the physiological functions of *Hc*-MITR-1 are partially divergent from those of *Ce*-MITR-1, or that the sequence difference is such that *Hc*-MITR-1 interacts differently from *Ce*-MITR-1 with interacting proteins in *C. elegans*. The latter factors could contribute to the lack of observed RNAi phenotype for *Hc-mitr-1* in *H. contortus* larvae.

## GENERAL DISCUSSION AND RECOMMENDATIONS

A comparative summary of the significant results for each dsRNA delivery technology that we have investigated in this study is provided in Table 3. With two exceptions, we achieved measurable reduction in transcript levels for each gene and for each technology tested. This implies that RNAi was successfully achieved and confirms the function of the RNAi pathway in *H. contortus*, as predicted by Dalzell *et al.* (2011). We did not, however, detect RNAi phenotypes for some of the genes tested by larval feeding, and detected no significant phenotypes for any of the genes tested in the larval electroporation or adult soaking experiments. It is clear that the larval feeding technology was the most successful in inducing RNAi under our experimental conditions and we recommend this technology for testing the functions of genes that are expressed during the larval stages.

### *Changes in transcript levels*

A general feature of successful RNAi in most organisms capable of RNAi is a measurable reduction in mRNA transcript levels for the targetted gene, although this has not always been the case for some parasitic nematodes tested (e.g. Samarasinghe *et al.* 2011). In this study, we achieved significant reductions in transcript levels for each technology investigated, and for most of the genes tested. Interestingly, many of the genes successfully knocked down are not expressed (in *C. elegans*) in the intestine, excretory cell or amphids – the 'accessible sites' considered by Samarasinghe *et al.* (2011). It would be of interest to examine their sites of expression in *H. contortus*.

In the bacterial feeding experiments (Fig. 1D,E,F) we detected reduced transcript levels for all eight genes tested, although the timing during the course of the 9-day exposure to dsRNA was variable: five genes exhibited an increase in levels in the first four days followed by a later reduction, whereas the remaining three genes showed an early reduction followed by an increase in the samples collected on days 7–9. There was no correlation of the timing of these changes with the presence or absence of a detectable phenotype. The early increase could potentially be due to PCR amplification of the administered dsRNA, because the primers used for RT-PCR could also have detected the administered dsRNA. However, this clearly was not the case for the experiments targetting *Hc-ubiquitin, Hc-tubulin* or *Hc-vha-19*, where transcript levels decreased early and increased later. For *Hc-tubulin* and *Hc-ubiquitin* we compared the results

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Table 3. Summary of results of the RNAi experiments completed in this project

| Target gene | Larval feeding | | Larval electroporation | | Adult soaking | |
|---|---|---|---|---|---|---|
| | Transcript levels[1] | Phenotype detected?[2] | Transcript levels[1,3] | Phenotype detected?[4] | Transcript levels[1] | Phenotype detected?[5] |
| *Hc*-ubiquitin | ⇓early,⇑late | Yes | ⇓(L1,L3) | No | ⇓ | No |
| *Hc*-tubulin | ⇓early,⇑late | Yes | | | ⇓ | No |
| *Hc*-paramyosin | – | – | ⇔ | No | – | – |
| *Hc*-tropomyosin | ⇑early,⇓late | No | ⇓(L3) | No | ⇓ | No |
| *Hc-vha-19* | ⇓early,⇑late | Yes | ⇓(L1,L3) | No | ⇓ | No |
| *Hc-noah-1* | ⇑early,⇓late | No | ⇓(L1) | No | ⇓ | No |
| *Hc-mitr-1* | ⇑early,⇓late | No | ⇓(L3) | No | ⇓ | No |
| *Hc-glf-1* | ⇑early,⇓late | Yes | ⇓(L1,L3) | No | ⇓ | No |
| *Hc-pat-12* | ⇑early,⇓late | Yes | ⇔ | No | ⇓ | No |

- not tested
[1] ⇓ reduced levels; ⇑ increased levels; ⇔ unchanged levels. A primer external and a primer internal to the administered dsRNA was employed for all genes except *Hc-vha-19*, *Hc-noah-1* and *Hc-mitr-1* in the larval feeding experiments, for which additional sequence external to the administered dsRNA was not available at the time. Therefore 'transcript levels' for the latter three genes in these experiments could potentially include the administered dsRNA.
[2] Development from L1 to L3; assessment of viability at each stage.
[3] Excludes eggs.
[4] Development from L1 to L3, or L3 migration assay.
[5] Survival; oral uptake of fluorescent dextran.

of RT-PCRs on the same cDNA preparations, using primers internal to the administered dsRNA, with reactions using an internal plus an external primer, and obtained similar results. Clearly, the processes responsible for the observed increases in transcript levels, both early and late, require further investigation.

In the electroporation experiments with free-living larval stages (Table 2), we observed significantly lower levels of transcripts for six of the eight genes targetted, and no significant change for the remaining two genes (*Hc-paramyosin* and *Hc-pat-12*). For three genes the transcript levels were reduced in both L1 and L3 stages, whereas only either L1 or L3 exhibited reduced levels for the other three genes. This may reflect differences in rates of mRNA transcription for these genes at different developmental stages, or could reflect physiological damage resulting from the electroporation procedure. Despite this, no significant RNAi phenotypes were recorded for any of the genes targetted.

The soaking experiments with adult worms (Fig. 3A) elicited reductions in the transcript levels of all eight targeted genes. This was, however, not a universal phenomenon with adult worms in these experiments, as only one out of three additional genes targetted as part of this study (which cannot currently be reported due to commercial-in-confidence considerations) showed reduced expression relative to control worms (data not shown). As for the electroporation experiments, no significant RNAi phenotype was detected for any of the targetted genes.

Thus, it appears that RNAi was successful, in terms of transcript knockdown, in most of our experiments, but we detected RNAi phenotypes only for some genes in the larval feeding experiments. It would provide greater insight in experiments such as these to have available specific antibodies or activity assays for the targetted proteins, which would enable comparison of protein expression or function with the observed changes in mRNA levels.

### *Analysis of RNAi phenotypes*

Those phenotypes that were observed in the larval feeding experiments were generally less intense than the RNAi phenotypes reported for silencing of the orthologues in *C. elegans*; this may represent any combination of factors that would include sub-optimal delivery of dsRNA, a fundamental biochemical/physiological difference in RNAi pathway function and regulation in *H. contortus* and our lack of established parameters for detailed phenotype analysis in *H. contortus* larvae. Thus, in our experiments we examined gross post-embryonic developmental progress in *H. contortus* larvae, gross motility in larvae or adults, and lethality. Additional processes important to screen for potential drug targets would include the moulting process, mating, egg production and early embryonic development. None of these can be successfully screened *in vitro* because the methods currently available for *in vitro* culture of the parasitic stages of *H. contortus* do not sufficiently support normal biological processes such as robust

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completion of the life cycle and production of viable eggs.

The science of phenotype analysis in parasitic nematodes is at an early stage of development in comparison with the repertoire of traits that are available to examine in *C. elegans*. It is likely that many of the more subtle, but potentially important, RNAi phenotypes in free-living larvae of *H. contortus* or other parasites will be overlooked unless very specific screening is developed. For example, it is unlikely that we could detect, say, a 10% decrease in worm size without specific size measurements of worms during the screening process. The 'clear' phenotype in *C. elegans* often indicates reduced accumulation of gut granules and/or lipid droplets; it is relatively easily observed for *C. elegans* on agar plates or microscope slides, but is more difficult to detect in less transparent worms swimming in liquid preparations. Similarly, important but subtle changes in motility, either in liquid or on a solid surface or matrix, will not necessarily be detected without development of more specific methods of assessment.

Therefore, the lack of observed phenotypic effects in the adult worm experiments, in particular, and possibly in the larval electroporation experiments as well, cannot necessarily be viewed as a failure to elicit RNAi, as there are a number of factors that could explain why a reduction in gene expression may have little consequence in the phenotype assessment assays described here. On top of the lack of established phenotypic traits to assess, other factors that could affect the development of an observable phenotype include: (1) Reductions in gene expression may have little effect on protein levels if the protein is quite stable in the organism, particularly given the short time scale for the adult worm experiments and the damaging effects of electroporation on the treated larvae, which could cause reduced protein synthesis and turnover in these dying or damaged worms; (2) The target protein may not be essential for the viability of the organism in the time frame of the experiments: investigation of more subtle biological processes might detect specific RNAi effects; (3) Not all phenotypes will be observable in either larval or adult worms *in vitro*, for example sterility, or changes in mating behaviour; (4) A degree of redundancy in biochemical pathways may operate for some genes, which would allow other proteins to substitute when the target protein is limiting; and (5) Limited longevity of any gene silencing effects may allow gene expression (and hence protein levels) to return to normal levels quite rapidly after initial reductions. This consideration would be more important in electroporation RNAi experiments than in feeding or soaking experiments, where dsRNA is administered over a longer time frame. Hence, the lack of phenotypic effects in the larval electroporation and adult soaking experiments is not *per se* an indication of the failure to elicit an RNAi response.

### *The problem of RNAi in parasitic stages of the life cycle*

In assessing the effects of dsRNA on adult worm viability we utilised a crude system of scoring worms as motile or immotile. This system will, of course, fail to measure subtle effects on motility which may be quite significant to the viability of a worm *in vivo*, and which therefore may be important when utilising the RNAi technique as a drug target validation tool. A mild effect on worm motility *in vitro* after exposure to dsRNA may indicate that the target is important despite having no profound effect on worm survival (motile vs immotile). Hence, for future studies examining the effects of dsRNA on adult worms we recommend that a more detailed approach to judging the effects of dsRNA on the movement of adult *H. contortus in vitro* be adopted, for example, visual assessment of the form and degree of movement of individual worms recently described by Kotze *et al.* (in press).

The maintenance of 'fit' worms for as long as possible is important for *in vitro* experiments with the adult life stages of *H. contortus*. In the present study, this was compromised to a significant degree by a perceived need to maintain the worms in a high concentration of dsRNA for at least the initial period of the incubation. For this reason, groups of five worms were held in 200 $\mu$l of medium for 24 h before an additional 0·8 ml of medium was added. This was considered necessary in order to maintain the dsRNA at a concentration of 0·5 $\mu$g/$\mu$l while not consuming too much of the RNA in each experimental sample as the use of *in vitro* transcription kits is an expensive exercise. This is quite different from our more recently-developed experimental procedures in which 5–15 adult *H. contortus* worms are maintained for periods in 3 ml of medium, and are observed to be in significantly better condition after 2–3 days than those examined in the RNAi experiments reported above (Kotze *et al.* in press). Further experimentation is required in order to obtain an adequate balance between assay volumes and dsRNA concentrations in order to minimise the impact of the incubation process on the viability of adult worms *in vitro*.

While the development of more effective RNAi methodology using short-term *in vitro* incubations of exsheathed-L3/L4/adult worms, coupled with improved methods of phenotype analysis, is likely to contribute to our understanding of gene function in the parasitic stages of *H. contortus*, there remains the significant problem of investigating the functions of genes important for development, survival and reproduction in the *in vivo* environment of the host. In an important study Samarasinghe *et al.* (2011) showed that it is possible to soak exsheathed-L3s

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of *H. contortus* in dsRNA that targets the intestinal aminopeptidase H11, then transfer the treated worms to sheep and observe apparent phenotypic effects in the adult worms. This is a very promising approach, though it remains to be shown that this procedure can be used successfully for other genes. Although *in vivo* assessment would be too expensive for routine screening for gene or protein function in the parasitic stages of *H. contortus*, it could prove very valuable for confirming function of genes of particular interest.

### *Recommendations*

(1) Adopt and further optimise the bacterial feeding method as the method of choice for RNAi of free-living stages of *H. contortus*; (2) Since bacterial feeding is not suitable for late larval and adult stages of *H. contortus*, it is necessary to optimise procedures for electroporation (L3) and soaking (adults) for *in vitro* RNAi of these stages of *H. contortus*; (3) Investigate the *C. elegans* RNAi phenotype for genes with orthologues in *C. elegans* and include this information in selecting traits to score in *H. contortus* RNAi experiments; (4) Develop a more extensive and specific repertoire of phenotype traits to assess in *H. contortus* RNAi experiments; (5) Consider preparing specific antibodies or functional assays for proteins encoded by the targetted genes, to improve interpretation of the results of RNAi experiments; (6) Ensure that the primer set used for RT-PCR to determine transcript levels includes a primer that targets mRNA sequence external to the administered dsRNA; (7) Where possible and desirable, include 'differential RNAi' or mutant rescue experiments in *C. elegans* (Britton and Murray, 2002) to assess whether the *H. contortus* protein of interest can rescue the *C. elegans* RNAi or mutant phenotype. *C. elegans* has been successfully used as a heterologous expression system in a number of laboratories (Kwa *et al.* 1995; Gilleard, 2004; Gillan *et al.* 2009; Glendinning *et al.* 2011) and (8) Consider including *in vivo* transfer experiments, as performed for the aminopeptidase H11 by Samarasinghe *et al.* (2011), to investigate protein function in the parasitic stages of *H. contortus*. While these experiments are expensive, they may provide valuable validation of observations from *in vitro* RNAi experiments.

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