final report

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RNA interference: characterisation of key components of the mechanism in the parasitic nematode *Haemonchus contortus*

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Abstract

To date, there has been considerable focus on the identification of biological targets in nematodes against which control agents can be developed. RNA interference allows determination of whether a specific gene is essential for development or survival and has potential as a technique for the high-throughput screening of genes for deleterious effects. RNAi as a technique has been applied to parasitic nematodes with variable success. This project aimed to characterise the pathway for RNAi in the parasitic nematode *Haemonchus contortus* as compared to the pathway already defined by studies in *Caenorhabditis elegans*.

A continual bioinformatics approach was utilised over the entire project as new *H. contortus* sequence was released into the public domain. Next-Generation Roche 454-Titanium sequencing of xL3 and adult nematodes was incorporated into the project as an extra deliverable and provided a great deal of very high quality data for *H. contortus*. All available *H. contortus* sequence (both in-house and public) was collected and compiled into a searchable database. This database was then queried using a variety of parameters in an attempt to increase the sequence information available for orthologues of the molecules involved in the RNAi mechanism. Considerable sequence information was obtained for ten of these genes (*sid-1*, *dcr-1*, *drh-1*, *eri-1*, *pir-1*, *rsd-3*, *rrf-1*, *rde-1*, *rrf-3* and *ego-1*). Sequence information was not obtained for two genes (*rsd-6* and *rde-4*), that do not appear to be present. However, sequence was identified for a possible family of argonaute proteins; these may fulfil the functional role of the missing *C. elegans* orthologues. A small amount of sequence was identified for the elusive *sid-2*, this gene appears to be rather divergent and has been extremely difficult to find. Primers were designed for seven of these genes (*sid-1*, *dcr-1*, *eri-1*, *pir-1*, *rsd-3*, *rde-1*, and *ego-1*), each representing a different processing branch in the RNAi mechanism. These primers were designed to amplify a small conserved coding region for each gene and were used to generate a stage-specific expression profile and localisation profile by quantitative PCR. All genes were expressed during the parasite life-cycle with variations in expression profiles. This provides evidence that the RNAi mechanism is functional in *H. contortus*, although it does differ from *C. elegans*. It should be noted that the compiled sequence library provides a strategic resource that will benefit discovery efforts in parasitic nematodes and may enable identification of new drug/vaccine targets in future projects.

Our RNAi assay that was originally developed in Project AHW.031 was significantly improved early in Project WP166. This improved RNAi assay was utilised in this project for a detailed functional characterisation of six genes, Nov0374, Nov0491, S3_0055, S1_452, ben-1 and ubq-1. These genes are expressed in all life cycle stages as determined by quantitative PCR. No RNAi phenotype was observed for Nov0374 and only slight mortality at L1 for ubq-1. A more dramatic developmental arrest was observed for ben-1 and S3_0055 with approximately 20% sick L1s at day 4 and 20% sick L1s and 20% sick L2s at day 8. Nov0491 had increased mortality at L1 and L2, while S1-452 had developmental arrest at L2 with 40% at day 8. In situ hybridisation experiments were undertaken to determine the localisation profiles for these genes. The differential gene silencing patterns did not correlate with the localisation of available mRNA transcripts for each gene. Alternatively, the variability of gene silencing by RNAi in *H. contortus* may be due to variable rates of protein turnover for the selected targets. Further studies would be required to investigate this possibility.

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1. Project objectives

The primary objective of this project was to characterise the potential of RNAi as a validation tool in parasitic nematodes. This project utilised the outcomes developed through the GINTIP project (AHW.031) and contributed to the outputs of project WP166 to provide a reliable method for the assessment of gene function that can be used for the validation of targets for testing as vaccine candidates or for drug design. The specific objectives were:

- The characterisation of the mechanism for RNAi in parasitic nematodes using *H. contortus*;
- Investigation of transcript down-regulation and protein abundance for silenced genes; and
- Localisation of RNAi effects for specific target constructs, both new and existing (from GINTIP).

The secondary objective of this project was to further develop scientific leadership qualities and collaborative networks by undertaking a researcher exchange. The specific objectives were:

- To spend time in a commercial animal health pharmaceutical company and participate in the Development side of Research & Development; and
- To develop the additional research skills required for localisation studies through a mentoring process using in house expertise.

2. Success in achieving milestone

The amended milestone achievement criteria are listed below. These milestones were negotiated and agreed to by both parties in October 2009. Furthermore, a 3 month extension was granted in December 2010 for the completion of milestone 6 due on the 30th March 2011.

This final report should replace the draft version that was submitted on the 30th January 2011. This final report discusses the completion of all milestones.

Milestone	Achievement Criteria	Due Date
2	RNAi Mechanism: To identify orthologues of the key genes for the RNAi machinery for the parasitic nematode, *H. contortus* using a next-Gen sequencing and cloning based approach. To determine mRNA and protein expression profiles for these genes. To specifically focus on those genes involved in the uptake and transport of dsRNA.	30.06.09
3	Commercial Perspective: To undertake a researcher exchange to a commercial animal health pharmaceutical company. To develop an understanding of high-throughput screening processes and the development of potential products for market.	30.01.09
4	RNAi Effects: At least 5 genes will be characterised by RNAi and phenotype, transcript down-regulation and protein abundance will be investigated for the silenced genes.	30.01.09
5	Development of Scientific Capability: Develop the additional skills required for localisation studies required in milestone 6. This will be completed through a mentoring process using in house expertise.	15.06.10
6	Localisation of RNAi: Localisation patterns of gene silencing effects will be determined for specific target constructs. Final report in MLA approved format and including the relevance of the outcome to the livestock industry to be submitted.	30.03.11

Milestone 2 – RNAi mechanism

The mechanism for RNAi has been well defined in *C. elegans*, and is widely utilised to determine gene function in this free-living nematode. For RNAi to be an effective validation tool in parasitic nematodes, a more thorough analysis of the RNAi mechanisms present in parasitic nematodes is required. Determination of whether the pathway is fully functional is essential to help define optimal delivery methods for the uptake of dsRNA and appropriate spread of dsRNA once within the nematode. Furthermore, knowledge of the stage-specific expression of these genes will determine whether RNAi can be utilised in all life cycle stages.

Figure 1 provides an overview of the mechanism for RNAi in *C. elegans*. RNAi is induced by the introduction of gene-specific dsRNA. Uptake of long dsRNA (>100 bp) into cells occurs via SID-1, a transmembrane channel. SID-2 is expressed in the gut and is believed to modify SID-1 activity in gut cells to enable efficient uptake of dsRNA from the environment or to bring dsRNA into vesicles for SID-1 dependent transport to the cytoplasm. Internalised long dsRNA is then recognised and cleaved by the DICER complex to produce siRNA fragments. The DICER complex consists of four proteins, DCR-1, DRH-1, RDE-1 and RDE-4. The siRNAs are then loaded into the RNA induced silencing complex (RISC) that mediates mRNA degradation to result in gene silencing. RDE-1 is the key component of the RISC complex and utilises a short segment of guide

RNA as a means to recognise or act upon a target transcript. The siRNAs can also act as primers for the amplification of target mRNA by the RNA dependent RNA polymerases (RdRPs) to produce secondary dsRNA. The RdRP complex consists of RRF-1 and EGO-1 and is competitively regulated by RRF-3. DCR-1 is also responsible for cleavage of the secondary dsRNA but requires PIR-1 for activity. Regulation of dsRNA occurs by the degradation of siRNA by ERI-1. Systemic RNAi or spreading is thought to involve the transport of long dsRNA to surrounding cells by the SID and RSD proteins, although this mechanism is not well understood. SID-1 is required for initial uptake and systemic spreading, while RSD-2, 3 and 6 are required for distribution to the germline.

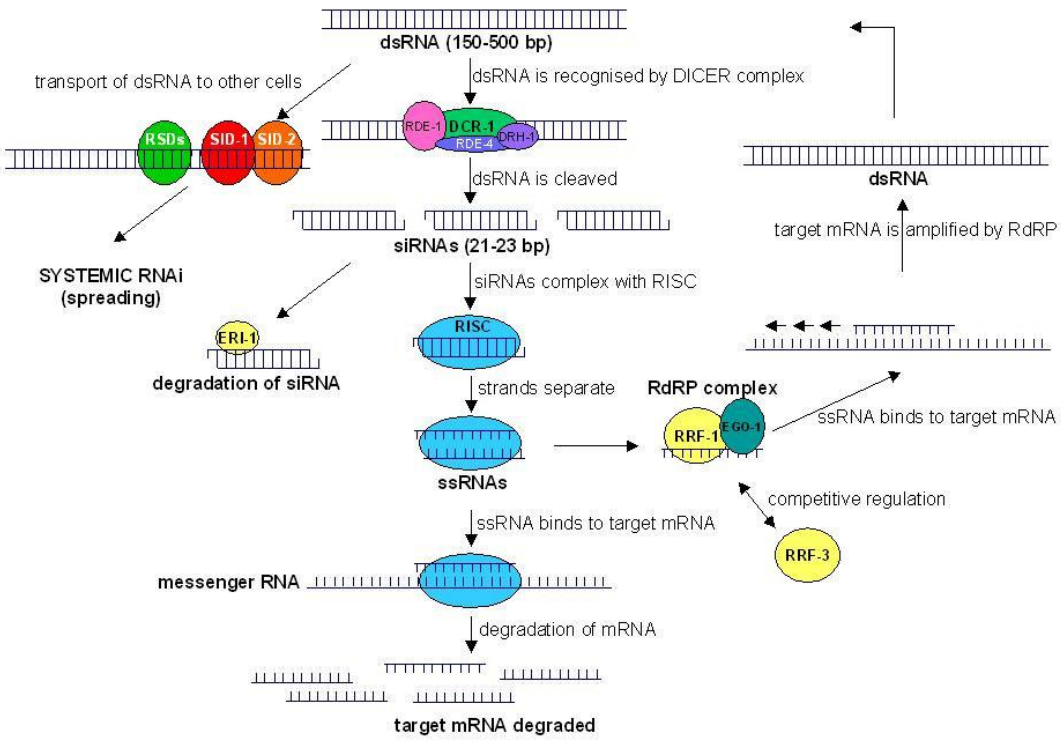

Figure 1. Mechanism for RNAi in *C. elegans*

Abbreviations: dsRNA, double-stranded RNA; RDE, RNAi deficient; Dcr, Dicer; Drh, Dicer helicase; siRNA, small interfering RNA; RISC, RNA induced silencing complex; ssRNA, single stranded RNA; RdRP, RNA-dependent RNA-polymerases; SID, systemic interference defect; RSD, RNAi spreading defect.

The results of past RNAi studies in *H. contortus* support the existence of a mechanism for RNAi and provide encouragement for the further development of RNAi as a validation method (Kotze and Bagnall, 2006; Geldhof et al., 2006).

In the first part of this project, a bioinformatics approach was utilised to mine the partial *H. contortus* genome sequence and EST sequences that were available within the public domain as well as within our in-house EST sequence database in an attempt to identify sequences that have homology to any of the molecules involved in the RNAi mechanism. At the time *H. contortus* sequence information was limited and we found it quite difficult to obtain useful sequence information. Small regions of sequence homology were identified for some of these molecules and gene-specific primers were designed and used in PCR optimisation experiments in an attempt to amplify a larger region of each specific gene. Unfortunately this approach was not successful and we were unable to PCR amplify gene-specific PCR products to use as probes to screen a cDNA library to find each full length gene.

As a new deliverable, we undertook a sequencing strategy in an attempt to collect additional sequence information for *Haemonchus*. Exsheathed L3 (xL3) and adult nematodes were collected and used to prepare cDNA for each of the stages, followed by nebulisation, polishing and an in house adapter ligation and amplification. The products were titrated onto 454 beads in emulsion PCR and the enriched beads were input for sequencing on the 454 Sequencer. We obtained 415 022 raw reads for the Adult library which converted to 406 322 reads following quality trimming. We obtained 331 083 raw reads for the xL3 library which converted to 148 066 reads following quality trimming. This new sequence information was assembled, annotated and combined with all other available sequence and a more intensive bioinformatics approach undertaken as described below.

All publically available *Haemonchus* sequences were downloaded; this included sequences from the Sanger and Blaxter sites, genome, ESTs and 454 reads. These sequences were combined with our in house ESTs and in house 454 sequences and converted into a searchable database. Blast searches (tblastn) for the RNAi spreading targets were performed using the *C. elegans* protein sequences as the query. DNA sequence output files were aligned using the CAP3 program. CAP3 assembly of all relevant sequence for each target gene yielded a number of contigs, which were then blasted (blastx) back against the NCBI database to ensure compatibility to the original target. Primers were designed against conserved regions for seven of these genes (*sid-1*, *dcr-1*, *eri-1*, *pir-1*, *rsd-3*, *rde-1*, and *ego-1*), each representing a different function of the RNAi processing mechanism (Table 1). L1, L2, L3, xL3, eL4, eL4bf, yAd and Ad life-cycle stages were collected and total RNA, mRNA and cDNA prepared. In addition, the Ad life-cycle stage was collected, dissected into body, ovary and gut components and total RNA, mRNA and cDNA prepared. The primer sets were used to determine the mRNA stage-specific expression profile as well as the mRNA localisation profile for each target gene by Quantitative PCR (Table 2 and 3). The amount of target has been normalised to an endogenous housekeeping gene and relative to the calibrator. These profiles indicate the stages that the RNAi genes are expressed and thereby predict which parasite stages should be amenable to gene silencing by RNAi. The localisation profiles indicate where the RNAi genes are expressed and may provide some insight into the potential for uptake of dsRNA from the environment and the potential for systemic spreading within the nematode.

Table 1. Quantitative PCR Primers. Sequences of forward and reverse primers for the selected RNAi mechanistic genes.

Primer name	Primer sequence 5'-3'	Fragment (bp)
Hc-Sid-1 F'	CCACAATTACGCATGCTCAG	294
Hc-Sid-1 R'	ACTTGCAAATGCCTCACACA	
Hc-Rsd-3 F'	TCCAACATCCTTTTCCAGAGA	209
Hc-Rsd-3 R'	CCGAAGCTGAAAATCTTGTCT	
Hc-Dcr-1a F'	AGTTCCGCTCACTGCAAAGT	222
Hc-Dcr-1a R'	CCTGGGGTGTTGACAGATTT	
Hc-Dcr-1b F'	GCCCTATCGCTGAAGACAAG	162
Hc-Dcr-1b R'	TTTTTGGCTTCCAGATGCTT	
Hc-Pir-1a F'	GCCTGACGAGCCTGAAATAC	210
Hc-Pir-1a R'	ACCCAGTTGGTCGAGATTTG	
Hc-Pir-1b F'	AGGCGAGAAGTTGATTGGTG	231
Hc-Pir-1b R'	CCCCGTGAGAACTCAAACAC	
Hc-Eri-1a F'	CCCTGCTGTGTTGATTGATG	165
Hc-Eri-1a R'	GGATGCAGAAAGGATCCAGA	
Hc-Eri-1b F'	CCAATGCATCTTGGTCAGTG	202
Hc-Eri-1b R'	TTCCTCACCAATGCTTACCC	
Hc-Rde-1 F'	ACCTTGTAGCAGCCAAATCG	208
Hc-Rde-1 R'	CGACGACGAATCTCAGTTCA	
Hc-Ego-1 F'	CATTGCATCGGTATGACAGC	249
Hc-Ego-1 R'	TATACGTCGCGGTAGCTCCT	

Table 2. Stage-specific mRNA expression profiles for the selected RNAi mechanistic genes in *H. contortus*.

H. contortus **Target**	***H. contortus*** **mRNA expression profile**							
Gene	**L1**	**L2**	**L3**	**xL3**	**eL4**	**eL4bf**	**yAd**	**Ad**
Hc-Sid-1	0	0.12	0	0.004	0.28	0.67	0.99	0.06
Hc-Dcr-1	0.49	0.22	0.09	0.28	0.24	0.23	0.17	0.95
Hc-Rde-1	nd	nd	nd	nd	nd	nd	nd	nd
Hc-Ego-1	0.34	0.09	0.16	0.17	0.07	0.10	1.19	0.02
Hc-Pir-1	0.33	0.003	0.07	0.01	0.01	0.29	0.008	0.02
Hc-Eri-1	0.24	0.82	0.12	2.43	3.47	1.61	1.97	1.36
Hc-Rsd-3	nd	nd	nd	nd	nd	nd	nd	nd

Abbreviations: L2; second stage larvae, L3; third stage larvae, xL3; exsheathed L3, eL4; early fourth stage larvae, eL4bf; eL4 blood feeding, yAd; young adult and Ad; adult, mRNA expression levels are indicated. nd; not determined.

Table 3. Localisation mRNA expression profiles for the selected RNAi mechanistic genes in *H. contortus*.

***H. contortus* Target Gene**	***H. contortus* mRNA expression profile**		
	Body	**Ovary**	**Gut**
Hc-Sid-1	+++	+++	++
Hc-Dcr-1	+++	++	+
Hc-Rde-1	+++	+	+
Hc-Ego-1	+++	+	+
Hc-Pir-1	+++	+	+
Hc-Eri-1	+++	++	+
Hc-Rsd-3	nd	nd	nd

Abbreviations: mRNA expression levels are indicated by -; none, +; low, ++; moderate, +++; high, nd; not determined.

The bioinformatic approach was then extended by downloading the Sanger supercontig database. A script was written that enabled the previously identified contigs to be queried against the supercontig database to identify which *H. contortus* supercontig each gene is likely to be located (Table 4).

Table 4. Summary of bioinformatic information for RNAi mechanistic genes.

Gene	Query seq	Top query blast hit (NCBI) blastx	E-value	Supercontig hit
sid-1	Contig1	ref|NP_001103253.1| Sid-1-related B [Tribolium castaneum] >gb...	7.00E-11	Supercontig_0000736
dcr-1	Contig1	ref|XP_002643058.1| C. briggsae CBR-DCR-1 protein [Caenorhabd...	2.00E-49	Supercontig_0005515
	Contig2	ref|XP_002643058.1| C. briggsae CBR-DCR-1 protein	4.00E-18	Supercontig_0002309
	Contig3	ref|NP_498761.1| DiCer Related family member (dcr-1) [Caenorh	1.00E-50	Supercontig_0005515
	Contig4	ref|XP_002643058.1| C. briggsae CBR-DCR-1 protein [Caenorhabd..	4.00E-15	Supercontig_0005515
	Contig5	ref|XP_002643058.1| C. briggsae CBR-DCR-1 protein [Caenorhabd...	5.00E-47	Supercontig_0002309
drh-1	Contig11	ref|XP_002633533.1| C. briggsae CBR-DRH-1 protein [Caenorhabd	5.00E-11	Supercontig_0006025
	Contig1	ref|NP_492161.3| Dicer Related Helicase family member (drh-3)..	6.00E-08	Supercontig_0028207
	Contig2	ref|XP_002633533.1| C. briggsae CBR-DRH-1 protein [Caenorhabd...	2.00E-04	Supercontig_0006025
eri-1	Contig1	ref|XP_002634999.1| C. briggsae CBR-ERI-1 protein [Caenorhabd...	5.00E-21	Supercontig_0002864
pir-1	haem-1375p20.q1k	ref|NP_495959.1| Phosphatase Interacting with RNA/RNP family .	5.00E-18	Supercontig_0027655
	Contig1	ref|NP_495959.1| Phosphatase Interacting with RNA/RNP family ..	5.00E-12	Supercontig_0050365
	Contig2	ref|XP_002629700.1| C. briggsae CBR-PIR-1 protein [Caenorhabd..	3.00E-06	Supercontig_0050365
				Supercontig_0027655
rsd-3	Contig1	ref|XP_001899173.1| ENTH domain containing protein [Brugia ma..	1.00E-16	Supercontig_0059706
rrf-1	Contig1	ref|NP_492131.1| RNA-dependent RNA polymerase Family family m...	1.00E-43	Supercontig_0002625
				Supercontig_0055722
	Contig3	ref|NP_492132.1| Enhancer of Glp-One (glp-1) family member (e...	3.00E-16	Supercontig_0040459
	Contig4	ref|XP_002638736.1| C. briggsae CBR-EGO-1 protein [Caenorhabd..	4.00E-19	Supercontig_0002625
	Contig5	ref|NP_492132.1| Enhancer of Glp-One (glp-1) family member (e..	3.00E-17	Supercontig_0049990
	Contig6	ref|NP_492132.1| Enhancer of Glp-One (glp-1) family member (e	3.00E-25	Supercontig_0067220
	Contig8	ref|NP_492132.1| Enhancer of Glp-One (glp-1) family member (e..	5.00E-08	Supercontig_0034585
				Supercontig_0025336
ego-1	Contig9	ref|NP_492132.1| Enhancer of Glp-One (glp-1) family member (e..	4.00E-07	Supercontig_0021190

Gene	Query seq	Top query blast hit (NCBI) blastx	E-value	Supercontig hit
	Contig10	ref|XP_002638736.1| C. briggsae CBR-EGO-1 protein [Caenorhabd	2.00E-15	Supercontig_0002625
rrf-3	Contig11	ref|NP_492131.1| RNA-dependent RNA polymerase Family family m.	3.00E-07	Supercontig_0002625
	Contig13	ref|XP_002638736.1| C. briggsae CBR-EGO-1 protein [Caenorhabd..	8.00E-05	Supercontig_0011646
	Contig14	ref|NP_495713.1| RNA-dependent RNA polymerase Family family m..	8.00E-07	Supercontig_0065267
	Contig16	ref|NP_495713.1| RNA-dependent RNA polymerase Family family m..	8.00E-28	Supercontig_0026772
	haem-202k04.p1ka	ref|NP_492132.1| Enhancer of Glp-One (glp-1) family member (e.	7.00E-17	Supercontig_0067220
	Contig2	ref|NP_495713.1| RNA-dependent RNA polymerase Family family m.	4.00E-18	unable to be located in the SC database
	Contig7	ref|NP_492132.1| Enhancer of Glp-One (glp-1) family member (e	1.00E-08	
	Contig12	ref|XP_002638736.1| C. briggsae CBR-EGO-1 protein [Caenorhabd.	2.00E-10	
rde-1	Contig1	F48F7.1b CE43299 WBGene00000105 locus:alg-1 status:Partially_con	1.00E-75	Supercontig_0001636
	Contig2	ref|NP_499191.1| Temporarily Assigned Gene name family member.	5.00E-35	Supercontig_0014820
	Contig3	ref|XP_002637670.1| C. briggsae CBR-RDE-1 protein [Caenorhabd.	3.00E-10	Supercontig_0027807
				Supercontig_0041222
	Contig4	ref|NP_492045.1| hypothetical protein R06C7.1 [Caenorhabditis.	2.00E-29	Supercontig_0000312
	Contig5	ref|XP_001901579.1| Piwi domain containing protein [Brugia m	8.00E-13	Supercontig_0030141
	Contig6	ref|NP_495151.1| hypothetical protein ZK1248.7 [Caenorhabditi	8.00E-20	Supercontig_0013213
				Supercontig_0008151
	Contig7	ref|XP_002399390.1| Cniwi protein, putative [Ixodes scapulari...	3.00E-31	Supercontig_0001843
Other argonautes	Contig8	ref|XP_002637670.1| C. briggsae CBR-RDE-1 protein [Caenorhabd..	0.029	Supercontig_0041222
				Supercontig_0040803
	haem-1330k04.p1k	ref|XP_001894256.1| argonaute 2 [Brugia malayi] >gb|EDP36908....	5.00E-38	Supercontig_0001636
	haem-1402p06.p1k	ref|NP_741611.1| RNAi DEfective family member (rde-1) [Caenor	6.00E-10	Supercontig_0027807

Our findings are summarised as follows:

Sid-1: One contig was obtained for *sid-1*, which had significant homology to a *sid-1* related gene from *Tribolium castaneum*, rather than to *C. elegans*. Primers were designed in the highly conserved coding region. This region of *sid-1* could be amplified and is expressed in most life-cycle stages, excluding L1 and L3.

Dcr-1: Five contigs were obtained for *dcr-1*, all with high homology to the *C. briggsae dcr-1* gene. These contigs localised over two supercontigs. Two sets of primers were designed in the conserved coding regions. Primer set a was used for the quantitative PCR analyses. *Dcr-1* is reasonably well conserved and is expressed in all life-cycle stages.

Drh-1: Three contigs were obtained for *drh-1*, two with high homology to the *C. briggsae drh-1*. These were located over two contigs; however, one is more likely to be *drh-3*.

Eri-1: One contig was obtained for *eri-1*, with high homology to the *C. briggsae eri-1* gene. Two sets of primers were designed in the conserved coding regions. Primer set a was used for the quantitative PCR analyses. *Eri-1* is reasonably well conserved and is expressed in all life-cycle stages.

Pir-1: Two contigs and one singleton were obtained for *pir-1*, two with high homology to *C. elegans* and one to *C. briggsae*. These localised over two supercontigs, one which is very short. Two sets of primers were designed in the conserved coding regions. Primer set a was used for the quantitative PCR analyses. *Pir-1* is not as well conserved but is expressed in all life-cycle stages. The second primer set may be required for a more consistent analysis.

Rsd-3: Two contigs were obtained for *rsd-3*, which when blasted back against NCBI database showed relevant hits, particularly to *Brugia malayi*. The second contig contained a more significant hit to Espin, although this could be relevant as *rsd-3* contains an N-terminal espin domain. Primers have been designed from contig 1 in the region which definitely correlates to coding region for *rsd-3* gene. This gene was difficult to work with and although it could be amplified using traditional PCR, it could not be consistently amplified using quantitative PCR. A new primer set would need to be designed for more detailed analysis.

Rrf-1, Rrf-2, Rrf-3 and Ego-1: Several contigs were obtained for these genes, although considerable overlap was observed. In *C. elegans*, these genes have highly conserved functional domains that are evident when an alignment is performed. Primers have been designed from contig 1 in the region which definitely correlates to coding region for *ego-1* gene. *Ego-1* is reasonably well conserved and is expressed in all life-cycle stages.

Rde-1: Eight contigs and a number of singletons were obtained for *rde-1*. At least one of these appears to be *rde-1* (contig 3). However, the others showed higher hits to other proteins when blasted back against the NCBI and wormbase databases. These other hits also contained the same domains as *rde1* (PIWI, PAZ and dicer protein domains). There is a family of Argonautes which play key roles in the RNAi silencing pathway using the small RNA guided strategy. Interestingly, the number or argonautes differs in each species (from 1 in *S.pombe* to 5 in mammals and fruit fly to 27 in *C.elegans*). The presence of these other argonautes may explain why *rde-4* and a couple of other genes important for *C. elegans* RNAi cannot be found in *H. contortus*. Primers were designed from contig 3. Interestingly, PCR consistently gave multiple products which may suggest that several argonautes are being amplified using this primer set.

Sid-2: Considerable effort has gone into the search for *sid-2* as it is a critical gene in the spreading pathway in *C. elegans*. A few different strategies were undertaken; however it is so divergent that it remains hard to locate. The N terminal region (190 a.a) only has 23% homology between 3 different *Caenorhabditis* species, and the C terminal region (100 a.a) has 53% homology. There is a TM domain (21 a.a) which has 86% homology, the blast parameters were manipulated to better look for this (decreased word size, increased e-

value and change the matrix etc) which yielded some positive results but not sufficient to continue.

This approach has yielded information for most of the genes required for effective RNAi in *C. elegans* and has provided strong evidence that an RNAi mechanism is functional in *H. contortus*. However, there are important genes that have not been identified (*sid-2*, *rsd-6* and *rde-4*) and it is likely that there are alternate genes (yet to be identified) that are responsible for performing the required RNAi functions in this nematode. Furthermore, the differential gene expression profiles suggest that RNAi is functional with all genes expressed at reasonable levels in the parasitic stages and at lower levels in the free-living stages. All RNAi mechanistic genes were expressed at their highest levels in the body of the adult nematode and at reduced levels in the ovary and gut. Interestingly, *sid-1* was the only gene that remained expressed at a moderate level in the gut consistent with a potential role in uptake. Unexpectedly, *sid-1* is expressed in L2 but is not expressed in L1 and L3, this would warrant further investigation. Interestingly, many of the *H. contortus* RNAi orthologues identified were more closely related to *C. briggsae* which provides further evidence that the RNAi mechanism does in fact differ from *C. elegans*.

Milestone 3 – Commercial perspective

The specific purpose of this milestone was to undertake a short visiting scientist position to one of the lead international animal pharmaceutical companies. Novartis Animal Health was approached and negotiations were entered to allow a visit to two different R&D sites within Switzerland. The first was Novartis headquarters located in Basel and the second was Novartis Centre de Recherche Santé Animale located in St Aubin. The focus of the trip was to gain an awareness of science as a business within a commercial company. In addition, this trip was coupled with a visit to the University of Zurich to visit Professor Peter Seeberger, a leading scientist in the research of carbohydrates as vaccines (for human medicine), to discuss the potential for the development of a collaborative project.

The visit to Novartis in Basel was hosted by Rolf Steiger and involved a tour of the Head Offices as well as time with the Chemistry and Formulations group. This visit provided insight into the processes required for a new animal health pharmaceutical product to progress from a validated target through the development phase and to determine the optimal formulation for stability and delivery. Surprisingly, this highlighted the high rate of failure experienced for converting a validated target to potential commercial product.

The visit to St Aubin was hosted by Jacques Bouvier. St Aubin is the location of the Novartis Parasitology Research group that have a major project on anthelmintic resistance and mode of action. A seminar was given that highlighted the research achievements from our GINTIP projects (AHW.031 and AHW.033) and discussions were held in relation to *in vitro* culture techniques and the potential application to mode of action studies. Participation in the later research phase of an anthelmintic project led to an increased awareness of the requirements that must be satisfied to get a potential therapeutic from the research phase through to the development phase for formulation as a potential commercial product.

While in Switzerland, I also took the opportunity to visit Professor Seeberger at the University of Zurich and present our H11 nematode vaccine research that had recently finished. The purpose of this was to discuss the possibility of a future collaboration for the synthetic synthesis of the H11 carbohydrate moiety. Follow-up discussions will be held with Professor Seeberger to attempt to set up a future collaboration to determine the feasibility of a carbohydrate-conjugated *Haemonchus* vaccine.

Overall, this trip was extremely rewarding, I had the opportunity to meet with several international parasitologists and discuss a range of research that is occurring in the area of both human and veterinary parasitology. This allowed me to assess the validity of our approaches and the direction of our future research. More specifically, the visit to Novartis

provided an overview of how a large Pharmaceutical company approaches new products, their formulation and marketing strategies. These areas can be considered earlier in our target identification and validation process to enhance the prospect of commercial uptake.

This travel formed part of the planned activities under the MLA Fellowship which has a strong focus on the development of both science and leadership skills with a particular emphasis on industry awareness and links with animal pharmaceutical companies. As Australia is so removed from the broader scientific community it is often difficult to form scientific networks and collaborative projects. The activities undertaken within this trip promoted recent Australian research to the wider international scientific community and aimed to increase scientific networks between Australia and Europe and should also facilitate future project development in the area of livestock parasitology. Subsequently, this trip coupled with my current postdoctoral project will lead to the development of RNAi as a method for target validation that is best suited to application in an industrial environment and thereby maximise the possible uptake of this science by a commercial company. An awareness and understanding of the processes involved in the commercialisation of a therapeutic will also lead to informed and focused research projects in the future that are more likely to result in a commercial product that will benefit the Australian Agricultural Industries.

Milestone 4 – RNAi effects

The focus of this milestone was to select at least five genes and characterise the RNAi phenotype and mRNA transcript profiles for the silenced genes. These genes were analysed in more detail in milestone 6, where the localisation of gene silencing effects was also considered. For this analysis to be informative, it was important to select genes that are known to be silenced by our RNAi assay as well as genes that are known to be unsusceptible to our RNAi assay.

Selection and Cloning of Target Genes

Four genes that were cloned in a previous project (AHW.031) were selected, Nov0374, Nov0491, S3_0055 and S1_452. In addition two genes, Ben-1 and Ubq-1 were selected because successful down-regulation by RNAi has been reported in the literature. This combination of genes (Table 5) will allow the comparison of our methods for a consistent RNAi effect with those already published.

Regions of 250-450 base pairs of gene-specific internal sequence were cloned into an RNAi feeding vector (pL4440). This vector is a modified version of Bluescript with a T7 promoter on each side of the multiple cloning site, driving transcription of each DNA strand. Positive clones were confirmed by sequence analysis. Target clones were transformed into HT115 (DE3), an RNase III-deficient *E. coli* strain with IPTG-inducible T7 polymerase activity. Transformants were prepared as discussed below for feeding to larvae in the RNAi Assay.

Table 5. Summary of *H. contortus* target genes: expected phenotypes are listed based on comparison to RNAi effects with the ortholog in *C. elegans*

H.contortus Target Gene	*C. elegans* Reported Phenotype	*C. elegans* Ortholog	*C. elegans* Localisation
Ben-1	LvL, EmbL, Lva, Rbs	Ben-1, C54C6.2	body muscle
Ubq-1	Lva, Ster, EmbL, Lvl	Ubq-1, F25B5.4	body muscle
Nov0374	EmbL, Gro, Lon, LvL, Unc	Ostb-1, T09A5.11, probable oligosaccharyl transferase	unknown
Nov0491	EmbL, Bmd, Gro, LvL, Clr, Sck	Nmt-1, T17E9, probable N-myristoyl transferase	pharynx, anal depressor muscle, rectal epithelium, reproductive system, body wall muscle, hypodermis and nervous system
S1-452	EmbL, Gro	Eef-1A.1, R03G5, eukaryotic translation elongation factor	pharynx, intestine, body wall muscle
S3_0055	Egl, Slu, Unc, Gro	Unc-78, C04F6.4 putative actin interacting protein	pharynx, body wall muscle, spermatheca and vulva

Abbreviations: Bmd; body morphology defect, Clr; clear, EmbL; embryonic lethal, Gro; slow growth, Lon; long body, Lva; larval arrest, LvL; larval lethal, Rbs; reduced brood size, Sck; sick, Ster; maternal sterility and Unc; uncoordinated.

Expression of Target Genes

The mRNA stage-specific expression profile for each target gene as determined by Quantitative PCR is compiled in Table 6. The amount of target has been normalised to an endogenous housekeeping gene and relative to the calibrator. These profiles indicate the stages that the target gene is expressed and allow an estimation of when the protein is required and consequently when an RNAi effect could be expected.

Table 6. Summary of mRNA expression profiles for *H. contortus* target genes

H. contortus	*H. contortus* mRNA expression profile						
Target Gene	**L2**	**L3**	**xL3**	**eL4**	**eL4bf**	**yAd**	**Ad**
Ben-1	ex	ex	ex	ex	ex	ex	ex
Ubq-1	ex	ex	ex	ex	ex	ex	ex
Nov0374	0.41	6.10	37.78	0.92	1.04	0.39	0.86
Nov0491	0.93	3.73	3.46	1.04	2.77	0.70	3.03
S1-452	0.95	7.11	3.76	1.99	3.25	1.32	0.90
S3_0055	1.96	39.66	2.57	1.95	1.75	0.68	1.08

Abbreviations: L2; second stage larvae, L3; third stage larvae, xL3; exsheathed L3, eL4; early fourth stage larvae, eL4bf; eL4 blood feeding, yAd; young adult and Ad; adult, ex; mRNA expressed but amount not quantified.

The mRNA localisation profile for each target gene was determined by Quantitative PCR (Table 7). The amount of target has been normalised to an endogenous housekeeping gene and relative to the calibrator. These profiles indicate where the target genes are expressed and may provide some insight into the potential for gene silencing following uptake of dsRNA from the environment.

Table 7. Localisation mRNA expression profiles for the RNAi target genes

***H. contortus* Target**	***H. contortus* mRNA expression profile**		
Gene	**Body**	**Ovary**	**Gut**
Hc-ben-1	+++	+++	+++
Hc-ubq-1	+++	+	+++
Hc-Nov0374	+++	++	++
Hc-Nov0491	+++	+++	++
Hc-S1_452	+++	+	+++
Hc-S3_0055	+++	+	+

Abbreviations: mRNA expression levels are indicated by, +; low, ++; moderate, +++; high.

RNAi in the free-living stages

The RNAi feeding method was used to introduce dsRNA to the larvae in a larval development assay. L1 are exposed to the internally expressed dsRNA through ingestion and digestion of recombinant *E coli*.

H. contortus eggs: A benzimidazole-resistant strain of *H. contortus* (Haecon-5) 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 1 month each animal was inoculated with 5,0007,500 *H. contortus* infective third stage larvae (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 µm sieve. Eggs were separated from faecal material by flotation in saturated sugar (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 µm nylon screen. Recovered eggs were washed to remove fine debris and reduce commensal bacteria, mould and fungi. Eggs were sterilised in an antibiotic-antimycotic solution (70 mM NaCl, 2.5 mM KCl, 5 mM glucose, 10 mM Na_2HPO4, 2.5 mM NaH_2PO4, 100/mL U Penicillin, 100 U/mL Streptomycin, 80 µg/mL Ciprofloxacin, 10 µg/mL Amphotericin B, 40 µg/mL Gentamicin, 100 µg/mL Ampicillin) for 5 min to kill remaining commensal bacteria, washed thoroughly to remove residual antibiotics and concentrated by centrifugation at 1,000 g for 5 min. Aliquots were taken to estimate recovery and the concentration adjusted to 15,000 eggs/mL for inclusion in the RNAi assay.

Bacteria: To induce the expression of dsRNA, a single *E. coli* colony was grown overnight at 37°C in LB supplemented with ampicillin (100 µg/ml) and tetracycline (12.5 µg/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 isopropyl-ß-D-thiogalactopyranoside (IPTG) and grown for a further three hours. The cells were pelleted by centrifugation at 2,000 g for 10 min, resuspended in Physiological Saline (PS) (0.85% NaCl) and adjusted to an OD_{600} of 0.2-0.25. *E. coli* expressing dsRNA was either used fresh or stored at 4 °C for subsequent use.

Cultures: Culture Medium (CM) was prepared to set up the assay in culture flasks (12.5 cm2). CM consists of 20 % Nutrient Medium (EBSS and 1 % yeast extract in sPS), eggs (17 %), bacteria (17 %), carbenicillin (100 µg/mL), amphotericin B (2 µg/mL) and 5-fluorocytosine (5 µg/mL) in sPS (46 %). 2.5 mL of the CM was added to each culture flask. The flasks were sealed and incubated at 27°C with 80 % relative humidity for 6 – 10 days. 3 replicate cultures in flasks were set up. Negative controls (no added bacteria) as well as positive controls (*E coli* strain HT115 + plant gene in pL4440) were included in each experiment. The success of egg hatch and development of the L1 through to L3 was monitored. Replicate cultures were harvested on Day 1, 3 & 8. This provided 2 -4,000 larvae to monitor transcript down-regulation by quantitative PCR. Subsamples were taken from these cultures and the larvae photographed in cell inserts. The images were used to differentiate between developmental stages and compare culture success of larvae fed the control or transformed bacteria. This enabled a more detailed examination of any phenotypic changes.

The criteria for an ideal experiment are as follows: An ‘egg hatch’ of 80 % is expected when the culture harvested on Day 1 is examined between 24 and 28 h after incubation. The bacterial lawn should not have progressed beyond light to moderate at this time. By Day 3, the L2 should have completed their moult and be active and growing. At Day 8, the majority of larvae should be active L3, with few L2 still in lethargus. This is the end-point of the experiment in the free-living stages; the larvae will remain at L3 provided that there are sufficient nutrients available in the media. In our experience larvae have been monitored up until 17 days post inoculation, they remained healthy and could be successfully exsheathed to xL3 when the appropriate triggers were provided.

Two experiments were conducted: The negative control had no bacterial lawn and the larvae hatched to L1 but remained small and pale as expected. The plant control gave results as described above for an ideal experiment. No RNAi phenotype was observed for Nov0374 and only slight mortality at L1 for ubq

1. A more dramatic developmental arrest was observed for ben-1 and S3_0055 with approximately 20% sick L1s at day 4 and 20% sick L1s and 20% sick L2s at day 8. Nov0491 had increased mortality at L1 and L2, while S1-452 had developmental arrest at L2 with 40% at day 8. These differential gene silencing patterns are considered in milestone 6, where the localisation of available mRNA transcripts for each gene has been determined by in situ hybridisation.

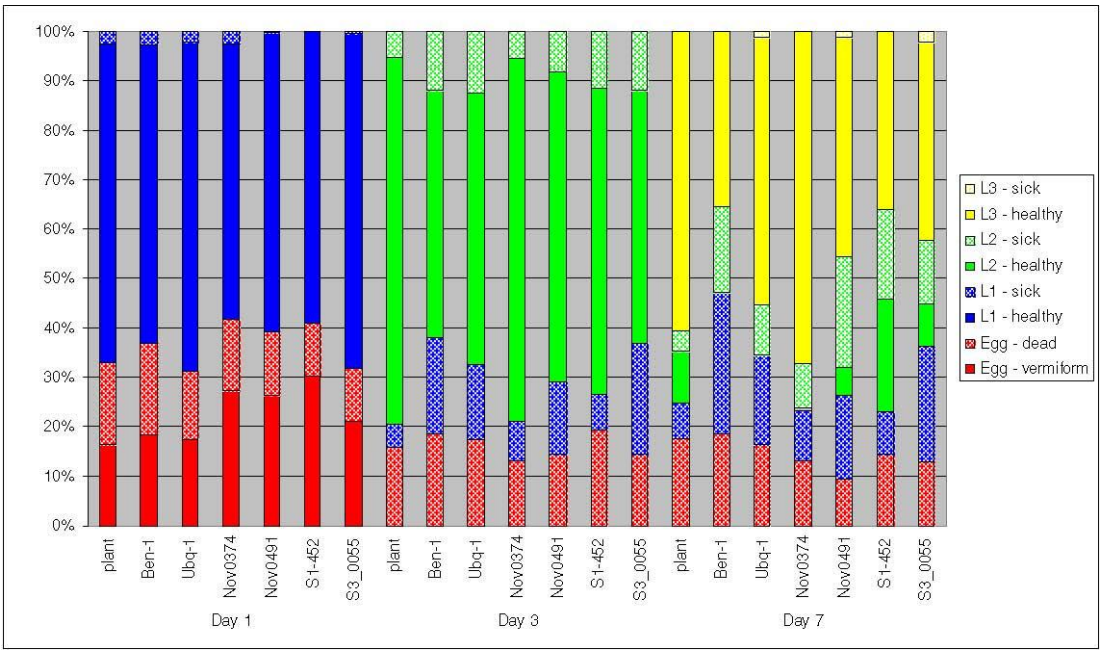

Figure 2. RNAi assay in free-living stages of *H. contortus* fed on *E. coli* expressing gene-specific dsRNA. Two consistent RNAi experiments were performed. All target genes were analysed in 3 replicates in each experiment.

Milestone 5 – Development of scientific capability

The focus of this milestone was to prepare a strategy for the localisation studies required in milestone 6 and to develop the scientific capability to undertake the required experiments. There were two possible strategies that could provide insight into the localisation of the RNAi mechanism in *H. contortus*.

The first would be to determine the localisation patterns of the proteins involved in the RNAi mechanism. This strategy would provide information on whether the mechanism is active organism wide or whether it is restricted to specific areas of the nematode such as the gut. This approach is dependent on the outcomes of milestone 2 and requires the identification of full length genes, cloning, recombinant protein expression, antibody production and *in situ* hybridisation experiments. This approach was our initial choice and we successfully applied to the Animal Ethics Committee for approval to produce antibodies in mice, we prepared the facility to undertake this work and developed protocols to perform this work. Unfortunately this approach was not possible due to the current limitations in available *H. contortus* sequence combined with the divergent gene sequences which enabled us to identify partial genes for the proteins involved in the RNAi mechanism but not full length genes. As such we have pursued our second strategy.

The alternative localisation strategy would be to undertake *in situ* hybridisation experiments with DNA or RNA probes. This technique can be used to detect and localise specific mRNAs within the nematode and define the patterns of gene expression. This technique does not require the full length gene sequence and therefore can be utilised in this project with the current amount of sequence information that we have obtained in the previous milestones. For this approach to be informative we will not look at the genes involved in the RNAi mechanism (milestone 2), but rather the target genes that were studied in the RNAi assay (milestone 4) as they will provide information on exactly where within the nematode the RNAi mechanism is actually able to act successfully. This approach requires probe design, probe labeling, nematode collection, preservation, sectioning, *in situ* hybridisation experiments and microscopy. In addition, as this approach does not require the production of antibodies it removes the need to undertake animal

experimentation. Ideally it would be most informative to compare the localisation patterns for these genes in nematodes with and without RNAi-induced gene silencing. However, this would require us to embed and section L1, L2 and L3 nematodes which are only up to ~ 680 µm in length. Adult nematodes are much larger with males between 5 and 5.5 mm in length and ~ 90 µm thick and females between 6.5 and 7.9 mm in length and ~ 90-130 µm thick. Although the adults are much larger than the larval stages they are still very difficult to manipulate. All of the gene targets are expressed in both the larval stages and the adult (Table 6); therefore, we decided to undertake localisation studies in the adult with the assumption that the expression profiles would be the same in the larval stages. Once the system was optimised and proven to be robust it may be possible to attempt to embed and section the larval stages following RNAi treatment; however this would not be feasible within the constraints of this project.

Milestone 6 – Localisation of RNAi

Differential gene silencing patterns were observed for the six target genes in milestone 4. It was hypothesised that differences in mRNA transcript localisation for each gene within the nematode may correlate with the amenability of the RNAi mechanism and consequently the effectiveness of gene silencing. From the bioinformatic study undertaken in milestone 2, it is possible that the RNAi transport mechanism in *H. contortus* differs from that in *C. elegans* as evidenced by the inability to find orthologues for *sid-2* and *rsd-6*. If the systemic transport of dsRNA is limited then it could be expected that effective RNAi may only occur for genes that are localised in areas that can be silenced during the uptake of environmental dsRNA by SID-1 such as the intestine, body wall, reproductive organs and excretory organs. In this milestone, mRNA transcript localisation patterns have been determined for the six target genes by *in situ* hybridisation and any correlations considered.

Oligonucleotide probes were designed for the six target genes (ben-1, ubq-1, Nov0374, Nov0491, S1_452 and S3_0055) (Table 7). Probes were labeled using a digoxigenin (DIG) oligonucleotide tailing system (Roche) under conditions specified by the manufacturer. Following the labelling reaction, DIG-probes were resuspended in DEPC-treated water to a final concentration of 1.25 pmoles/µl. and incorporation of label and sensitivity levels confirmed.

Table 7. Probe sequences for RNAi target genes to be used in *in situ* hybridisation experiments.

Probe name	Sequence (5' _ 3')
Hc-ben-1	GACCCGCCTTTTCGACAGGTATGCCAGGTCTTGCTTGCCTCAGGTAGC
Hc-ubq-1	CGCTTCTAGGTTCTGTTCCTTCCTTAGGGGGTCTGGTCGTTTCCGAGT
Hc-Nov0374	ATGAGCCAAACAGCTACTTCCTCCATTAGATAACCACCGACCTCCGAG
Hc-Nov0491	GGTAACACCGTCGGTAGGTCCGTAAACGTGTCGGGTGAAGTTCTTTCGA
Hc-S1_452	CCTATGTTAGGGTTCCGCCAGCGTAAGCAGGGATAAAGACCGAAG
Hc-S3_0055	CGAAGTGTAAACGTAGGACAGGTTGTGGACTCGTCGGAGTAGTCGTGG

Parasite material was collected and prepared for sectioning as follows. Merino sheep (6–12 months of age) were inoculated orally with 5000–7500 *H. contortus* L3, humanely killed 28–35 days p.i. and the nematodes recovered. The mature parasites (mixed population of female and male) were collected from the abomasal content in warm (37°C) PBS and washed several times in warm PBS to free them from plant debris.

For cryostat embedding: Nematodes were then placed in cold PBS to relax, 100 were aligned side-byside, in a cryomould containing optimum cutting temperature (OCT) freezing medium and snap frozen by floating the cryomould on liquid nitrogen, then stored at −70°C in airtight containers. Immediately prior to probing, blocks were removed from the mould, mounted in a cryostat and allowed to warm to −20°C before cutting 8 µm sections. Each section was collected on a poly- -lysine coated slide, airdried at room temperature, then fixed in 4% (v/v) paraformaldehyde [prepared in diethyl pyrocarbonate (DEPC)-treated PBS] for 30 min at 4°C, and then washed gently in DEPC-treated PBS, twice for 5 min.

Several labelling experiments were performed using cryostat sections; however numerous difficulties were encountered with this approach. Although we were eventually able to obtain reasonable sections, they would detach from the slides during the labelling process. We attempted to optimise this process; however with tight time constraints this was put on hold. Paraffin embedding was then trialled as a more robust system.

For paraffin embedding: Nematodes were then placed in cold PBS to relax, 40-80 of mixed sex were placed in histology cassettes with pre-soaked sample sponges and immersed in cold PBS. Cassettes were then rinsed in 25% Histochoice fixative (sigma), 2% DMSO, 20% ethanol [prepared in DEPCtreated PBS] then soaked for 3 days at 4°C. Cassettes were then washed in cold DEPC-treated PBS overnight at 4°C and then twice for 2 hours at 4°C with gentle rocking. Nematodes were then embedded into paraffin blocks and 3 µm sections cut using a microtome. Each section was collected on a poly- -lysine coated slide, air-dried at room temperature, then de-paraffinated in xylene twice for 5 mins followed by rehydration in decreasing concentrations of ethanol in DEPC-treated PBS (99%, 95%, 70%, 50%, 30%), and then washed gently in DEPC-treated PBS, twice for 5 min.

Several labelling experiments were performed using paraffin sections with much better results.

Sections (cryostat or paraffin) were prehybridised in 100 µl of hybridisation solution [DIG Easy Hyb (Roche)], covered with a plastic coverslip, and incubated at 42°C for 2 h. The prehybridisation solution was drained from the sections, and 100 µl of hybridisation solution (containing 5 pmols probe) added directly to the slides. Sections were covered with a plastic coverslip, and hybridised at 42°C overnight. Following hybridisation, sections were washed in 1×SSC, once for 5 min and then twice for 10 min at 30°C. The detection of bound probe was performed using anti-DIG antibody conjugated to alkaline phosphatase under the conditions specified by the manufacturer (Roche). Slides were counterstained with Bismark Brown, mounted and allowed to dry at room temperature overnight.

Images were captured on an Olympus CKX41 microscope with a blue filter using SPOT4.6 software (Figure 3).

Our findings are summarised as follows:

Nov0374: For Nov0374 silencing was not observed in the RNAi assay. Signal was detected in the intestine and the muscle cells lining the cuticle in *in situ* experiments. This localisation profile should be amenable to silencing by RNAi as the transcript should be reached during environmental uptake without additional transport.

Ubq-1: Ubq-1 showed very low levels of silencing in the RNAi assay. Signal was detected in the body wall muscle in *in situ* experiments.

S3_0055: S3_0055 showed partial silencing in the RNAi assay. Signal was detected in the body wall muscle in *in situ* experiments. This result is in contrast to the results obtained for *ubq-1* which has the same transcript expression profile but only low levels of silencing.

Ben-1: Ben-1 showed partial silencing in the RNAi assay. In the *in situ* hybridisation experiments signal was detected in the muscle cells lining the cuticle, the body wall

muscle and the ovaries. It is possible that this wide distribution of transcript is responsible for incomplete silencing if the systemic transport of dsRNA is not fully functional.

Nov0491: Nov0491 showed reasonable silencing in the RNAi assay. Signal was detected in most body parts except for the intestine. The effective silencing of this gene suggests that the systemic transport of dsRNA is functional. This is in contrast to the result obtained for *ben-1* which is distributed widely but not effectively silenced.

S1_452: S1_452 showed the highest level of silencing in the RNAi assay. Signal was detected in the intestine, body wall muscle and the muscle cells lining the cuticle.

Taken together, these results do not show any correlation between the localisation of specific gene transcripts with the amenability of that gene to silencing by RNAi in *H. contortus*. It remains possible that the ability to silence some genes but not others by RNAi could be attributed to differences in the rates of protein turnover. This is outside the scope of this project and further studies would be required to investigate this possibility.

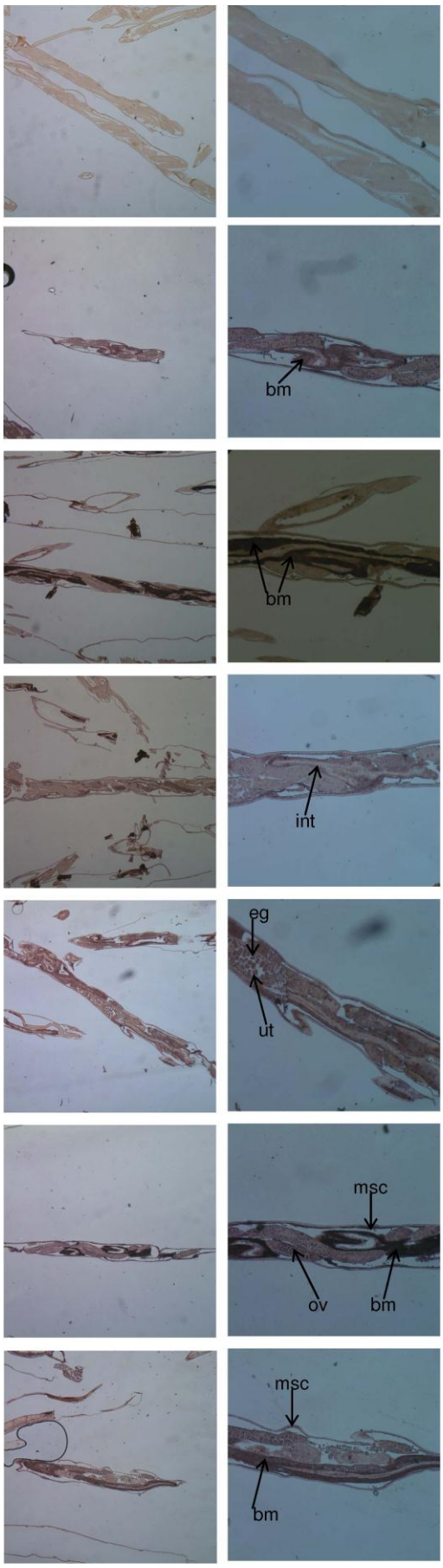

Figure 3. In situ localisation of mRNA transcripts for the genes investigated in the RNAi assay.
The morphology of nematode sections analysed consist of intestine (int); intestinal microvilli (mv); female reproductive organs comprising ovaries (ov) and uterus (ut) with eggs (eg); the outer cuticle (cu) with underlying platymyrian muscle cells (msc) and body muscle (bm). Panel 1 at x40 and panel 2 at x100 magnification.

3. Success in achieving objectives and impact on meat and livestock industry

The major outcome of this project is progress towards the characterisation of the RNAi mechanism in *H. contortus* and further justification of the potential of RNAi as a validation tool in parasitic nematodes. RNAi can be utilised for the validation of target genes from *H. contortus* target identification projects that could then move forward into medium throughput screening. When utilising this silencing approach it will be important to consider the localisation of the specific target gene. Further work into the correlation of protein turnover rates with effective silencing would be warranted.

Our compiled sequence library (extensive in-house Novartis EST collection, 454 sequence as well as all publically available genome, EST and 454 sequences) provides a strategic resource that will benefit discovery efforts in parasitic nematodes. This resource will be of great value because it will facilitate the cloning of full-length genes and the expression of recombinant proteins. This genomic information together with our procedure for RNAi will provide the ideal set up for target discovery, validation and drug development and may enable identification of new drug/vaccine targets in future projects.

An additional outcome of this project was the development of scientific leadership qualities and collaborative networks. At the completion of this project I am recognised both nationally and internationally as a leading Molecular Parasitologist. I have served on the Committee of the Australian Society for Parasitology as the Executive Secretary and the Victorian Representative and the Committee of Management for the ARC, NHMRC Network for Parasitology. I collaborate formally with Carolyn Behm from the Australian National University, Canberra and Andrew Kotze from CSIRO Livestock Industries, Queensland. I am currently developing a collaboration with Robin Gasser from the University of Melbourne, Victoria and have begun discussions with Kita Kiyoshi from the University of Tokyo, Japan. Following this Fellowship I am well positioned to continue scientific discovery in the field of Animal Health, particularly with a focus on Molecular Parasitology.

4. References

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