Characterisation of the *Fasciola hepatica* miRNome and an evaluation of its role in the host-parasite relationship

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Doctor of Philosophy

under the supervision of

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Certificate of original authorship

I, Alison Mae Ricafrente declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Sciences, Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by the Australian Government Research Training Program.

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Supplementary Material

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Abbreviations

Abbreviation	Term
AcCoA	Acetyl-CoA
Ad	Adult
AFBI	Agri-Food Biosciences Institute
AGO	Argonaute
ANOVA	Analysis of variance
ASCT	Acetate:succinate CoA transferase
ATP	Adenosine triphosphate
BAN	4-Bromoanisole
BCL10	B-cell lymphoma/leukemia 10 signaling adaptor
BMDM	Bone marrow-derived macrophages
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
cDNA	Complimentary DNA
CITR	Citrate
CoA	Coenzyme A
CPM	Counts per million
CREB1	CAMP responsive element binding protein 1
DAVID	Database for Annotation, Visualization and Integrated Discovery
DC	Dendritic cell
DGCR8	DiGeorge syndrome critical region 8 gene
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
dpi	Days post-infection
ELISA	Enzyme linked immunosorbent assay
EV	Extracellular vesicle
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit
FBP	Fructose 1,6-bisphosphate
FBS	Foetal bovine serum
FC	Fold change
FEST	Fluke egg sedimentation test
FhCL	Fasciola cathepsin
FP6	Fructose 6-phosphate
FRD	Fumarate reductase
FUM	Fumarate
G1P	Glucose 1-phosphate
GM-CSF	Granulocyte macrophage colony stimulating factor receptor
GO	Gene ontology
GP6	Glucose 6-phosphate
h	Hour(s)
HDAC	Histone deacetylase

Abbreviation	Term
HSP	Heat shock protein
HSPA4	HSP Family A Member 4
IFN	Interferon
IKZF3	Ikaros Family Zinc Finger 3
IL	Interleukin
ILC	Innate lymphoid cell
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
ITS2	Internal transcribed spacer 2
JAK1	Janus kinase 1
JUV	Juvenile/Immature
KEGG	Kyoto encyclopaedia of genes and genomes
LCP1	Lymphocyte cytosolic protein 1
LPS	Lipopolysaccharide
MAL	Malate
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
Methymal-CoA	Methylmalonyl-CoA
MFE	Minimum free energy
MHC	Major histocompatibility complex
miRISC	miRNA induced silencing complex
miRNA	MicroRNA
mRNA	Messenger RNA
NEJ	Newly excysted juvenile
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells.
NO	Nitric oxide
NOD	Non-obese diabetic
nt	Nucleotide
NTC	Non- template control
OXAC	Oxaloacetate
PBS	Phosphate buffer solution
PCA	Principal components analysis
PCR	Polymerase chain reaction
PEP	phosphoenolpyruvate
pi	Post-infection
PoC	Point-of-care
PRDM1	Positive regulatory domain I-binding factor 1
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
PRKCB	Protein kinase C beta
PROP	Propionate

Abbreviation	Term
Prop-CoA	Propionyl-CoA
PTEN	Phosphatase and tensin homolog
PYR	pyruvate
RAD50	Double strand break repair protein
RELA	REL proto-oncogene, NF-κB subunit
RMPI	Roswell Park Memorial Institute
RNA	Ribonucleic acid
RT	Reverse transcription
RT-qPCR	Reverse transcription-quantitative PCR
RXRA	Retinoid X Receptor Alpha
SAC	Spindle assembly check point
SAP	Sin3A Associated Protein
SD	Standard deviation
SDH	succinate dehydrogenase
SOAP	Short oligonucleotide alignment program
SP1	Specificity protein 1
STAT	Signal transducer and activator of transcription
SUCC	Succinate
Succ-CoA	Succinyl-CoA
TCA	The citric acid
TCBZ	Triclabendazole
TDE	Thermodynamic ensemble
Th1/2	T helper 1 /2
TLR	Toll like receptor
TNF	Tumor necrosis factor
TPM	Transcripts per million
UDP-G	Uridine biphosphate glucose
Uninf	Uninfected
UTR	Untranslated region
UTS	University of Technology Sydney
w	Week(s)
WHO	World Health Organisation
wpi	Weeks post-infection
XPO	Exportin

Abstract

The liver fluke, *Fasciola hepatica*, is recognised as one of the most successful parasites worldwide due to its remarkable capacity to infect every mammal it encounters. For this reason, liver fluke disease, or fasciolosis, has the widest geographical spread of any parasite disease and contributes to significant animal loss, particularly within the agricultural sector. Since the discovery of the post-transcriptional regulation of genes by micro(mi)RNA in the free-living worm *Caenorhabditis elegans*, a myriad of processes within worm biology are now linked to miRNAs. These concepts have catalysed interest in the contribution that miRNAs have on the dynamic shifts of the *F. hepatica* transcriptome and parasite survival within the host.

In Chapter 1, the miRnome assemblies of early miRNA discovery projects were compared to determine knowledge to date. Examination of 38 miRbase miRNAs revealed that the revised miRNome was highly associated to the regulation of inflammatory events and innate mechanisms of pathogen recognition and expulsion by the host. These preliminary explorations were experimentally challenged in Chapter 2. Sequencing of miRNAs isolated from the peritoneal macrophages of *F. hepatica* infected mice revealed that specific Fasciola miRNAs were internalised by host macrophages. In particular, fhe-miR-125b was uncovered as a potent immune regulator due to its capacity to suppress the expression of a central signal transduction molecule Traf6 within the host, after functionalisation by mammalian Ago.

The realisation of the complete *F. hepatica* miRnome in Chapter 3 expanded the number of *F. hepatica* miRNAs to 124 within intra-mammalian life stages; newly excysted juveniles (NEJs), immature and adult fluke, exposing a wider collection of isomiRs, life stage specific novel miRNAs and genomic clustering. By integrating the life stage miRnomes with their predicted targets within the transcriptomes of each life stage identified the key biological processes in metabolism, parasitism, and growth that were systematically targeted during parasite development within the host. With the expanded miRnome, the utility of parasite miRNAs as biomarkers of fasciolosis was explored, with diagnostic capabilities examined through RT-qPCR analysis of sera from infected sheep (Chapter 4).

The collective outcomes of this research project have fostered new perspectives in *F. hepatica* research. These include, evolving methods of miRNA discovery; re-thinking the biogenesis of microRNAs; mapping the molecular events of parasite development; unveiling new mechanisms of host-parasite interplay, and advancing diagnostic techniques.

Chapter 1:

An evaluation of the *Fasciola hepatica* miRNome predicts a targeted regulation of mammalian innate immune responses

1.1. Introductory Statement

The work presented in this chapter significantly contributed to the publication of a Hypothesis and Theory Article:

Ricafrente A, Nguyen H, Tran N, Donnelly S. An evaluation of the *Fasciola hepatica* miRnome predicts a targeted regulation of mammalian innate immune responses. ***Frontiers in Immunology***. 2021 Jan 29;11:3623.

1.2. Abstract

Understanding mechanisms by which parasitic worms (helminths) control their hosts' immune responses is critical to the development of effective new disease interventions. *Fasciola hepatica*, a global scourge of humans and their livestock, suppresses host innate immune responses within hours of infection, ensuring that host protective responses are quickly incapacitated. This allows the parasite to freely migrate from the intestine, through the liver to ultimately reside in the bile duct, where the parasite establishes a chronic infection that is largely tolerated by the host. The recent identification of micro(mi)RNA, small RNAs that regulate gene expression, within the extracellular vesicles secreted by helminths suggest that these non-coding RNAs may have a role in the parasite-host interplay. To date, 77 miRNAs have been identified in *F. hepatica* comprising primarily of ancient conserved species of miRNAs. We hypothesized that many of these miRNAs are utilised by the parasite to regulate host immune signalling pathways. To test this theory, we first compiled all of the known published *F. hepatica* miRNAs and critically curated their sequences and annotations. Then with a focus on the miRNAs expressed by the juvenile worms, we predicted gene targets within human innate immune cells. This approach revealed the existence of targets within every immune cell, providing evidence for the universal management of host immunology by this parasite. Notably, there was a high degree of redundancy in the potential for the parasite to regulate the activation of dendritic cells, eosinophils and neutrophils, with multiple miRNAs predicted to act on singular gene targets within these cells. This original exploration of the Fasciola miRnome offers the first molecular insight into mechanisms by which *F. hepatica* can regulate the host protective immune response.

1.3. Introduction

Fasciolosis is a major production limiting disease of ruminant livestock globally [(1)]. Infection with *F. hepatica*, the liver fluke parasite, results in substantial delays in animals reaching slaughter weight with increased levels of worm burden in the liver directly correlating with reduced growth rates of animals [(2)]. The impact of infection on the production of meat, wool and milk is estimated to result in economic losses over US$3.2 billion annually [(3)]. Due to the close proximity of people with their livestock, humans are incidental hosts and fasciolosis is now recognised as an emerging human disease. The World Health Organisation has estimated that at least 2.4 million people are infected in more than 70 countries worldwide, with several millions at risk, and have thus classified liver fluke infection as one of the food-borne trematode priority diseases [(4)]. Despite this status, the only option to treat the infection is Triclabendazole and although effective at reducing worm burden, it does not prevent re-infection. Furthermore, over reliance on this single drug and its frequent widespread use has resulted in the emergence of resistant flukes [(5)]. The global scale of *F. hepatica* infection, combined with limited treatment options, raises an urgent need to develop novel control strategies. To achieve this, a deeper understanding of the parasite's mechanisms of invasion and colonisation are necessary.

1.4. *Fasciola hepatica* manipulates the host immune response to support successful invasion

All mammalian hosts of *F. hepatica* become infected by ingestion of vegetation that is contaminated with the encysted dormant larvae (metacercariae). In the duodenum, the newly excysted juveniles (NEJ) emerge and penetrate the intestinal epithelium to migrate through the peritoneal cavity to reach the liver. Within the liver these parasites spend many weeks feeding on tissue and blood to mature, after which, they migrate to the bile duct, where they take up residence, often for decades, producing thousands of eggs, which are excreted from their mammalian host to continue their life cycle [(6)].

In naturally infected animals, there is no evidence of the typical host protective, pro-inflammatory Th1 type, immune response that would be expected in response to infection with a pathogen [(7)]. Instead, the *Fasciola*-specific immune response is predominantly Th2, which becomes more potent as the parasite migrates through the liver. Once the worm is established in the bile duct and the infection becomes chronic, the parasite-specific immune response switches to a combination of regulatory T cells and anergic effector T cells [(8-12)]. Notably,

vaccine trials have shown that when a parasite-specific Th1 response is activated, significant levels of protection against infection are achieved [13, 14]. Collectively, these observations suggest that by inhibiting the immediate host protective immune response, the parasite ensures survival of the NEJs, permitting their safe passage from the intestine, across the peritoneal cavity and on to the liver, at which point the host response switches to a Th2 phenotype to mediate tissue repair mechanisms. Indeed, in mice deficient in Th2 immunity, worm burden and size were unaffected, suggesting there was no impact to the maturation of the parasite. However, the infected mice displayed significantly more damage to liver tissue and succumbed to premature deaths [15].

While the host innate immune response is activated by the presence of the parasite, resulting in an influx of dendritic cells (DC), eosinophils, neutrophils and macrophages to the peritoneal cavity immediately after infection [16, 17], evidence suggests that the anti-pathogenic, pro-inflammatory activities of these immune cells are inhibited by the NEJs. The DCs display low expression of CD80, CD40, MHC class II, and CD86 and high expression of CCR5 [17, 18], a phenotype that is indicative of an immature DC. Functionally, these DCs, are unable to promote the differentiation of Th1 cells, and instead induce the expansion of anergic T cells [17]. Similarly, macrophages have a low expression of MHC-II and are impaired in their ability to produce pro-inflammatory mediators such as iNOS and TNF, instead adopting a regulatory profile by secreting IL-10 and TGFβ [17, 19, 20]. Although not demonstrated *in vivo*, the exposure of bovine neutrophils to the intra-mammalian life stages of *F. hepatica in vitro*, failed to induce significant production of reactive oxygen species or NETosis, suggesting that the parasite was impairing the antimicrobial activities of neutrophils [21]). Likewise, there is no evidence of eosinophil degranulation in the peritoneal cavity, which indicates that these cells are not activated or have an alternative phenotype that is not contributing to parasite killing [16].

Understanding the mechanisms that *F. hepatica* employs to disarm the host's response during the early stages of infection offers the opportunity to counteract these strategies to target the NEJs, which would prevent penetration of the liver capsule and thus the disease pathology. While research to date has largely focused on the characterisation of immune modulating proteins/glycans secreted by helminths during infection (reviewed in [22], [23]), it has recently become apparent that parasitic worms also actively release micro(mi)RNAs which may have a role in the regulation of host immune cells.

1.5. Helminth-derived miRNAs regulate mammalian gene expression to modulate host immune responses

MicroRNAs are small (~22 nucleotides long) non-coding RNA[24], that function to regulate gene expression at the posttranscriptional level through specific binding and subsequent silencing of target messenger RNA (mRNA; Figure 1.1). Target recognition is a highly specific process with complementary binding between the seed region (2-8 nt) at the 5' end of the miRNA and the 3' untranslated region (UTR) of the target mRNA [25]. This binding activity eventually leads to either inhibition of the initiation step of translation or promotes mRNA decay through the deadenylation of the poly(A)-tail of the mRNA target [25]. With many hundreds of human miRNAs identified to date, it is not surprising that miRNAs are recognised to take part in virtually every biological process [26]. The first indication that miRNAs were involved in the regulation of immune responses, emerged from a study in 2004, which demonstrated the selective expression of a small number of miRNAs in immune cells [27]. Since then, numerous miRNAs have been characterised as having roles in the regulation of both innate and adaptive immune responses, in which they control the development of immune cell progenitors, maintenance and differentiation and mature immune cell function (reviewed in [28]).

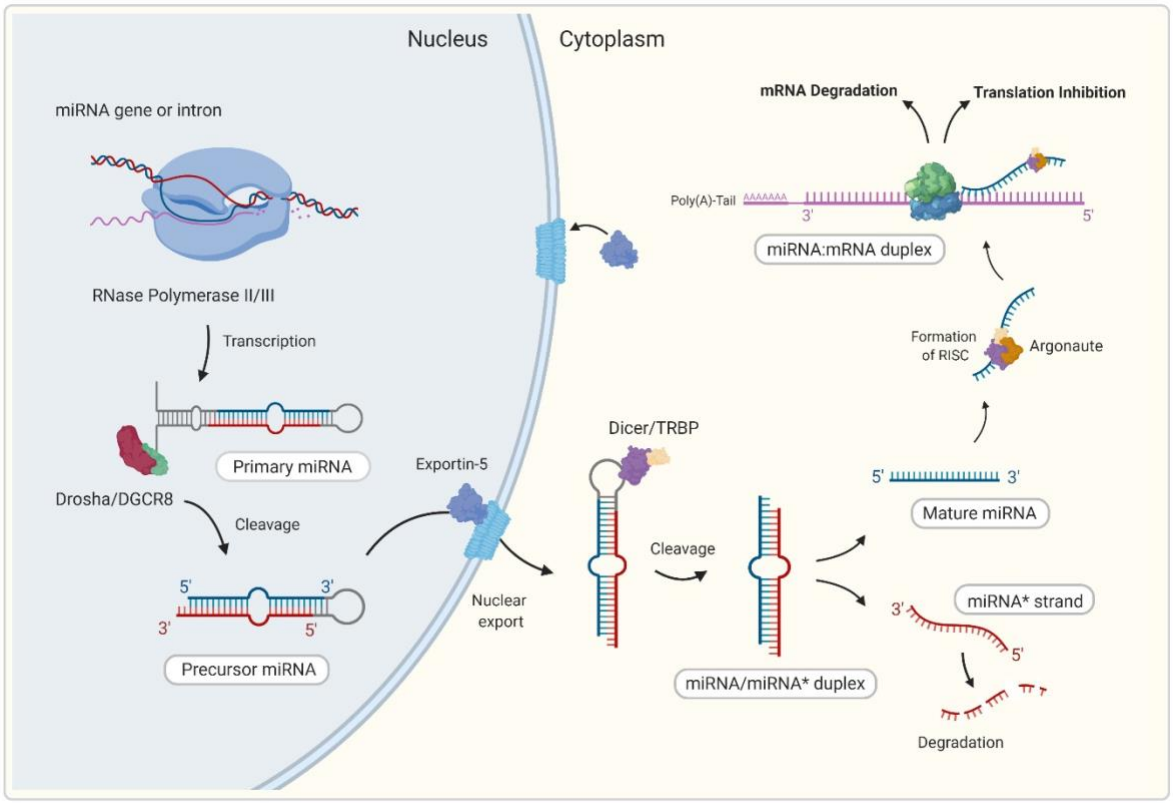


Figure 1.1. The canonical biogenesis of mammalian mature microRNAs. Generally, through canonical processes miRNAs are first transcribed in the nucleus by RNase polymerase II (Pol-II) to form a stem loop structure that is the primary miRNA (pri-miRNA). Similar to mRNAs, pri-miRNAs present a 5' 7-methylguanosine (m7G)-cap and polyadenylated 3' end. The pri-miRNA is then cropped by RNase III Drosha and the double stranded RNA-binding protein DGCR8 (DiGeorge syndrome critical region 8 gene) to form the precursor miRNA (pre-miRNA), which is then exported to the cytoplasm by Exportin-5 (XPO5). In the cytoplasm, the stem loop of the pre-miRNA is cleaved by RNase III Dicer to form a short miRNA duplex (miRNA/miRNA*) comprising of the guide strand and the passenger strand (miRNA*). The miRNA* is degraded while the guide strand is stably loaded onto an Argonaute protein (AGO) which forms the core of the miRNA induced silencing complex (miRISC). Once bound to the miRISC, the mature miRNA finally has the capacity to bind to a target mRNA and form a miRNA/mRNA duplex. Image created by Biorender.

Importantly, miRNAs are highly conserved through metazoan evolution and are thus considered to be a vital ancient component of gene regulation [29, 30]. Comparative analyses of parasite genomes revealed that a number of helminth miRNAs are widely conserved across diverse organisms and share sequence identity with mammalian species known to have an immune regulatory role [31, 32]. These observations led to the suggestion that parasite-derived miRNAs could target mammalian genes within the immune cells of their hosts to modulate immune responses [33]. Further support for this hypothesis was provided with the discovery that miRNAs, encapsulated in extracellular vesicles (EVs), secreted by parasitic worms can be delivered to host immune cells [34, 35]. While definitive proof for worm-derived miRNAs acting on host cells *in vivo* remains to be obtained, enticing evidence has been provided by *in vitro* studies [34, 36]. Of particular relevance to *F. hepatica*, it has been recently reported that EVs, derived from the closely related trematode worm *Schistosoma japonicum,* were internalised by murine macrophages resulting in the release of parasite-derived miRNAs intracellularly. One of these schistosome miRNAs, *sja-miR-125b,* incorporated into the host AGO protein resulting in the regulation of the host Toll Like Receptor (TLR) signalling pathway, which consequently modulated the production of cytokines by the macrophages [36]. Despite this growing evidence for a functional role for helminth miRNAs in the host-parasite relationship, proper characterisation of the complete miRNomes of these pathogens is lacking and very little exploration of their putative role in host immune modulation has been performed.

1.6. An evaluation of the *Fasciola hepatica* miRNome identified to date

Currently, the identification of helminth microRNAs is performed from sequencing reads by applying various algorithms based on sequence structure, evolutionary conservation, thermodynamic stability, and machine learning. Consequently, the output from every discovery pipeline is highly dependent on specific input requirements critical to producing reliable miRNA candidates.

The characterisation of *F. hepatica* miRNAs has been reported across three primary discovery projects and was achieved using three distinct pipelines mandated by the availability of the *F. hepatica* genome, the sequencing input, and the use of different analytical tools. Initial explorations by Xu et al. [37], employed strategies that compensated for the absence of an assembled *F. hepatica* genome. The availability of a reference genome is required for predicting the candidate pre-miRNAs (characterised by complementary sequences separated

by a hairpin loop). These pre-miRNAs give rise to the mature miRNAs that partake in gene regulation. Therefore, without a reference genome, the ability to predict miRNAs from sequencing data is diminished. To fill this gap, Xu et al., utilised the genome of *S. japonicum* in combination with the Short Oligonucleotide Alignment Program (SOAP) to map putative miRNAs within the RNA sequencing data obtained from adult *F. hepatica*. This approach produced the initial repertoire of 16 *F. hepatica* miRNAs. Matching these miRNA candidates with the known miRNAs of *S. japonicum* from miRBase (version 16.0) revealed that 8 were conserved miRNAs between the trematode species (Tables 1.1 & 1.2), which suggested that the remaining 8 miRNAs were unique to *F. hepatica*.

The subsequent study by Fromm et al. [38], also worked with the lack of an available *F. hepatica* genome, and instead utilised the miRCandRef tool to develop assembled contigs from *F. hepatica* genomic data (as part of the 50 Helminth Genomes Initiative) to use as the reference genome. This study used a modified version of the miRDeep2 algorithm to permit a higher sensitivity for predicting miRNA loci within the assembled contigs. Using this approach, the sequencing data from the initial study by Xu et al. was re-analysed in addition to the sequenced miRNA content of extracellular vesicles (EVs) isolated from adult liver fluke. This analysis produced an expanded list of 55 miRNAs, all of which were found in both the adult fluke and the EVs. This list included the eight conserved miRNAs identified by the earlier study (Table 1.1 & 1.2). However, the 8 novel miRNAs proposed in that study were not found by Fromm et al., suggesting that in fact, they may not be *bone fide* miRNAs and thus we propose should be removed from the listed miRNAs for *F. hepatica*.

Table 1.1 Comparison of miRBase *Fasciola hepatica* mature miRNAs to published sequences.

miR BasemiRNA	Mature miRNASequence on miRBase	Other Proposed Annotation	Other Proposed Mature miRNA Sequence	Study					Sample
				Xu	Fontenla	Fromm 2015	Fromm 2017	Ovchinnikov	
fhe-bantam	TGAGATCGCGATTAAAGCTGGT	Fhe-Bantam		+	+	+	+	+	NEJ, Ad, EV
fhe-miR-2b	GTATCACAGCCCTGCTTGGGACA	miR-2b-3p	_TATCACAGCCCTGCTTGGGACAC	+	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-2-P2b	_TATCACAGCCCTGCTTGGGACACA						
		Fhe-Mir-2-P2c							
fhe-miR-2e	GTATCACAGTCCAAGCTTTGG	miR-2e-3p	_TATCACAGTCCAAGCTTTGG	+	+	+	+	+	NEJ, Ad, EV
		fhe-mir-2c-A	_TATCACAGTCCAAGCTTTGGT						
		Fhe-Mir-2-P3b	_TATCACAGTCCAAGCTTTGGTAAA						
fhe-miR-10	AACCCTGTAGACCCGAGTTTGCA	miR-10-5p	AACCCTGTAGACCCGAGTTTG:	+	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-10-P1	AACCCTGTAGACCCGAGTTTGC_						
fhe-miR-71a	TGAAAGACGATGGTAGTGAGATG	Fhe-Mir-71-P1b	TGAAAGACGATGGTAGTGAGAT_	+	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-71-P1b							
fhe-miR-71b	TGAAAGACTTGAGTAGTGAG	Fhe-Mir-71-P1a	TGAAAGACTTGAGTAGTGAGACG	+	+	+	+	+	NEJ, Ad, EV
fhe-miR-124	TTAAGGCACGCGGTGAATGTCA	Fhe-Mir-124	_TAAGGCACGCGGTGAATGTCA	+	+	+	+	+	NEJ, Ad, EV
fhe-miR-2a	GTCACAGCCAGAATTGATGAACG	Fhe-Mir-2-P1b	_TCACAGCCAGAATTGATGAACG	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-2c	ATATCACAGCCGTGCTTAAGGGCT	Fhe-Mir-2-P3a	_TATCACAGCCGTGCTTAAGGGCTT	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-2d	GTATCACAGTCCTGCTTAGGTG	Fhe-Mir-2-P2a	_TATCACAGTCCTGCTTAGGTGACGA	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-2f	GTCACAGCCAATATTGATGCC	Fhe-Mir-2-P1a	_TCACAGCCAATATTGATGCCTG	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-7	TGGAAGACTGGTGATATGTTGTT	Fhe-Mir-7-P1		–	+	+	+	+	NEJ, Ad, EV
fhe-miR-8	CTAATACTGTTTGGTAAAGATGCC	Fhe-Mir-8	_TAATACTGTTTGGTAAAGATGCC	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-9	TCTTTGGTTATCAAGCAGTATG	Fhe-Mir-9	TCTTTGGTTATCAAGCAGTATGA	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-31	TGGCAAGATTATGGCGAAGCTGA	Fhe-Mir-31		–	+	+	+	+	NEJ, Ad, EV
fhe-miR-36a	GTCACCGGGTAGACATTCATTCAC	Fhe-Mir-36-P1	_TCACCGGGTAGACATTCATTCAC	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-61	ATGACTAGAAAGTGCACTCACTT	Fhe-Mir-279	_TGACTAGAAAGTGCACTCACTTC	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-87	GGTGAGCAAAGTTTCAGGTGTGA	Fhe-Mir-87	_GTGAGCAAAGTTTCAGGTGTGA	–	+	+	+	+	NEJ, Ad, EV
fhe-mir-96	CTTGGCACTTTGGAATTGTCA	Fhe-Mir-96	CTTGGCACTTTGGAATTGTCAC	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-125a	TCCCTGAGACCCTAGAGTTTC	Fhe-Mir-10-P2b	TCCCTGAGACCCTAGAGTTTCC	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-125b	CCCCTGAGACTGATAATTGCTC	Fhe-Mir-10-P2a	CCCCTGAGACTGATAATTGCT_	–	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-10-P2a	CCCCTGAGACTGATAATTGCTCC						
fhe-miR-190	AGATATGTTTGGGTTACTTGGTG	Fhe-Mir-190-P1		–	+	+	+	+	NEJ, Ad, EV
fhe-miR-219	TGATTGTCCATTCGCATTTCTTG	Fhe-Mir-219	TGATTGTCCATTCGCATTTCTT_	–	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-219							

fhe-miR-277	GTAAATGCATTTTCTGGCCCG	Fhe-Mir-277-P1	_TAAATGCATTTTCTGGCCCGTAA	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-745b	GAAAGCTGCCAAGCGAAGGGC	Fhe-Mir-22-P2	:AAGCTGCCAAGCGAAGGGCCAA	–	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-22-P2	:AAGCTGCCAAGCGAAGGGCCAAG						
fhe-miR-2162	GTATTATGCAACATTTCACTCT	Fhe-Mir-1993	_TATTATGCAACATTTCACTCT	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-3479	GTATTGCACTTTCCTTCGCCTTA	Fhe-Mir-92-P1	_TATTGCACTTTCCTTCGCCTTA	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-11584	CCATTATATAAGATTGAGGCTCT	Fhe-Mir-NOV-1	_CATTATATAAGATTGAGGCTCT	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-46	ATGTCATGGAGTTGCTCTCTACA	Fhe-Mir-281	_TGTCATGGAGTTGCTCTCTACA	–	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-281	AGGAGGGCAATTTTATGACTTT						
fhe-miR-307	ATCACAACCTACTTGATTGAGGGG	Fhe-Mir-67	_TCACAACCTACTTGATTGAG:_	–	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-67	CCTCAACAAGAAGGCTGTTGGATG						
fhe-miR-745a	ATGCTGCCTTATAAGAGCTGTG	Fhe-Mir-22-P1	_TGCTGCCTTATAAGAGCTGTGA	–	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-22-P1	TCAGTTCTCATTAGGCATGACATG						
fhe-miR-1	ATGGAATGTGGCGAAGTATGGT	Fhe-Mir-1-P2	_TGGAATGTGGCGAAGTATGG_	–	+	–	+	+	NEJ, Ad, EV
		Fhe-Mir-1-P2	_TGGAATGTGGCGAAGTATGGTCT						
fhe-miR-36b	ACCACCGGGTAGACATTCATC	Fhe-Mir-36-P3	_CCACCGGGTAGACATTCATCCGC	–	+	–	+	+	NEJ, Ad, EV
fhe-miR-750	ACCAGATCTGACTCTTCCAGCTCT	Fhe-Mir-750	_CCAGATCTGACTCTTCCAGCTCT	–	+	–	+	+	NEJ, Ad, EV
		Fhe-Mir-750	_CCAGATCTGACTCTTCCAGCTCTT						
fhe-miR-11585	GACCGGTTTCGTCGTTCAACAC	Fhe-Mir-NOV-6	_ACCGGTTTCGTCGTTCAACACC	–	+	–	+	+	NEJ, Ad, EV
		Fhe-Mir-NOV-6	CGTTGCACCGTTCGGAATTCGGGCA						
fhe-let-7	GAGAGGTAGTGACTCATATGACT	fhe-let-7c	_AGAGGTAGTGACTCATATGACT	–	+	–	–	+	NEJ, Ad, EV
		Fhe-Let-7-P2							
fhe-miR-11586	TGTAAGACGATCGTAGTTGACG			–	+	–	–	–	NEJ
fhe-miR-11587	ATTCCGGCAGCTTAGTACAGCT			–	+	–	–	–	NEJ

Adult *F.hepatica* (Ad); Adult *F. hepatica* extracellular vesicles (EVs); Newly excysted juveniles (NEJ); featured in study (+); not featured in study (-). Sequences that are featured in a study with identical annotation but a non-identical sequence are characterised with a yellow box. Grey highlighted nucleotides represent nucleotide variability and/or missing nucleotides when compared to respective *F. hepatica* miRBase miRNA. *F. hepatica* miRBase miRNAs as featured on miRBase database (version 22.1) and hyperlinked to respective miRNA profile on mirbase.org. Other proposed annotation represent the most up to date annotation of the respective other proposed mature miRNA sequence, colour coded based on the study, Xu et al., 2012 (pink), Fromm et al., 2015 (blue), Fromm et al., 2017 (orange) and Ovchinnikov et al., 2020 (green). Sequences sorted based on presence throughout the featured studies

Table 1.2. Published *Fasciola hepatica* mature miRNAs not featured in miRBase database.

Most Recent Published Annotation	Other Published Annotation	Mature miRNA Sequence(s)	Study					Sample
			Xu	Fontenla	Fromm 2015	Fromm 2017	Ovchinnikov	
Fhe-Let-7-P1	let-7 Fhe-Let-7	GGAGGTAGTTCGTTGTGTGG_ GGAGGTAGTTCGTTGTGTGGT	+	–	+	+	+	Ad, EV
Fhe-Mir-1-P1	fhe-mir-1	TGGAATGTTGTGAAGTATGTAC	–	–	+	+	+	Ad, EV
Fhe-miR-2-P4	fhe-mir-2a-B	TATCACAGCCCTGCTTGGAACA: TATCACAGCCCTGCTTGGAACACA	–	–	+	+	+	Ad, EV
Fhe-Mir-7-P2	fhe-mir-7b	TGGAAGACTTGTGATTAAGTTGT_ TGGAAGACTTGTGATTAAGTTGTT	–	–	+	+	+	Ad, EV
Fhe-Mir-10-P3	fhe-mir-10*	CAAGCTCGGGTATACAGGAGCAG	–	–	+	+	+	Ad, EV
Fhe-Mir-12	fhe-mir-12	TGAGTATTTCATCAAGTAGTG_ TGAGTATTTCATCAAGTAGTGA	–	–	+	+	+	Ad, EV
Fhe-Mir-71-P2	fhe-mir-71b	TGAAAGACATGGGTAATGAGGT	–	–	+	+	+	Ad, EV
Fhe-Mir-184-P1 Fhe-Mir-184-P2	fhe-mir-184	TGGACGGAGATTTGTTAAGAGC	–	–	+	+	+	Ad, EV
Fhe-Mir-1175	fhe-mir-1175	TGAGATTCAACTACTTCAGCTG	–	–	+	+	+	Ad, EV
Fhe-Mir-1992	fhe-mir-1992	TCAGCAGTTGCACCATTGACG	–	–	+	+	+	Ad, EV
Fhe-Mir-1989	fhe-mir-1989	TCAGCTGTGTTCATGTCTTCGA	–	–	+	+	+	Ad, EV
Fhe-Mir-2160-P1	fhe-mir-novel-5 Fhe-Mir-2160	TGGCGCTTAGTTATATGTCATCG	–	–	+	+	+	Ad, EV
Fhe-Mir-NOV-2	fhe-mir-novel-6	AGTGGTGATGGTCGAGTGGTTTAG AGTGGTGATGGTCGAGTGGTTTAG_	–	–	+	+	+	Ad, EV
Fhe-Mir-NOV-3	fhe-mir-novel-7	TCAGCACCGGCCGAAACGACAC TCAGCACCGGCCGAAACGACA	–	–	+	+	+	Ad, EV
Fhe-Mir-92-P2	fhe-mir-92b Fhe-Mir-92-P2	GATTGCACTACTCATAGCCTTC AGGCTGTGTGTAGAGCAAGTTG	–	–	+	+	+	Ad, EV
Fhe-Mir-210-P2	fhe-novel-3 Fhe-Mir-219-P2	TAGTCACTGGGCTACGAACACG TGTGCGTAGTTTCAGTGATTAGC	–	–	+	+	+	Ad, EV
Fhe-Mir-NOV-4	fhe-mir-novel-8	ACCCTCATTTAGATCGAAGGT	–	–	+	+	–	Ad, EV
Fhe-Mir-NOV-5	fhe-mir-novel-10	AGACACTCAGAGGACGATCAGT	–	–	+	+	–	Ad, EV
Fhe-Mir-36-P2		TCACCGGGTGTTTTTCACCCTC GGGTGGATACAGTCGGTTATG	–	–	–	+	+	Ad, EV
Fhe-Mir-190-P2		TGATATGTATGGTTTTCGGTTG	–	–	–	+	+	Ad, EV
Fhe-Mir-277-P2		AAAATGCATCATCTACCCGAGA	–	–	–	+	+	Ad, EV
Fhe-Mir-210-P1		TTGTGCGTCGTTTCAGTGACCGAA	–	–	–	+	+	Ad, EV
fhe-let-7-5p		TGAGGTAGTAGGTTGTATAGT	–	+	–	–	–	NEJ
miR-2e-5p		TACCAACTTAGACTGCGTTAT	–	+	–	–	–	NEJ
miR-61-5p		TGTGAGTCTCTTTCTTGTCCATG	–	+	–	–	–	NEJ
miR-190-3p		CCAGTGACCAAACATATTCTC	–	+	–	–	–	NEJ
miR-10-3p		AAATTCGAGTCTACAAGGAAC	–	+	–	–	–	NEJ
miR-205		CGAGGACGTTCAATGGGTTCT	–	+	–	–	–	NEJ
miR-562		TCTAGTCCGACTTTGTGAGGA	–	+	–	–	–	NEJ
miR-598		CGCTGTACGATGATGATGATTT	–	+	–	–	–	NEJ
miR-920		AGGTGTAGAAGTGGTAACACT	–	+	–	–	–	NEJ
miR-1985		TAAAGTGACTGTTAGAATGGT	–	+	–	–	–	NEJ
miR-2478		TCGTATCCCACCTCTGACACCA	–	+	–	–	–	NEJ
miR-3487		TCCCCGTAATCGAACTGTTGT	–	+	–	–	–	NEJ
fhe-miR-3064		CTGGCTGTTGCGGTTAAACC	–	+	–	–	–	NEJ
fhe-novel-5		TAGAGTACCTGTAGATTTAG	–	+	–	–	–	NEJ
fhe-mir-novel-1		GTGGCCTCGTAGCTCAGCTGGTAG	–	–	+	–	–	Ad, EV
fhe-mir-novel-2		TTTGCATATCTAAGTCGGACA	–	–	+	–	–	Ad, EV
fhe-mir-novel-9		GTCAGCGAAGACGTCGGGAA	–	–	+	–	–	Ad, EV
fhe-mir-novel-11		TCCGAAAACGCGATGGAACCT	–	–	+	–	–	Ad, EV
fhe-mir-novel-12		ATGAGACGGTGAGTGATGAATT	–	–	+	–	–	Ad, EV
fhe-mir-novel-13		GACCGGTGGTGGTCGAGTGGGTT	–	–	+	–	–	Ad, EV
Fhe-Mir-36-P2		TCACCGGGTGTTTTTCACCCTC	–	–	–	+	–	Ad, EV
Fhe-Let-7-P3		GAGGTAGTGAGTTGTATGTCTG	–	–	–	–	+	Ad, EV
Fhe-Mir-36-P2		GGGTGGATACAGTCGGTTATG	–	–	–	–	+	Ad, EV
Fhe-Mir-133		TTGGTCCCTATCAACCAGCTAT	–	–	–	–	+	Ad, EV
Fhe-Mir-278		TCGGTGGGAGTATCATTCGTGC	–	–	–	–	+	Ad, EV
Fhe-Mir-2160-P2		AGGCGCTTTGATTGTCCACACTGA	–	–	–	–	+	Ad, EV

Ad (adult fluke); Adult fluke extracellular vesicles (EV); Newly excysted juveniles (NEJ) featured in study (+); not featured in study (-). Annotation of published miRNA as most up to date of that sequence across studies. Other published annotation represent studies by Xu et al,. 2012 (pink), Fromm et al., 2015 (blue) Fromm et al., 2017 (orange) and Ovchinnikov et al., 2020 (green). Sequences sorted based on presence throughout the featured studies.

The most recent discovery project by Fontenla et al. [39], focused on the miRNA content of newly excysted juveniles (NEJ) 6 hours post-excystment as opposed to the adult life stage utilised in the previous two studies. Initially, the conservation of *F. hepatica* miRNAs across mammalian and platyhelminth species was determined via a series of filtering processes which compared the NEJ sequencing data against published sequences and databases of miRNA and non-coding RNA such as miRBase (version 20.0), Rfam [40] and the functional RNA database fRNAdb (www.ncrna.org/frnadb).

This identified a total of 46 miRNAs. However, although six of these were classified as miRNAs due to high similarity (>85%) with other helminth miRNAs reported in literature, as they were not found within any of the databases the authors removed these from their final list of proposed miRNAs, thus producing a total of 40 (Tables 1.1 & 1.2). Of these, 34 shared sequence identity to the miRNAs discovered in the previous analyses of the adult miRNA [37, 38], which suggests that the other six miRNAs may be specific to the NEJs. Only three of the eight putative novel miRNAs identified by Xu et al. were identified within this sequencing data but were found to correspond to repetitive sequences within the *Fasciola* genome, confirming the earlier proposition that they are not genuine miRNAs. To differentiate the *Fasciola* specific miRNAs from conserved sequences within the panel of 40, a genome assembly was generated using genomic sequence reads downloaded from the Welcome Trust Sanger Centre and used as the reference genome for analysis by miRDeep2. Novel mature miRNA candidates were then aligned to *F. hepatica* genomes PRJEB6687 [41] and PRJNA179522 [42] to confirm the presence and location of mature miRNAs. This analysis resulted in the identification of five *F. hepatica* specific miRNAs, of which four are now officially annotated as fhe-miR-11584 to 11587 on miRBase.

Combined, these studies resulted in the identification of 72 miRNAs (excluding the 8 proposed novel miRNAs by Xu et al). Of these, 38 are currently featured in the miRBase database (F_hepaticav1; Table 1.1). Of the remaining 34 miRNAs (Table 1.2), 13 were characterised as novel to *F. hepatica* (i.e. unidentified within the miRNA profile of any other species on miRBase). Although the other 21 sequences have been annotated, within their respective publications, based on their similarity with known miRNAs, they are not listed on the miRBase database, suggesting they were not deemed as authentic miRNAs. This is most likely due to expression and biogenesis criteria for miRNA identification. It is possible that miRNAs identified in the adult flukes were not accepted due to the genome assembly that was used in those discovery pipelines. Although several miRNAs in the NEJ study were localised

within the *Fasciola* genome according to the 2015 genome, these miRNAs may not have satisfied the specific criteria for precursor hairpin structures employed by miRBase [43].

However, these should not be discounted as likely miRNAs, as it has more recently been proposed that the hairpin structures of miRNAs may be more variable than originally proposed [44]. This suggestion has influenced the development of a new set of criteria for the annotation of metazoan miRNAs, which is employed by the curated miRNA gene database MirGeneDB. Utilising these criteria to re-evaluate the *F. hepatica* small RNA sequencing data from all 3 of the discovery projects, Fromm et al., (2017) determined that the annotated miRNAs, not present in miRBase were genuine miRNAs (Table 1.2) [33]. However, 7 of the putative novel miRNAs from the Fromm et al., 2015 study (Table 1.2) and 3 novel miRNAs proposed within the NEJ study were no longer considered as *bona fide* miRNAs (Table 1.1 and 1.2). This outcome highlights the impact of using different criteria for the assessment of miRNAs, as two of these NEJ miRNAs are currently annotated as genuine miRNAs in miRBase as fhe-miR-11586 and fhe-miR11587 (Table 1.1). In addition to the assessment of previously identified miRNAs, this study also discovered 8 new conserved miRNAs within the adult parasites (Fhe-mir-1-P2, Fhe-mir-36-P2, Fhe-mir-36-P3, Fhe-miR-190-P2, Fhe-mir-210-P1, Fhe-mir-210-P2, Fhe-mir-277-P2, Fhe-mir-750). Of these, 3 were also present in the NEJs (Fhe-mir-1-P2, Fhe-mir-36-P3, Fhe-mir-750) and are listed in miRBase as fhe-miR-36b and fhe-miR-750, with the sequences for both Fhe-mir-1-P2, Fhe-mir-36-P3 aligning to fhe-miR-36b (Table 1.1).

As well as using different criteria for assessment, MirGeneDB employs an internal annotation, which differs from the nomenclature utilised by MirBase. Whereas, the miRBase system assigns the next number in succession (i.e. miR-10 was reported after miR-9 etc.) to new sequences, with paralogs indicated by a letter (if there is a difference of a single nucleotide) or a number (if the mature sequences are identical), the MirGeneDB nomenclature was developed to capture the phylogenetic relationship between miRNAs, where genes of common descent are assigned the same miRNA family name [45]. Using this system resulted in the re-classification of Mir-novel-5 [33] as a member of the MIR-2160 family (Table 1.2). Similarly, fhe-miR125 has been assigned to the eumetazoan MIR-10 gene family resulting in the nomenclature Fhe-MiR-10, with the paralogs identified as Fhe-Mir-10-P2a and Fhe-Mir-10-P2b (Table 1.2). Likewise, fhe-miR-745a has been named Fhe-Mir-22-P1.

Most recently, the small RNA sequencing data sets from the adult parasites [37] and their EVs [38] were re-evaluated again using an improved version of MirMiner [46]. This study

reported the discovery of 4 conserved miRNAs (Fhe-Let-7-P3, Fhe-Mir-133, Fhe-Mir-278 and Fhe-Mir-2160-P2) and 4 *Fasciola*-specific miRNAs. Of the parasite-specific miRNAs, the sequences for Fhe-Mir-NOV-1 and Fhe-Mir-NOV-6 were near identical to miRNAs within the NEJ miRBase dataset, listed as fhe-miR-11584 and fhe-miR-11585 respectively (Table 1.2). Although the other 2 (Fhe-Mir-NOV-2 and Fhe-Mir-NOV-3) may be classified as new sequences, they each differ in only one nucleotide from the previously identified adult parasite miRNAs fhe-mir-novel-6 and fhe-mir-novel-7 respectively (Table 1.2).

Compiling the findings from all of these studies suggests that in addition to the 38 miRNAs listed on miRBase, *F. hepatica* expresses an additional 39 miRNAs (excluding the 7 sequences deemed not to be genuine according to the MirGeneDB criteria for miRNA annotation [33], as described above. Of the 77 miRNAs, 36 were identified in both NEJ and adult parasite, 15 were specific to NEJ and 26 were specific to adult parasites (Tables 1.1 and 1.2).

Despite the similarities in the profiles of miRNAs identified across all studies, it is important to note the number of variations in the recorded sequences for many of the mature miRNAs (Table 1.1). Differences in nucleotides are particularly evident towards the 5' or 3' end for 26 of the common mature miRNAs. Studies of mammalian miRNAs have indicated that variations in the 3′- and/or 5′-end(s) of canonical miRNA-sequence represent IsomiRs (sequence variants) created either due to imprecise cleavage of miRNA sequence by drosha or dicer enzymes or through the addition of nucleotides at 3′ end during miRNA-biogenesis [47]. Whether the variations within the *Fasciola* miRNA sequences represent IsomiRs that correlate with different life stages is an issue that will only be resolved with continued analysis of the fluke's miRNA. Nonetheless, as these changes do not alter the 2-8 nt seed region of these miRNAs, they are not likely to have a significant effect on the specificity of gene that they target.

In contrast, the sequences reported for let-7, miR-1 and miR-71b are quite different between the studies, with nucleotide variations evident throughout the entire sequence of the mature miRNA. Further examination of these sequences suggests that the annotations are correct, but the different sequences may in fact reflect distinct members of the miRNA families (Table 1.3). The sequence of miR-1 identified in the NEJs more closely aligns to the *S. mansoni* miR-1a and other species of fluke, whereas the miR-1 in adult fluke is more closely conserved to *S. mansoni* miR-1b and species of tapeworm. Similarly, miR-71b in NEJs is near identical to only other species of trematode, whereas the adult fluke miR-71b closely resembles other

parasitic and non-parasitic helminths outside of the trematode class. Likewise, the sequence of the let-7 within the NEJs is conserved with Planaria while the adult fluke let-7 is closer to other parasitic trematodes and mammalian sequences. This is of particular interest as let-7 is the miRNA that regulates the expression of *Lin41* in *C. elegans*, a gene that controls the transition to adulthood [48]. This suggests that perhaps the differences in the listed sequences for *Fasciola* adult and NEJ miRNAs may in fact reflect the evolution of variants of the same miRNAs specific to the different life stages of *Fasciola* to ensure the regulation of different gene targets as necessary for maturation of the worm and modulation of host responses in different tissue environments.

Table 1.3. Mature miRNA sequence, study and conservation between fhe-let-7, fhe-miR-1 and fhe-miR-71b.

Source	Species	miRNA	Mature sequence
Fontenla et al	***Fasciola hepatica***	**fhe-let-7**	**GAGAGGUAGUGACUCAUAUGACU**
miRBase	*Melibe leonina*	mle-let-7-5p	_UGAGGUAGUGACUCAUUUUGUU
miRBase	*Schmidtea mediterranea*	sme-let-7c-5p	_UGAGGUAGUGACUCAAAAGGUU
miRBase	*Schmidtea mediterranea*	sme-let-7d	_AGAGGUAGUGAUUCAAAAAGUU
Fromm et al	***Fasciola hepatica***	**fhe-let-7**	**GGAGGUAGUUCGUUGUGUGGU**
miRBase	*Schistosoma japonicum*	sja-let-7	GGAGGUAGUUCGUUGUGUGGU
miRBase	*Schistosoma mansoni*	sma-let-7-5p	GGAGGUAGUUCGUUGUGUGGU
miRBase	*Ovis aries*	oar-let-7b	UGAGGUAGUAGGUUGUGUGGU
miRBase	*Homo sapiens*	hsa-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU
Fontenla et al	***Fasciola hepatica***	**fhe-miR-1**	**AUGGAAUGUGGCGAAGUAUGGU**
miRBase	*Schistosoma japonicum*	sja-miR-1	_UGGAAUGUGGCGAAGUAUGGUC
miRBase	*Gyrodactylus salaris*	gsa-miR-1-3p	_UGGAAUGUGGCGAAGUAUGGUC
miRBase	*Schistosoma mansoni*	sma-miR-1a-5p	_UGGAAUGUGGCGAAGUAUGG_
Fromm et al	***Fasciola hepatica***	**fhe-miR-1**	**UGGAAUGUUGUGAAGUAUGUAC**
miRBase	*Schistosoma mansoni*	sma-miR-1b-3p	UGGAAUGUUGUGAAGUAUGUGC
miRBase	*Echinococcus granulosus*	egr-miR-1-5p	UGGAAUGUUGUGAAGUAUGU_
miRBase	*Echinococcus multilocularis*	emu-miR-1-3p	UGGAAUGUUGUGAAGUAUGU_
Fontenla et al	***Fasciola hepatica***	**fhe-miR-71b**	**UGAAAGACUUGAGUAGUGAG**
miRBase	*Schistosoma japonicum*	sja-miR-71b-5p	UGAAAGACUUGAGUAGUGAGACG
miRBase	*Schistosoma mansoni*	sma-miR-71b-5p	UGAAAGACUUGAGUAGUGAGACG
Fromm et al	***Fasciola hepatica***	**fhe-miR-71b**	**UGAAAGACAUGGGUAAUGAGGU**
miRBase	*Gyrodactylus salaris*	gsa-mir-71a	UGAAAGACAUGGGUAAUGAGU_
miRBase	*Schmidtea mediterranea*	sme-mir-71c	UGAAAGACAUGGGUAGUGAGAU
miRBase	*Haemonchus contortus*	hco-mir-71	UGAAAGACAUGGGUAGUGAGAC
miRBase	*Heligmosomoides polygyrus*	hpo-mir-71	UGAAAGACAUGGGUAGUGAGAC

Nucleotides highlighted in grey represent mismatches when compared to the *F. hepatica* mature miRNA sequences (shown in bold text).

Of interest, miR-281, miR-279, miR-67 and miR-1993 identified in adult fluke by Fromm et al. [38], are alternatively annotated as miR-46, miR-61, miR-307, and miR-2162 respectively, by Fontenla et al. [39]. An analysis of miR-281 and miR-46 showed that the sequences are in fact very similar, but have been classified as two distinct miRNAs in miRBase. In both fluke miRNA studies; this particular sequence was published as miR-46/miR-281. However, due to increased availability of miRNA sequences within databases, it is now evident that miR-46 is generally found within helminths, and while miR-281 can be found in some species of parasitic helminth it is more prominent in other invertebrates (Table 1.4). This was likely a miRbase consideration to finalise the annotation of this sequence to miR-46 in *Fasciola*. The same scenario can be applied to the miRNAs characterised as miR-279, miR-67, and miR-1993, which are now listed as miR-61, miR-307 and miR-2162 respectively on miRBase.

This compilation and comparative analysis of the *Fasciola* miRNome has highlighted the impact and need for appropriate annotation of all flatworm miRNAs. The complexity of miRNA biogenesis giving rise to isomiRs will add to these challenges in annotation and subsequent curation. It must be noted that confirmation and authenticity of miRNAs is largely dependent on available sequencing data, and therefore will improve as more sequencing data is generated and made available. There are currently 38 *Fasciola* miRNAs listed in miRBase, which is presently the only repository of flatworm miRNAs. As more sequences become available, it is likely that *Fasciola* will feature within other curated databases such as MirGeneDB and miROrtho. However, it is clear from this compilation of studies, that a universal system of naming needs to be accepted by the wider *Fasciola* research community for the sake of clarity. The exciting outcome of adopting a uniform system of annotation is the release of a fully curated and annotated *Fasciola* miRnome for all life stages.

Table 1.4. Species conservation of miR-46/281.

Species	miRNA	Mature miRNA Sequence	Phylum			
			Platyhelminth	Nematoda	Anthropoda	Mollusc
Fasciola hepatica	**fhe-miR-46**	**ATGTCATGGAGTTGCTCTCTACA**	+			
Schistosoma mansoni	sma-miR-281-3p	_TGTCATGGAGTTGCTCTCTATA	+			
Echinococcus granulosus	egr-miR-281-3p	_TGTCATGGAGTTGCTCTCTATA	+			
Echinococcus multilocularis	emu-miR-281-3p	_TGTCATGGAGTTGCTCTCT__	+			
Ascaris suum	asu-miR-46-3p	_TGTCATGGAGTTGCTCTCTTCA		+		
Panagrellus redivivus	prd-miR-46-3p	_TGTCATGGAGT_GCTCTCTTA_		+		
Haemonchus contortus	hco-miR-46	_TGTCATGGAGTCGCTCTCTTCA		+		
Heligmosomoides polygyrus	hpo-miR-46-3p	_TGTCATGGAGTCGCTCTCTTCA		+		
Caenorhabditis elegans	cel-miR-46-3p	_TGTCATGGAGGCGCTCTCTTCA		+		
Caenorhabditis briggsae	cbr-miR-46	_TGTCATGGAGGCGCTCTCTTCA		+		
Caenorhabditis brenneri	cbn-miR-46	_TGTCATGGAGGCGCTCTCTTCA		+		
Caenorhabditis remanei	crm-miR-46-3p	_TGTCATGGAGTCGCTCTCTTC_		+		
Pristionchus pacificus	ppc-miR-46	_TGTCATGGAGTCGCTCTCTTC_		+		
Tribolium castaneum	tca-miR-281-3p	_TGTCATGGAGTTGCTCTCTTT_			+	
Acyrthosiphon pisum	api-miR-281	_TGTCATGGAGTTGCTCTCTTT_			+	
Bombyx mori	bmo-miR-281-3p	ACTGTCATGGAGTTGCTCTCTT__			+	
Branchiostoma floridae	bfl-miR-281	_TGTCATGGAGTTGCTCTCTTTT			+	
Tetranychus urticae	tur-miR-281-3p	_TGTCATGGAGTTGCTCTCTTTC			+	
Manduca sexta	mse-miR-281	CTGTCATGGAGTTGCTCTCTTT_			+	
Culex quinquefasciatus	cqu-miR-281-3p	_TGTCATGGAATTGCTCTCTTT_			+	
Lottia gigantea	lgi-miR-281-3p	_TGTCATGGAGTTGCTCTCTTTA				+

Nucleotides highlighted in grey represent mismatches when compared to the Fontenla *et al*/miRbase *F. hepatica* mature miRNA sequence (shown in bold text).

1.7. Predicted Gene Targets for *Fasciola hepatica* miRNAs reveal a high degree of redundancy in the regulation of immune cell activation

Despite the characterisation of *F. hepatica* miRNAs, beyond an acknowledgement that some share homology to mammalian miRNAs involved in the regulation of immune responses [33-39], there has been no detailed exploration of the possible mammalian genes that might be targeted by parasite miRNAs as a mechanism for controlling the host immune response. Given that a single miRNA can have multiple targets and that the 3'UTR of the mRNA can include various binding sites for multiple miRNAs, predictive tools provide an *in silico* method for screening potential targets followed by experimental validation.

As the miRBase *F. hepatica* miRNAs were identified within the NEJ stage, we specifically explored potential mammalian gene targets that were specific to innate immune cells. It is important to acknowledge that highly characterised species such as human, mouse and rat dominate the curated literature that support these predictive tools and databases. Although *F. hepatica* is typically regarded as a parasite of sheep and cattle, due to the lack of information on the biological contribution of genes in these species it is not possible to accurately apply the predictive tools against these hosts. Accordingly, predictions of mammalian gene targets for *Fasciol*a derived miRNAs were performed against a *Homo sapiens* background. Given that the bovine genome is regarded as 80% similar to the human genome [49], and that the profile of immune response to *F. hepatica* is common across all host species [50], it is probable that the predicted gene targets are common to the regulation of immune response during infection for multiple mammalian hosts of *Fasciola*.

We analysed the mature sequences of the 38 miRBase *Fasciola* miRNAs for custom target prediction using miRDB (mirDB.org.) This database was selected as it utilises the bioinformatic tool MirTarget, which is a compendium of experimentally validated miRNA targets [51]. Genes with a target score of >60 were selected for further analysis using InnateDB (innatedb.com), an integrated analysis platform that has been specifically designed to facilitate systems-level analyses of pathways and genes specific to mammalian innate immune cells [52]. Thus, gene targets were further filtered according to their association with immunological responses of innate cells including dendritic cells (DCs), eosinophils, innate lymphoid cells (ILCs), macrophages, monocytes and neutrophils. Within the miRBase *Fasciola* miRNome, 26 of the 38 miRNAs were predicted to target genes within all of the innate immune cells examined (Figure 1.2). Of these cells, DCs, eosinophils and neutrophils were the most targeted

with 17, 14 and 8 miRNAs, respectively, identified as having gene targets within these cells (Figure 1.2). Furthermore, this mapping revealed a high degree of redundancy as many of the miRNAs were predicted to act on the same targets, suggesting a certain selectivity to the genes that are targeted and thus the modulation to immune response that consequently occurs.

Of the 14 miRNAs acting on eosinophils, 8 were predicted to target colony stimulating factor receptor (CSF2R). This receptor is a heterodimer comprised of an alpha and beta chain. The alpha subunit contains a specific binding site for granulocyte macrophage colony-stimulating factor (GM-CSF;). The beta chain triggers signal transduction and is also present in the receptor complexes for IL-5 and IL-3 [54-56]. Many aspects of eosinophil biology are controlled by GM-CSF and IL-5, acting through the CSF2R complex [57-59]. Of relevance to infection with *F. hepatica,* and in particular to the excystment and migration of the NEJs, in the context of intestinal inflammation GM-CSF and IL-5 foster the survival of peripheral eosinophils, but only GM-CSF promotes the activation of effector functions [57]. The prediction that multiple *Fasciola* miRNAs target the expression of both subunits, proposes a heightened capacity to regulate the recruitment and functionality of eosinophils, and therefore reduce parasite killing.

Within DCs, the parasite miRNAs primarily targeted three host genes, Histone deacetylase-2 (HDAC2), PRDM1 and SP1. By regulating their expression, the parasite miRNAs would significantly impact the maturation of DCs, as both HDAC and PRDM1 promote the expression of costimulatory molecules (such as CD40, CD86 and MHC-II; [60-62]) and the acquisition of CD1a [63], the hallmark of an IL-12 producing, pro-inflammatory DC. Considering the requirement for DC-derived cytokines and co-stimulatory molecules for efficient T-cell activation, regulating the expression of these genes would also inhibit the development of an effector adaptive immune response, as seen during infection with *F. hepatica*. Indeed knockdown of PRDM1 in DCs resulted in a reduced ability for these cells to induce efficient allogeneic T cell proliferation [62], and inhibition of HDACs in DCs, led to the differentiation of T cells with an anergic phenotype [64]. All 8 of the *Fasciola* miRNAs that were determined to target neutrophils, were predicted to target only a single gene; CREB1. This gene encodes a transcription factor that has a central role in the regulation of the functional response of neutrophils [65-68]. Specific mutation of this gene, results in a decreased ability of neutrophils to generate inflammatory chemokines and cytokines [65], which reflects the reduction in neutrophil activity when exposed to the parasite.

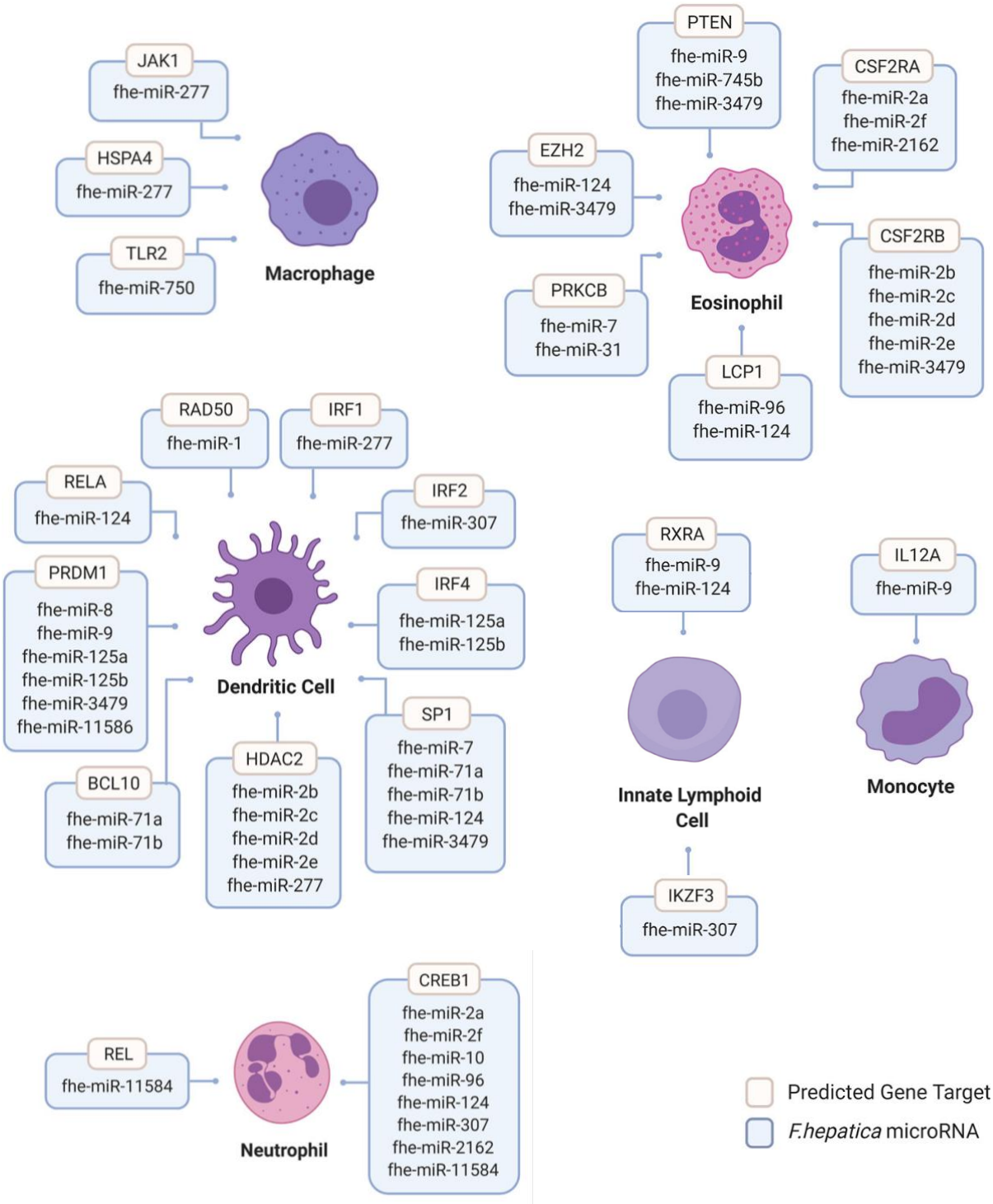

JAK1
fhe-miR-277
HSPA4
fhe-miR-277
TLR2
fhe-miR-750
Macrophage
PTEN
fhe-miR-9
fhe-miR-745b
fhe-miR-3479
CSF2RA
fhe-miR-2a
fhe-miR-2f
fhe-miR-2162
EZH2
fhe-miR-124
fhe-miR-3479
CSF2RB
fhe-miR-2b
fhe-miR-2c
fhe-miR-2d
fhe-miR-2e
fhe-miR-3479
PRKCB
fhe-miR-7
fhe-miR-31
Eosinophil
LCP1
fhe-miR-96
fhe-miR-124
RAD50
fhe-miR-1
IRF1
fhe-miR-277
RELA
fhe-miR-124
IRF2
fhe-miR-307
PRDM1
fhe-miR-8
fhe-miR-9
fhe-miR-125a
fhe-miR-125b
fhe-miR-3479
fhe-miR-11586
IRF4
fhe-miR-125a
fhe-miR-125b
Dendritic Cell
BCL10
fhe-miR-71a
fhe-miR-71b
HDAC2
fhe-miR-2b
fhe-miR-2c
fhe-miR-2d
fhe-miR-2e
fhe-miR-277
SP1
fhe-miR-7
fhe-miR-71a
fhe-miR-71b
fhe-miR-124
fhe-miR-3479
RXRA
fhe-miR-9
fhe-miR-124
Innate Lymphoid Cell
IKZF3
fhe-miR-307
IL12A
fhe-miR-9
Monocyte
REL
fhe-miR-11584
Neutrophil
CREB1
fhe-miR-2a
fhe-miR-2f
fhe-miR-10
fhe-miR-96
fhe-miR-124
fhe-miR-307
fhe-miR-2162
fhe-miR-11584
Predicted Gene Target
F.hepatica microRNA

Figure 1.2. Human innate immune cell genes predicted to be targeted by *Fasciola hepatica* microRNAs. Human genes targeted by *F. hepatica* miRNAs were predicted using the miRNA target prediction tool and database miRDB (mirdb.org). Gene targets are considered with >60 target score produced by miRTarget in miRDB with Homo sapiens background using *F. hepatica* miRNA sequences featured in miRbase database version 22 (mirbase.org). Gene targets filtered for innate immune cell background including dendritic cells (DCs), eosinophils, innate lymphoid cells (ILCs), macrophages, monocytes and neutrophils were considered. Dendritic cell targeted genes include B-Cell Lymphoma/Leukemia 10 Signaling Adaptor (BCL10), Histone Deacetylase 2 (HDAC2); Interferon Regulatory Factor (IRF) 1, 2, and 4; Positive Regulatory Domain I-Binding Factor 1 (PRDM1); RAD50 Double Strand Break Repair Protein (RAD50); REL Proto-Oncogene, NF-KB Subunit (RELA) and Sp1 Transcription Factor (SP1). Eosinophil targeted genes include Colony Stimulating Factor 2 Receptor Subunit Alpha (CSFR2A), Colony Stimulating Factor 2 Receptor Subunit Beta (CSFR2B), Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2), Lymphocyte Cytosolic Protein 1 (LCP1), Protein Kinase C Beta (PRKCB) and Phosphatase and Tensin Homolog (PTEN). Innate lymphoid cell gene targets include Ikaros Family Zinc Finger 3 (IKZF3) and Retinoid X Receptor Alpha (RXRA). Macrophage gene targets include Heat Shock Protein Family A Member 4 (HSPA4), Janus Kinase 1 (JAK1) and Toll Like Receptor 2 (TLR2). Monocyte targeted genes include Interleukin 12A (IL12A). Neutrophil targeted genes include CAMP Responsive Element Binding Protein 1 (CREB1) and REL Proto-Oncogene and NF-KB Subunit (REL). Image created by Biorender.

This analysis strongly supports the hypothesis that parasite-derived miRNAs can regulate host genes, and by doing so can manipulate the functional activity of all immune cells. While, the use of a single miRNA target prediction tool (miRDB) has its limitations, particularly on non-model organisms such as the liver fluke, using it in combination with other databases such as (InnateDB and Reactome) captured preliminary insights to the immunoregulatory functions of *Fasciola* miRNA. More so, the revelation that multiple parasite miRNAs targeted the same genes within eosinophils, DCs and neutrophils suggest that *Fasciola* miRNAs have been conserved to regulate specific anti-parasitic immune pathways during an infection. With knowledge of the miRNA sequences, this hypothesis can be experimentally tested. In the first instance, the standard approach of reporter gene expression after the *in vitro* transfection of cells with synthetic miRNA mimics, would provide some validation of the host genes that parasite derived miRNAs are predicted to target. The ability to knockout parasite miRNAs from the juvenile parasite and subsequently trace the development of host immune responses post infection would be ideal. However, the capacity to silence gene expression in Fasciola, while reportedly possible [69-72], is in its infancy. As this and similar technologies, such as miRNA inhibitors and CRISPR, advance in helminth biology, opportunities will arise to provide highly controlled experimental environments that will reveal distinct associations between miRNAs and their targets.

As this review was being finalised, a similar consideration of the biological role of the 46 of the most abundant miRNAs in adult parasites and EVs was published. Using a combination of TargetScan and PITA to predict gene targets within the genome of cattle and humans, and applying Reactome and KEGG pathway analysis to these targets, identified 44 and 23 genes respectively, that were characterised as having a role in the immune system [46].

1.8. Discussion

We specifically focused on the innate immune cells as the miRBase list of miRNAs has been validated for the NEJs, a stage of the parasite that is most closely associated with the immediate host immune response. However, based on this analysis the identification of gene targets within every cell suggests that the parasite's ability to modulate host cell behaviour is widespread. Therefore, by characterising the expression of specific miRNAs at each stage of the parasite's life cycle, this analytical workflow could be extended to other biologically relevant host cells, such as intestinal epithelium, liver cells, B and T lymphocytes, and the bile duct. However, in addition to the relative abundance of miRNA, the source of the parasite miRNAs within the host should be considered. It has now broadly acknowledged that helminths communicate with their host cells through the active secretion of extracellular vesicles, which contain miRNA cargo that has been selectively loaded for functional activity [34, 35, 73]. Furthermore, a growing body of evidence is reporting detection of parasite-derived miRNAs, with predicted immune regulatory ability, in the sera of infected hosts [74, 75]. It has been proposed that circulating miRNAs maintain their stability through binding to Ago proteins, an interaction which mediates the uptake of the miRNAs by host cells [76, 77]. Thus, an understanding of both the abundance of different miRNAs during the progression of the parasite's development combined with knowledge of whether they are delivered into host cells (or released by dying parasites for cellular uptake) will provide a holistic analytical approach to the host-parasite crosstalk.

Before undertaking this type of analysis, a definitive characterization of the *Fasciola* miRNome is required. Compiling the sequencing data and annotations from all five independent studies reporting the identification of the parasite's miRNAs revealed a surprising level of variation in the sequences and annotation. This outcome illustrates the importance of the research community working together to compare data and to submit these analyses for verification by an independent body. In this manner, the parasite's miRNome will be correctly catalogued (and reviewed), to be used in future studies as a reference point for comparison and continued expansion. At this stage, the *Fasciola* research field requires the accurate curation of a completed *Fasciola* genome and robust guidelines for processing of miRNA sequencing data. If we can generate these tools, variations in sequencing will be reduced and more importantly, it will permit the precise identification of parasite miRNAs within immune cells in vivo during an infection. This is an essential piece of defining evidence to fully support the hypothesis that *Fasciola* miRNAs are manipulating host immune cell function. Furthermore,

accurate knowledge of the parasite miRNA sequences would also support the targeted knockdown of specific miRNAs within the NEJs, to determine the relative importance of each in supporting the safe passage of these juvenile parasites to the liver.

One additional consideration is the verification that different isomiRs within the same family of miRNAs may be differentially expressed according to the life stage of the parasite. This possibility was only uncovered as we compared all of the sequences for each annotated miRNA across all of the published studies. It has been shown that the transcriptome of the worm varies greatly between the NEJ and adult stage [78]. It is thus, not surprising that the parasite may require different miRNAs to regulate the expression of different parasite genes as it matures. If these isomiRs regulate different targets, this would also represent an adaptation to different immunological environments as the parasite migrates from intestine, through the liver to the bile duct. Further, the fine-tuning of single nucleotide in parasitic miRNAs for immune regulation would be a novel area of gene regulation.

In conclusion, the continued characterization and functional analysis of miRNAs in *F. hepatica* will create a new mechanistic framework for the regulation of host immune responses by parasite-secreted miRNAs. This information will also reveal the molecular biological pathways that are unique to parasitism and will be of enormous benefit to the development of novel strategies for infection control.

Chapter 2:

Fasciola hepatica hijacks host macrophage miRNA machinery to modulate early innate immune responses

2.1. Introductory Statement

The work presented in this chapter significantly contributed to the publication of a Research Article:

Tran N, Ricafrente A, To J, Lund M, Marques TM, Gama-Carvalho M, Cwiklinski K, Dalton JP, Donnelly S. *Fasciola hepatica* hijacks host macrophage miRNA machinery to modulate early innate immune responses. ***Scientific Reports***. 2021 Mar 24;11(1):1-1.

2.2. Abstract

Fasciola hepatica, a global worm parasite of humans and their livestock, regulates host innate immune responses within hours of infection. Host macrophages, essential to the first-line defence mechanisms, are quickly restricted in their ability to initiate a classic protective pro-inflammatory immune response. We found that macrophages from infected animals are enriched with parasite-derived micro(mi)RNAs. The most abundant of these miRNAs, *fhe-miR-125b*, is released by the parasite via exosomes and is homologous to a mammalian miRNA, *hsa-miR-125b*, that is known to regulate the activation of pro-inflammatory M1 macrophages. We show that the parasite *fhe-miR-125b* loads onto the mammalian Argonaute protein (Ago-2) within macrophages during infection and, therefore, propose that it mimics host *miR-125b* to negatively regulate the production of inflammatory cytokines. The hijacking of the miRNA machinery controlling innate cell function could be a fundamental mechanism by which worm parasites disarm the early immune responses of their host to ensure successful infection.

2.3. Introduction

Helminths, or worms, are multicellular parasites that can live for many years within their vertebrate hosts. Of prime importance is the regulation of the host immune cell signalling pathways to prevent the parasite's elimination before they can produce their off-spring in the form of eggs. Many worm parasites inactivate innate cell detection systems, such as macrophage and dendritic inflammatory responses, and direct the adaptive immune response towards a tolerant or hyporesponsive state [23, 79-81]. Understanding how worm parasites do this will inform the design of new anti-helminthic treatments, such as drugs or vaccines.

Fasciola hepatica, or liver fluke, is a global zoonotic food-borne parasite that infects humans and their livestock. The parasite is one of the most successful helminths as it is found on every inhabited continent and infects the widest variety of mammalian hosts infected by any worm, including some that it has encountered only in relatively recent times (e.g. camelids and marsupials) [82, 83]. This implies a superior adaptation to the mammalian host and suggests that *F. hepatica* parasites have evolved a universal process of invasion, tissue migration and immune modulation.

Mammalian hosts become infected with *F. hepatica* following ingestion of vegetation contaminated with encysted parasites (metacercariae). Within the intestine, juvenile parasites emerge from their cysts (termed newly excysted juveniles, NEJ) and within ~2 to 6 hours traverse the intestinal wall and enter the peritoneal cavity. During the next 2-3 days of infection they rely on stored glycogen resources to migrate from the peritoneal cavity into the liver parenchyma where they begin to rapidly grow and develop. Their migratory activity in the liver causes the extensive haemorrhaging and tissue destruction associated with acute disease. During the invasion and pre-hepatic migratory stage, animals display no clinical signs of infection, and, histologically, no significant inflammatory changes are observed in the intestinal wall or peritoneal cavity [84, 85]. This suggested that the parasites employ a mechanism(s) to disarm the host's innate 'early-response' immune system to prevent their detection at a vulnerable time in their invasion.

Within the peritoneal cavity macrophages are the primary immune cell contributing to the immediate response to invading pathogens [86] and are the predominant innate immune cell present during the first days of infection with *F. hepatica* [20, 87]. Macrophages are major regulators of the inflammatory response as they monitor blood and tissues for signs of infection or damage and alert adaptive immune cells to any incursions. In response to invading

pathogens, macrophages are typically 'classically activated' by signals from the pathogen itself and from damaged host tissue. This 'M1' phenotype of macrophage displays enhanced phagocytic and antigen presentation capacity and drives host protective immune responses by producing pro-inflammatory cytokines (such as TNF, IL-6 and IL-12) and anti-microbial effector molecules (like nitric oxide; NO) [88]. Despite the presence of migrating *F. hepatica* parasites, the levels of NO produced by peritoneal macrophages isolated from infected sheep are the same as macrophages from uninfected sheep [87, 89]. Furthermore, peritoneal macrophages from infected sheep show no significant increase in the expression levels of pro-inflammatory cytokines (TNF, IL-12, IFNγ, iNOS) [16]. Importantly, this modulation of pro-inflammatory immune responses is also evident in experimental infections in mice [90-92]. The absence of a classical host protective inflammatory response to invading *F. hepatica* implies that the parasite possesses an effective mechanism of control on macrophage activity.

Helminth parasites secrete a range of soluble proteins/glycans that have been shown to suppress the ability of host macrophages to differentiate to an M1 phenotype [93-95]. More recently, a role for miRNAs in the regulation of host immune responses by parasitic worms has been suggested [38, 96, 97]. Comparative analysis of parasite genomes has shown that some helminth miRNAs are widely conserved while others are specific to particular helminths [37, 98]. Moreover, a number of the conserved miRNAs share sequence identity with mammalian miRNAs that are known to have an immune regulatory function. Target prediction software suggests that helminth miRNAs may act to regulate host immune responses to enable their development and survival [38, 96, 97]. However, to date, the evidence to support this proposed miRNA-mediated mechanism of immune modulation has come from *in vitro* analysis of transfected cells [34, 36] or the identification of parasite miRNAs in sera or tissue of infected hosts [74, 99, 100]. Here we show that *F. hepatica* releases miRNAs, that are homologous to host miRNAs, that regulate macrophage function *in vivo* and propose that worms may hijack this process to control their host's early immune responses.

2.4. Methods

2.4.1. Murine and sheep infections.

Six to eight week old female BALB/c mice (Australian Resource Centre, Perth, Australia) were orally infected with 20 metacercariae of *F. hepatica* (Baldwin Aquatics Inc, USA). Peritoneal exudate cells were collected by washing the peritoneal cavity with 5 ml of sterile saline. Ethical approval for this study was granted by the University of Technology Sydney (UTS) Animal Care and Ethics Committee (Approval Number: 2012-080) and experiments were conducted in accordance with the approved guidelines to be compliant with The Australian Code for the Care and Use of Animals for Scientific Purposes.

Ten 6 month-old male Dorset cross sheep (UK) were orally infected with 150 *F. hepatica* metacercariae (South Gloucester isolate, Ridgeway Research Ltd) administered in water. Animals were euthanized at selected time points post infection by captive bolt and peritoneal cells harvested as previously described [16]. These experimental procedures were reviewed and approved by the Agri-Food Biosciences Institute (AFBI) Animal Ethics Committee, Northern Ireland, UK and carried out in accordance with the conditions of the operating license issued from the Department of Health, Social Services and Public UK by the Animal (Scientific Procedures) Act 1986 (License No. PPL 2771). All animal work was performed and reported according to the ARRIVE guidelines.

2.4.2. Preparation of murine macrophages.

Peritoneal macrophages were separated from the complete exudate cell population by negative selection using magnetic beads in accordance with the manufacturer's instructions (Miltenyi, USA). Bone marrow cells isolated from BALB/c mice were differentiated for 6 days in RPMI 1640 supplemented with FBS (10%v/v) and recombinant M-CSF (50 ng/ml; eBioscience, San Diego, CA, USA).

2.4.3. Small RNA library preparation and bioinformatics analysis.

The quality of the raw fastq files was assessed using FastQC and the adapters were removed using Cutadapt v1.13. The sequences with length smaller that 18bp were discarded also using Cutadapt. The reads were then pre-processed with an in-house perl script that filters homopolymers, sequences that contain Ns and low-quality reads. The mature miRNA

sequences of *Mus musculus* were retrieved from miRbase and used to build the index for the alignment using bowtie. After the pre-processing, the reads were aligned against the mouse mature miRNAs. The reads that did not align against the mouse mature miRNAs were aligned against the *F. hepatica* known miRNAs. After the alignments, an in-house script was used to count the reads that were successfully mapped and normalised to counts per million (CPM).

2.4.4. Preparation of parasite RNA

Fasciola hepatica metacercariae were sourced from Ridgeway Research Ltd, UK. The South Gloucester isolate was used to infect sheep which allowed the recovery of adult worms from the bile ducts. BALB/c mice aged 6-8 weeks were orally infected with 30 metacercariae of *F. hepatica* (Italian isolate, Ridgeway Research Ltd) to recover the 21 day old immature flukes. All animal experimental procedures were carried out at Queen's University Belfast, UK under license from the Home Office by the Animal (Scientific Procedures) Act 1986 (License No. PPL/2806) after ethical review by the Queen's University Belfast Animal Welfare and Ethical Review Body. The Italian isolate was also used for the excystment of newly excysted juveniles (NEJ) [78] and parasite culture for the recovery of extracellular vesicles (EVs) [101]. Total RNA from all parasites stages (NEJ, NEJ EV, immature and adult worms) was extracted using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, eluted in 30μl RNase-free water.

Caenorhabditis elegans were maintained in 35 mm diameter petri dishes containing Nematode Growth Medium agar, (3 g NaCl,17 g agar, 2.5 g peptone, 1 mL 1M CaCl2, 1 mL 5 mg/mL cholesterol in ethanol, 1 mL 1M $MgSO_4$, 25 mL 1M KPO_4 buffer (pH 6.0, 108.3g KH_2PO_4, 35.6g K_2HPO_4, H2O to 1 L)) seeded with a lawn of *Escherichia coli* OP50. After 3-4 days, when all life stages of worms were present (embryo, larvae stages 1-4, and adult), worms were sub-cultured into new plates containing NGM agar and *E. coli* OP50. Once adequate numbers were achieved, worms were harvested by washing petri dishes with S buffer (129 mL 0.05M KH_2PO_4, 871 mL 0.05M KH_2PO_4, and 5.85 g NaCl), and scraped off agar with a sterile glass rod. The worm suspension was then centrifuged at 1000 x g for 5 min, and the pellet containing the worms washed with sterile PBS. The final worm pellet was resuspended in 1 mL RNAzol RT (Molecular Research Centre Inc, USA) and homogenised with mortar and pestle for RNA extraction.

Adult males and female *S. mansoni* worms (n=166) in RNAlater stabilisation solution (Life Technologies, USA) was kindly donated by Prof. Donald McManus (Queensland Institute of Medical Research). Worms were washed with sterile PBS to remove RNAlater by centrifugation at 1000 x g for 5 min at 4°C. The final worm pellet was resuspended in 2 mL of RNAzol RT and homogenised with mortar and pestle for RNA extraction.

2.4.5. Preparation of macrophage extracellular vesicles

RAW 264.7 macrophages were cultured to confluency in RPMI + 10% FBS (v/v) at 37°C and 5% CO_2. Cells were collected by scraping and cellular debris removed by sequential centrifugation steps of 2000 x g for 20 min at 4°C followed by 10,000 x g for 30 min at 4°C. To obtain the extracellular vesicles the supernatant was subjected to ultracentrifugation at 104,492 x g for 2h at 4°C and the pelleted material re-suspended to maximum volume depending on the size of the centrifuge tube. This resuspension was then ultracentrifuged at 104,492 x g overnight at 4°C to separate the soluble proteins (now in the supernatant) and vesicles (now in the pellet).

2.4.6. miRNA target prediction

To determine possible gene targets of selected *F. hepatica* miRNA, the miRNA sequence of *fhe-miR-125b* was initially analysed using custom prediction with a murine background on the online miRNA target prediction tool, miRDB (mirdb.org). Additionally, mouse mRNA 3'UTR sequences were analysed for fhe-miR-125b target pairing using miRNA target predictive tools miRanda (http://www.microrna.org/) and TargetScan (http://www.targetscan.org). miRNA:mRNA pairings with a target score of >60 were considered in miRDB, target score >155 and minimum free energy (MFE) <-20 (Energy-Kcal/Mol) were considered in miRanda, and a seed sequence match of 7mer-m8 site in TargetScan. Only common genes determined by all predictive tools were considered for further analysis. The acquired list of gene targets was analysed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (david.ncifcrf.gov) and significant pathways targeted by *fhe-miR-125a* were determined using the murine Kyoto Encyclopaedia of Genes and Genomes (KEGG) database [102](40).

2.4.7. Microarray analysis

Microarray analyses were performed using the Illumina whole genome microarray platform (Illumina, USA) as previously described [103]. Quality control of data was performed using GenomeStudio (version 1.1.1, Illumina) by examining intensity histograms of hybridisation efficiency and noise. GeneSpring GX version 11 (Agilent Technologies, Foster City, USA) was used for all subsequent data analyses. All data were entered into GeneSpring GX 11 and normalised to the 75th percentile. Data for each replicate were normalised to respective uninfected controls and filtered for significance on the basis of detection score, a GenomeStudio generated measure of signal intensity relative to background. At least half of all hybridisations had to have a detection score >0.949, (which equates to a confidence value of $p \leq 0.05$) for a gene to be accepted. Gene expression values for commonly up- and down-regulated genes were averaged and a Students t-test was performed to identify significant changes in gene expression compared with controls ($p \leq 0.05$). An additional cut-off of ±2 fold change in expression was applied to identify changes in gene expression with likely biological significance.

2.4.8. Transfection of bone marrow derived murine macrophages

Bone marrow derived macrophages (1×10^6 cells/500 µL) were transfected with synthetic miRNAs (20 nM; (IDT, USA) combined with Lipofectamine RNAiMAX (Life Technologies, USA) over a period of 24h in accordance to manufacturer's protocol. Transfected cells were washed with sterile PBS before stimulation with *E. coli* lipopolysaccharide (LPS; 10 ng/mL) for 6h at 37°C and 5% CO_2 to induce an inflammatory response. Non-transfected cells, and cells treated with Lipofectamine RNAiMAX alone, without LPS stimulation, were also analysed to control for contamination and possible immune responses induced by transfection reagents. Non-transfected cells were stimulated with LPS to determine the baseline inflammatory response of cultured macrophages. After LPS stimulation, the culture media was collected from all treatment groups for cytokine assays, and the cells washed with sterile PBS and collected for RNA extraction.

2.4.9. RNA extraction from mammalian cells

Cells or extracellular vesicles were homogenised with the RNAzol RT at 1 mL to 10^7 cells. Ultrapure DNAse/RNase free distilled water (dH_2O) (Life Technologies, USA) was added to the homogenate, at 0.4 mL of dH_2O per 1 mL of RNAzol RT used, and then centrifuged at 12,000 x g for 15 min at 4°C, to pellet DNA, proteins, and polysaccharides. Supernatant containing soluble RNA was collected and combined with 4-bromoanisole (BAN; Sigma-Aldrich, USA) at 0.5% of supernatant volume, and then centrifuged at 12,000 x g for 10 min at 4°C to pellet residual DNA, proteins, and polysaccharides. To precipitate RNA, the supernatant was collected and combined with isopropanol (100% v/v; Sigma-Aldrich, USA) and 5 µL glycogen (5 mg/mL; Life Technologies, USA), and incubated overnight at -20°C. The RNA precipitate was pelleted by centrifugation at 12,000 x g for 10 min at 4°C, washed with 500 µL ethanol (75% v/v; Sigma-Aldrich, USA) twice and re-solubilised in 25 µL dH_2O. Concentrations and quality of isolated RNA was measured using the NanoDrop One/One^c UV-Vis spectrophotometer (Life Technologies).

2.4.10. Pull-down of Argonaute-2 (Ago-2)-bound miRNA from peritoneal macrophages

The MagCapture microRNA isolation kit specific for mouse Ago-2 (FUJIFILM Wako Pure Chemical, JPN) was used to pull-down Ago-2 protein from extracts of peritoneal macrophages according to manufacturer's instructions. Ago2 was harvested, resuspended in 200 µL dH_2O and bound miRNA purified using RNAzol RT and used as a template for RT-qPCR.

2.4.11. RT-qPCR

After extraction of RNA, all samples were normalised to 100 ng/uL prior to cDNA synthesis. cDNA synthesis was carried out using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, USA) according to manufacturer's instructions. Taqman primers were designed using fhe-miR-125b (5'- CCCCUGAGACUGAUAAUUGCUC-3'), hsa/mmu-miR-125b (5'-UCCCUGAGACCCUAACUUGUGA-3') and mmu-miR-21 (5'- UAGCUUAUCAGACUG AUGUUGA-3') mature sequences, with respective Taqman hydrolysis probes incorporating 5'-FAM or 5'-JUN reporter dye. This ensured specificity for each miRNA and negated the possibility of cross amplification. Synthesised cDNA was further diluted 1:4 dH_2O. For RT-qPCR, 1 µL of diluted cDNA was combined with 5 µL of SensiFAST Probe Hi-ROX Kit

(Bioline, UK), 3.5 µL dH_2O, and 0.5 µL of TaqMan Probe for gene expression or miRNA. Samples were prepared on a MicroAmp optical 96-well reaction plate as technical triplicates (Life Technologies, USA) and analysed using the QuantStudio 6 flex real-time PCR system (Life Technologies, USA). Data was analysed using LinRegPCR (v2017.1), and presented as absolute expression of genetic material prior to amplification (N0).

2.4.12. Cytokine assay

The quantities of IL-6 and TNF secreted by transfected bone marrow derived macrophages was determined using ELISA kits (BD Biosciences, USA) according to manufacturer's instructions.

2.4.13. Statistical analysis

Results were analysed using Graphpad prism software (version 8). Comparisons between samples were made using one-way ANOVA. Differences were considered significant when $P \leq 0.05$.

2.5. Results

2.5.1. Parasite-derived miRNAs are located within macrophages during infection with *Fasciola hepatica*.

To investigate whether miRNAs produced by *F. hepatica* contributed to the inhibition of macrophage activation in early infection, we orally infected BALB/c mice with *F. hepatica* metacercariae and searched for parasite miRNAs in peritoneal macrophages at selected time-points (6h, 12h, 24h, 3 days and 5 days after infection) using RNASeq (deposited in NCBI's GeneExpression Omnibus; accession number GSE145597; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145597). After first cleaning the datasets obtained, reads were mapped against the mouse database from miRBase (mmu). Then, to identify *F. hepatica*-specific miRNAs the remaining 15,796,193 reads were screened against the miRBase list of 38 *F. hepatica* miRNAs [39]. Using this approach, we identified 10 *F. hepatica* miRNAs that were present within peritoneal macrophages of infected mice (Table 2.1).

This compares to an average of 447 mouse miRNAs that were identified within the macrophages across all infected and uninfected mice. Furthermore, as expected the endogenous mouse miRNAs were more abundant than the exogenous Fasciola miRNAs, with read counts ranging from 1 – 241,373 CPM, as compared to a range of 1 – 16 CPM respectively (Table 2.1). Of the parasite miRNAs, *F. hepatica* miR-125b (fhe-miR-125b) was the most abundant observed in peritoneal macrophages during the first 24h of infection, with expression peaking at approximately 12h after infection. This pattern of abundance is coincident with an increase in the expression of endogenous murine miR-125b, which occurs as soon as 6h after infection and is maintained for the first three days of infection (Table 2.1). The timing of this increase in both mouse and parasite miR-125b is coincident with the migration of NEJs through the intestine and into the peritoneal cavity, and with the observed early suppression of host innate immune responses, as described above [16, 20, 89], suggesting a coordinated mechanism of immune regulation by host and parasite.

This rapid and transient expression pattern of *fhe-miR-125b* in peritoneal macrophages was corroborated by RT-qPCR (Figure. 2.1a). Consistent with these observations, the expression of *fhe-miR-125b* was also observed in peritoneal cells isolated from sheep after an experimental infection with 150 metacercariae (Figure. 2.1b). These miRNAs were not detected in peritoneal macrophages taken from non-infected mice or sheep (Figure. 2.1a, b).

Table 2.1. Total read numbers after normalisation of known *Fasciola hepatica* miRNAs detected by miRNASeq in peritoneal macrophages harvested from infected mice over a time-course of 5 days.

miRs	Time after infection											
	Uninfected[a]		6 hrs		12 hrs		24 hrs		3 days		5 days	
fhe-bantam	–[b]	–	2	1	–	–	1	–	–	–	–	–
fhe-miR-125b	–	–	1	16	6	16	1	2	–	1	–	–
fhe-miR-36a	–	–	1	–	–	–	–	–	–	–	–	–
fhe-miR-61	–	–	–	3	1	5	–	3	–	–	–	–
fhe-miR-125a	–	–	–	1	–	–	–	–	–	–	–	–
fhe-miR-46	–	–	–	1	–	1	–	–	–	–	–	–
fhe-miR-277	–	–	–	2	–	3	–	–	–	–	–	–
fhe-miR-10	–	–	–	1	–	–	–	–	–	–	–	–
fhe-miR-219	–	–	–	–	1	1	–	–	–	–	–	–
fhe-miR-71b	–	–	–	–	–	1	–	–	–	–	–	–
mmu-miR-125b	793	1395	3121	2394	2642	2123	1958	2371	2390	2038	880	836

[a] Two biological samples were sequences at each time point with read count for each sample presented in columns below. [b] "-" no read counts detected. Reads represented as CPM

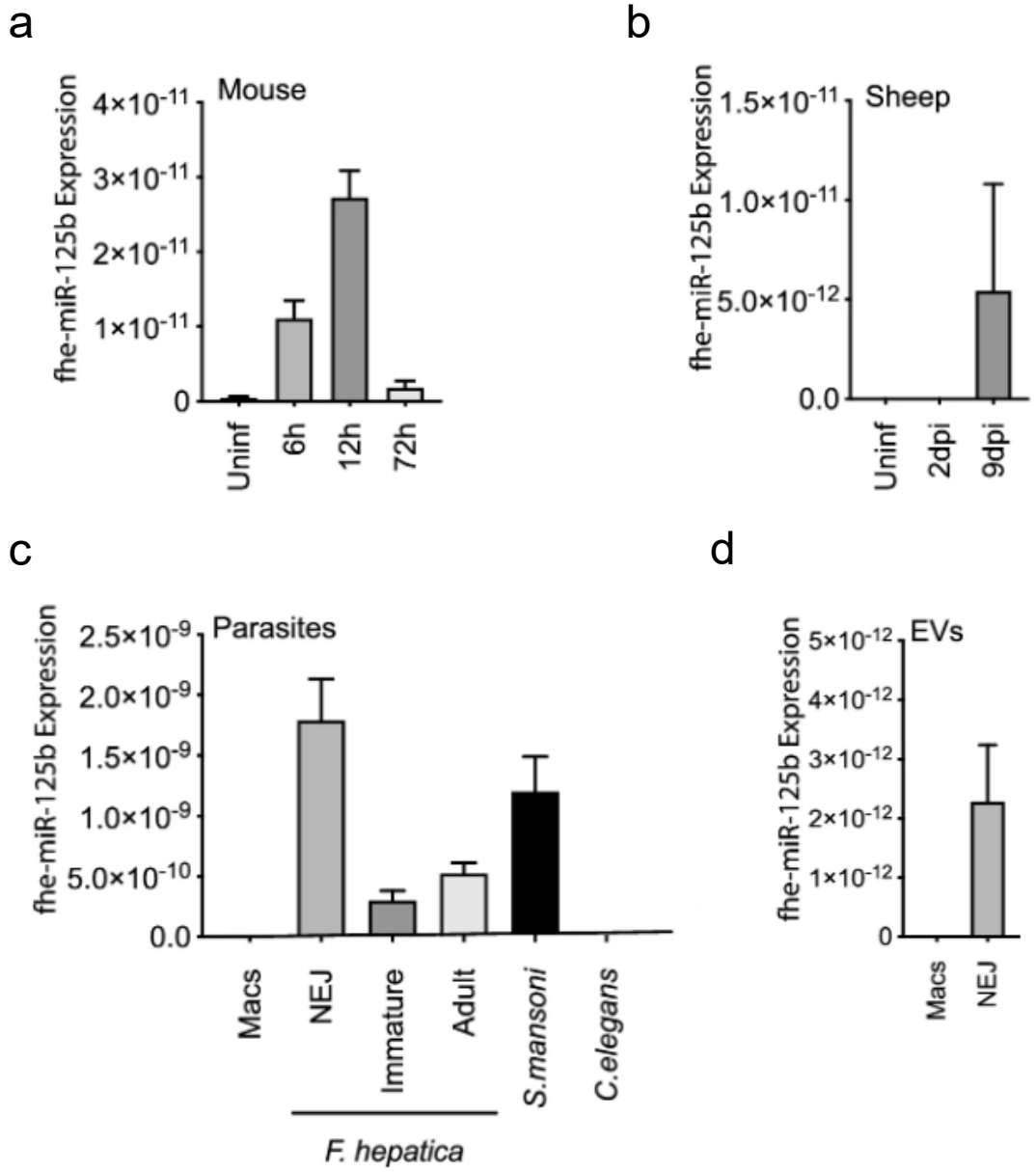


Figure 2.1. Worm-derived miRNA fhe-miR125b is detected within the peritoneal macrophages of mice and sheep infected with *Fasciola hepatica*. The presence of worm fhe-miR-125b was (a) validated in the peritoneal macrophages that had been harvested from mice at 0 h, 6 h, 12 h and 72 h after an oral infection with 20 *F. hepatica* metacercariae and subjected to small RNA sequence analysis; and examined in (b) peritoneal cells harvested from uninfected sheep and from sheep at 2 and 9 days after an oral infection with 150 metacercariae. (c) The expression of fhe-miR-125b was measured in RNA extracted from the *F. hepatica* newly excysted juveniles (NEJ), 21-day old worms (immature) and adult worms and compared to expression levels in adult (male and female mixed population) *Schistosoma mansoni* and *Caenorhabditis elegans*. (d) RT-qPCR using primers specific for fhe-miR125b was carried out on RNA extracted from NEJ extracellular vesicles (EVs). In these experiments, levels of fhe-miR-125b expression determined by RT-qPCR are represented as the starting quantity of genetic material prior to amplification (N0) as determined by LinRegPCR (v.11). The data is presented as the mean ± SD of triplicate biological samples across mouse/sheep studies and cell/EV experiments.

To verify that the primers were specifically detecting a miRNA of parasite origin, infectious metacercariae were experimentally excysted in vitro and the RNA of the emerging newly excysted juvenile (NEJ) subjected to RT-qPCR. This confirmed that *fhe-miR-125b* is expressed by the NEJ parasites (Figure. 2.1c). RT-qPCR also demonstrated that *miR-125b* could be amplified from RNA isolated from *Schistosoma mansoni*, a trematode parasite like *F. hepatica*, but not from the free-living nematode *Caenorhabditis elegans*. This result concurs with reports demonstrating that the expression of a *miR-125b* is specific to trematode parasites [(104)]. Comparison of the expression of *fhe-miR-125b* across the different intra-mammalian life-stages of *F. hepatica* revealed strict temporal expression (Figure. 2.1c), supporting the idea that worm miRNAs govern developmental processes. In the context of a role in the immune modulation of innate immune responses after infection, it was notable that the expression of *fhe-miR-125b* was most abundant in NEJs, the stage that initiate infection.

Several studies suggest that extracellular vesicles (EVs) are the main reservoir of exogenous miRNAs. In this study, EVs were harvested from in vitro cultured NEJs and shown to contain *fhe-miR-125b* (Figure. 2.1d). Since EVs released during helminth infections are primarily taken up by macrophages [(34, 37)] this provides a potential mechanism by which *fhe-miR-125b* and other miRNAs may be delivered into the macrophages.

2.5.2. *Fasciola hepatica* miR-125b targets innate immune signalling pathways

The secondary structure of *fhe-miR-125b* indicates a typical miRNA with the mature strand located on the 5′ arm of the precursor (Figure. 2.2a). Previous studies have documented human *hsa-miR-125b* as a miRNA that regulates the inflammatory response of macrophages by controlling the activation of M1 macrophages [(105-107)]. Using a phylogenetic approach, we compared the structure of fhe-miR-125b to its human homologue and to *miR-125* sequences from the closely related trematodes, *S. japonicum* and *S. mansoni* (Figure. 2.2.b). This analysis revealed that *miR-125b* in *Fasciola* is closely related to the human miRNA but is distinct from *miR-125b* found in the *Schistosome* parasites. This may suggest *fhe-miR-125b* could target the same host genes as *hsa-miR-125b* and thus play a significant role in the control of host immune responses during early infection.

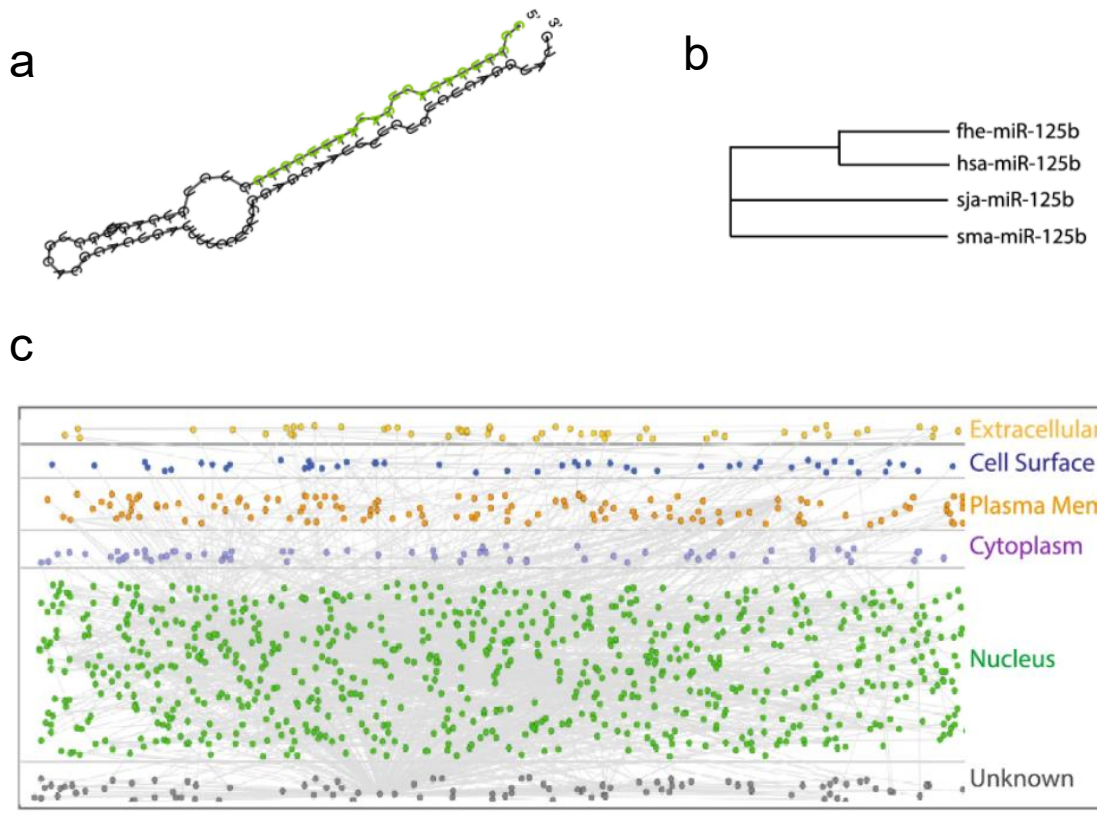

a
b
fhe-miR-125b
hsa-miR-125b
sja-miR-125b
sma-miR-125b
c
Extracellular
Cell Surface
Plasma Membrane
Cytoplasm
Nucleus
Unknown
d
MicroRNAs in cancer
Epstein–Barr virus infection
Toxoplasmosis
Salmonella infection
Proximal tubule bicarbonate reclamation
Neurotrophin signaling pathway
TNF signaling pathway
RIG–I–like receptor signaling pathway
NOD–like receptor signaling pathway
Pathways for pluripotency of stem cells
Adherens junction
Hippo signaling pathway
Osteoclast differentiation
Notch signaling pathway
Adrenergic signaling in cardiomyocytes
Endocytosis
Protein processing in endoplasmic reticulum
Sphingolipid signaling pathway
MAPK signaling pathway
Sphingolipid metabolism
P Value
0.075
0.050
0.025
2.5
5.0
7.5
10.0
12.5
Number of Genes
e
f
Traf6 mRNA Expression
2.5×10-10
2.0×10-10
1.5×10-10
1.0×10-10
5.0×10-11
0.0
***
***
Uninf
6hr
12hr

Figure 2.2 TRAF6 is the primary gene predicted as a target for fhe-miR-125b in peritoneal macrophages. (a) The hairpin structure of fhe-miR125b, produced by RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) (b) Phylogram *of miR-125* generated using T-Coffee (http://tcoffee.crg.cat), based on the hairpin structures. (c) Illustration of a Protein–Protein Interaction map for the 798 immune-related genes predicted from gene target analysis (created by Innate DB; https://www.innatedb.com). Of these, the nucleus showed the highest number of interactions account for greater than 50% of all the interactions. (d) GO analysis of pathways represented by the predicted gene targets for *fhe-miR-125b*, produced using bioconductor (https://www.bioconductor.org). (e) Mapping predicted gene targets to a mouse interactome produced 66 nodes (image created by Innate DB; https://www.innatedb.com). Within these, Traf6 (red box) mapped to 35–40% of the nodes (Blue dots). All other nodes not connected to Traf6 are shown in yellow and red. A detailed map of these interactions can be viewed in Supplementary Figure 2.1. (f) The expression of TRAF6 was measured in RNA extracted from peritoneal macrophages harvested from uninfected mice (Uninf) or from mice at 6h and 12h after an oral infection with *F. hepatica*. The level of expression is represented as the starting quantity of genetic material prior to amplification (N0) as determined by LinRegPCR (v.11). The data is representative of three independent experiments and is presented as the mean ± SD of triplicate biological samples. The data was analyzed using one-way analysis of variance (ANOVA).

To address this possibility experimentally, a list of putative cellular targets for mature *fhe-miR-125b*, (CCCCUGAGACUGAUAAUUGCUC) was initially generated using the online miRNA target prediction tool miRDB (https://mirdb.org). Only those genes assigned a target score >60 (a predictive score generated by MirTarget; [108]) were regarded as authentic and selected for further analysis. The resultant 511 genes (Supplementary Table 2.1) were mapped for protein-protein interactions using the innate immune data reference set [52] and provided 798 immune-related protein-protein interactions with an enrichment in the nucleus (Figure 2.2c). This dataset was then filtered down to cell specific associations restricted to peritoneal macrophages. Surprisingly, from this analysis, only two gene targets emerged; Traf6 and Nlrc5. Pathway analysis of the interactions showed that Traf6-mediated events were over-represented (Figure 2.2e).

To further scrutinise if Traf6 could be the prime target candidate for *fhe-miR-125b,* two additional predictive tools were then used, miRanda and TargetScan (Supplementary Table 2.1). Supporting the miRDB initial analysis, TRAF6 was one of only 43 genes that were commonly predicted as targets for *fhe-miR-125b* by all three tools. Furthermore, the significant reduction in the expression of TRAF6 in peritoneal macrophages harvested from mice at the same timepoints that *fhe-miR-125b* is most abundant (Figure 2.2f), provides experimental evidence to support the predictive analysis. As a signalling transducer that is central to several immune signalling pathways, TRAF6 is regarded as an indispensable regulator of inflammatory responses [109]. Indeed, gene ontology analysis of the predicted gene target list

conducted against a *Mus musculus* background using GO tool DAVID (http://david-d.ncifcrf.org) identified major signalling pathways known to control innate inflammatory responses (MAPK signalling, TNF signalling, NOD-like receptor signalling) within the top ten generated target KEGG pathways [102] (Figure. 2.2d and Supplementary Table 2.2) thus providing more evidence for a role for *fhe-miR-125b* in the regulation of immune responses.

A central role for *fhe-miR-125b* in the regulation of these signalling pathways was also corroborated by expression profiling of macrophages harvested from the peritoneal cavity of mice 18h after an oral infection with *F. hepatica*. Of the 610 genes that were identified to be significantly ($p > 0.05$) downregulated (>2-fold change in expression), a large number of these were pro-inflammatory/innate cytokines and chemokines or transcription factors that regulate cytokine-signalling pathways (Supplementary Table 2.3). This is illustrated in the global KEGG analysis of these genes which created a profile suggesting involvement predominantly in MAPK, JAK-STAT, TNF and TLR signalling (Supplementary Figure 2.2) and which was almost identical to that predicted for *fhe-miR125b* using GO tool DAVID above (Figure. 2.2).

To provide experimental proof of the ability of *fhe-miR-125b* to directly engage in the regulation of pro-inflammatory pathways in macrophages, primary murine macrophages were transfected with synthetic versions of both the *F. hepatica* and human *miR-125b*. While neither miRNA had any apparent effect on macrophages when transfected under basal culturing conditions, both transfections significantly inhibited the ability of macrophages to express TRAF6 in response to a challenge with bacterial LPS (Figure. 2.3a) and, as a consequence, the production of both IL-6 and TNF were significantly reduced (Figure. 2.3b, c). This outcome was specific to the *miR-125b* as transfection with a scrambled non-targeting miRNA had no effect. We propose, therefore, that the *F. hepatica fhe-miR-125b*, like its mammalian counterpart, functions to regulate the ability of macrophages to respond to pro-inflammatory signalling, thus preventing the differentiation to an M1 phenotype.

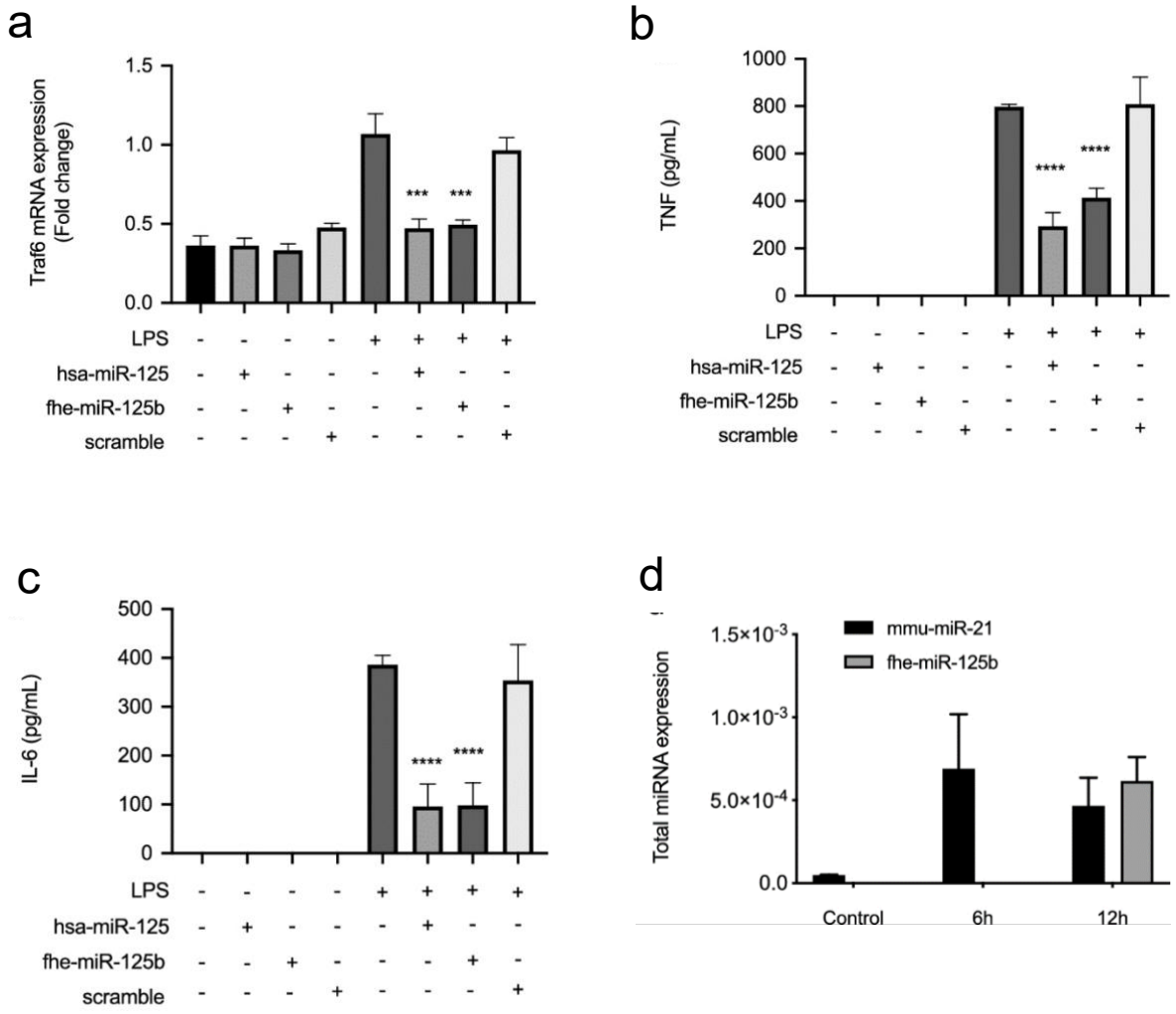


Figure 2.3. TRAF6 signaling and downstream production of IL-6 and TNF by macrophages is suppressed by fhe-miR-125b by binding the mammalian argonaute protein-2. Bone marrow derived macrophages were transfected with the parasite-derived *fhe-miR-125b*, the human *miR-125b* or a scrambled miRNA (20nM) and stimulated 24h later with bacterial lipopolysaccharide (LPS; 10 ng/ml) for 6h. (a) The expression of TRAF6 was measured using RT-PCR and is presented the fold change in expression as compared to the LPS stimulated cells. The data is the mean ± SD of three independent experiments, each with biological triplicate samples. (b) The quantity of TNF and (c) IL-6 secreted by the macrophages into culture medium was measured by ELISA. The data shown is the mean ± SD of two independent experiments, each with biological triplicate samples. (d) Argonaute (Ago)-2 proteins were pulled down from extracted murine peritoneal macrophages harvested from uninfected BALB/c mice (n=3) and from mice (n=3) at 6h and 12h post-infection with *F. hepatica*. Ago-2-bound RNA was extracted and analysed using RT-qPCR with primers specific for fhe-miR-125b. The quantity of miRNA detected is presented as the starting quantity of genetic material prior to amplification (N0) as determined by LinRegPCR (v.11). For all experiments, data was analyzed using one-way analysis of variance (ANOVA).

2.5.3. *Fasciola hepatica* miR-125b complexes with the mammalian Argonaute-2 protein in host macrophages to regulate mammalian gene expression.

The mechanism(s) by which the exogenous worm miRNAs operate in mammalian immune cells to alter their function remains enigmatic. However, studies in mammalian cells have shown that in the first step miRNAs must form a complex with an Argonaute protein (Ago). This Ago-miRNA association forms the core of the miRNA-induced silencing complex (miRISC), which is then guided by the complexed miRNA to bind complementary (target) sequences in the messenger RNA (mRNA) [110]. Helminth Ago proteins have been detected within secreted EVs and may be involved in the regulation of mammalian target genes [34]. However, given the similarity of *fhe-miR-125b* to its mammalian homolog we surmised that helminth miRNAs delivered into immune cells could directly load onto mammalian Ago2. In this way worm miRNAs would compete with the mammalian miRNA machinery to influence host gene expression. To test this idea, murine Ago2 was isolated by immunoprecipitation from the peritoneal macrophages taken from infected mice at 6h and 12h post infection (IP; Supplementary Figure 2.3) and the miRNA extracted and subjected to RT-qPCR. We used the detection of miR-21 as a positive pulldown control since this miRNA operates within macrophages to regulate host gene expression and is expected to be loaded onto Ago2 (Figure. 2.3d). Our result showed that that *fhe-miR-125b* was bound to Ago2 in macrophages taken from mice infected at the 12h time point (a time when *fhe-miR-125b* expression was at its peak; Table 2.1).

2.6. Discussion

On invasion of the host by the parasite *F. hepatica* protective pro-inflammatory cytokine expression in macrophages is quickly blocked. Thus, a major player in the innate cell immune system is inactivated at a stage when the parasite is most vulnerable and depends on its glycogen stores for energy to power its way from the intestine to the liver. Our study suggests that this trematode parasite, and perhaps others, exploit homologs of the immune-regulatory miRNAs of the host to hijack miRNA gene regulatory pathways to disarm their function.

Several parasitic worm miRNAs share homology with mammalian miRNAs. Thus, they have the capacity to regulate the expression of an array of host genes within macrophages and other innate cells to effectively evade expulsion and continue their growth and development to maturity. Our gene target analysis and *in vitro* studies pinpoint a specific mechanism whereby *F. hepatica* could interrupt the MAPK signalling pathway of innate immune responses via the regulation of TRAF6 by *fhe-miR-125b*. This mechanism is supported by the presence of a single *miR-125b* binding site on the 3′ UTR of TRAF6 [111]. The identification of mammalian genes and immune pathways targeted by parasite miRNAs has major implications for our understanding of the interaction between parasite and host.

It is highly significant that *miR-125b*, the most predominant parasite miRNA found in host macrophages of mice infected with *F. hepatica*, is conserved amongst the trematode clade of helminths, including the blood flukes of the genus *Schistosoma*, as it points towards a common mechanism for the anti-inflammatory control amongst these important parasites of humans and their livestock. Indeed, recent studies have shown that the *miR-125b* orthologue is highly enriched in EVs released by *S. mansoni* and *S. japonicum*, representing 40% and 65% respectively of the total miRNA reads [36, 112]. However, in contrast to the immune modulatory effect of *fhe-miR-125b* we have shown here, transfection of murine macrophages with *sja-miR-125b* resulted in the induction of pro-inflammatory immune responses as assessed by an increased production of TNF [36]. Predictive analysis of this parasite miRNA suggests that *sja-miR-125b* was targeting the expression of Pros1 [113], a gene known to inhibit TLR-driven pro-inflammatory responses. Neither of the three predictive tools used in our study, predicted this gene as a target for the *F. hepatica miR-125b,* nor was it identified amongst the genes down-regulated in macrophages isolated from infected mice. These contrasting observations could suggest that *miR-125b* plays different functions for different parasites. Unlike *F. hepatica*, a pro-inflammatory Th1-type immune response dominates during the first 3–5 weeks of infection

with *Schistosome* parasites. As the parasites mature and females become fecund, parasite eggs trapped in tissues induce potent Th2 responses that suppress Th1 responses [114]. The *sja-miR-125b* was identified within EVs isolated from parasites collected from mice at 28 days post-infection [36], a pre-patent time point, coincident with a strong Th1 pro-inflammatory immune response.

A single miRNA can influence the expression of a multitude of different genes. mRNA silencing efficiency is determined by the sequence of the seed region, free energy produced by the miRNA-target duplex and stability of the miRNA 5' terminal and seed region [115]. While high specificity of the miRNA seed sequence has been largely reported as the crucial factor to miRNA target recognition, nucleotide pairings outside of the seed are also known to affect gene target recognition and overall thermodynamic stability of the miRNA-target duplex [116]. These key concepts are integrated in predictive tools such as miRDB and MiRanda that scrutinise whole mature miRNA sequences. Examining the overall structure of miR-125 from various species, phylogenetic analysis indicates that fhe-miR-125b is closely related to its human counterpart and distal to that of *miR-125b* found in *Schistosome*. This may explain why Pros1 was not predicted to be a target for *fhe-miR-125b* and only regulated by *sja-miR-125b*. It would be interesting to determine which exact nucleotides outside the seed region may account for this specificity.

miRNAs are not functional until they are loaded onto an Ago protein, a process that facilitates base-pairing to mRNA targets and ultimately translational repression and mRNA degradation. Here we provide proof that exogenous, worm-derived miRNA is delivered into macrophages, probably via exosomes, during infection and loads onto host Ago2. Coupled with our *in vitro* transfection studies that shows *fhe-miR-125b* can alter macrophage phenotype we can deduce that parasite miRNAs are functional within mammalian cells. We have to assume that this binding mimics that of host *hsa-miR-125b* and, therefore, operates in the same way, to regulate the pro-inflammatory response of macrophages [105-107].

It has been reported that miRNAs that have homologs in mammalian hosts dominate the miRNA cargo of *F. hepatica* EVs. However, a number of miRNAs which are novel to the parasite have also been identified here and elsewhere[24]. Predictive analysis suggests that these too have the capacity to regulate immune-related mammalian genes [37, 38, 98]. As the loading of miRNAs to Ago protein is a very precise process with the requisite thermodynamics and base-match composition, it remains to be determined whether homology to a mammalian miRNA is

essential for a parasite miRNA to associate with mammalian Ago and annex the mammalian miRNA machinery.

The ability of *F. hepatica* to invade, infect and produce progeny in a very broad range of species indicates a strong universal compatibility between the parasite and its mammalian host, particularly in terms of attuning the immune response. This study provides direct *in vitro* and *in vivo* evidence for the role of helminth-derived miRNAs in this complex parasite-host immune-interplay. Further elucidation of how mechanisms of immune modulation, involving nucleic acids, proteins and glycans, are employed by the parasite, especially during the early stage of infection, is critical for development of effective anti-helminth therapies to prevent infection and pathogenesis.

Chapter 3:

Stage-specific miRNAs regulate gene expression associated with growth, development and parasite-host interaction during the intra-mammalian migration of the zoonotic helminth parasite *Fasciola hepatica*

3.1. Introductory Statement

The work presented in this chapter significantly contributed to the publication of a Research Article:

Ricafrente, A., Cwiklinski, K., Nguyen, H., Dalton J.P., Tran, N. & Donnelly, S. Stage-specific miRNAs regulate gene expression associated with growth, development and parasite-host interaction during the intra-mammalian migration of the zoonotic helminth parasite *Fasciola hepatica*. ***BMC Genomics***. 2022 Dec;23(1):1-9.

3.2. Abstract

MiRNAs are small non-coding RNAs that post-translationally regulate gene expression in organisms ranging from viruses to mammals. There is great relevance in understanding how miRNAs regulate genes involved in the growth, development, and maturation of the many parasitic worms (helminths) that together afflict more than 2 billion people. Here, we describe the miRNAs expressed by each of the predominant intra-mammalian development stages of *Fasciola hepatica*, a foodborne flatworm that infects a wide range of mammals worldwide, most importantly humans and their livestock. A total of 124 miRNAs were profiled, 72 of which had been previously reported and three of which were conserved miRNA sequences described here for the first time. The remaining 49 miRNAs were novel sequences of which, 31 were conserved with *F. gigantica* and the remaining 18 were specific to *F. hepatica*. The newly excysted juveniles express 22 unique miRNAs while the immature liver and mature bile duct stages each express 16 unique miRNAs. We discovered several sequence variant miRNAs (IsomiRs) as well as miRNA clusters that exhibit strict temporal expression paralleling parasite development. Target analysis revealed the close association between miRNA expression and stage-specific changes in the transcriptome; for example, we identified specific miRNAs that target parasite proteases known to be essential for intestinal wall penetration (cathepsin L3). Moreover, we demonstrate that miRNAs fine-tune the expression of genes involved in the metabolic pathways that allow the parasites to move from an aerobic external environment to the anerobic environment of the host. These results provide novel insight into the regulation of helminth parasite development and identifies new genes and miRNAs for therapeutic development to limit the virulence and pathogenesis caused by *F. hepatica*.

3.3. Introduction

Micro(mi)RNAs are a conserved class of small non-coding RNAs (18-25 nucleotides) that regulate gene expression post-transcriptionally [117]. Highly specific binding of the miRNA seed region (nucleotides 2-8) onto a target mRNA 3'UTR can induce complete degradation of the mRNA or obstruct ribosomal scanning to prevent translation [118, 119]. This interaction was first described in the free-living nematode *Caenorhabditis elegans* where the let-7 miRNA was found to be temporally expressed and necessary for the developmental progression of the worm [120], a function that was later found to be conserved in higher organisms [121]. However, with the completion of numerous genome sequences and the identification of many miRNAs in uni- and multi-cellular plants and animals that did not exhibit temporal expression concomitant with development it became evident that miRNAs perform more complex biological functions in regulating gene expression [122-124]. It is now recognised that most mRNAs are regulated by one or more miRNAs [125] and that they play central roles in co-ordinating gene expression during growth, development, differentiation, metabolism, reproduction, and pathogenesis [124, 126-129].

There is increasing interest in the identification and functional characterisation of miRNA in parasitic worms (helminths). Soil-transmitted intestinal nematodes such as hookworms (*Ancylostoma duodenale* and *Necator americanus*), *Trichuris trichiura* and *Ascaris lumbricoides* infect over two billion people worldwide [130] while flatworms like *Schistosoma spp*. cause ~200 million infections per year [131]. Recent studies have shown that miRNAs are essential not only for the development of parasites within their hosts but for regulation of the complex interactions between them [31, 132, 133]. The release of miRNAs by parasites, either freely or as part of their extracellular vesicle cargo, is likely to have been important in the co-evolution of the host-parasite relationship, particularly when the parasites exploit their miRNome to modulate host immune responses [134-136]. The detection of altered expression of unique parasite miRNAs in blood or other tissues could be exploited for the development of novel biomarkers and/or diagnostics of disease [137-140], or may possibly predict or explain the emergence of drug resistance [31].

The flatworm *Fasciola hepatica* (liver fluke) is a zoonotic parasite with a remarkable global prevalence due to its unique capacity to infect and mature in a broad range of mammals [141]. Approximately 2.4 – 17 million are infected with the parasite and 180 million are at risk of infection which recently impelled the World Health Organisation to classify fasciolosis as a food-borne trematode priority disease [3]. The global economic burden imposed by liver fluke

onto the agriculture industry and by human and animal healthcare costs is likely to be in the many billion dollars (US) each year [142].

Fasciola hepatica is a very tractable model parasite for investigating the regulation of parasite development and parasite-host interaction because it progresses through distinct stages of growth that are associated with defined host tissues. Moreover, updated draft genomes and extensive stage-specific transcriptomics as well as proteomics (somatic, secretions, tegument and extracellular vesicles) have laid a solid foundation on which to attribute a molecular explanation to processes linked to development [143, 144]. Infection with *F. hepatica* occurs following the ingestion of pasture contaminated with the infective metacercariae that rapidly emerge in the small intestine as newly excysted juveniles (NEJs). Within hours, the NEJs alter their metabolic activities, penetrate the gut wall tissue and migrate to the liver [78, 145]. While migrating within the liver parenchyma the parasite matures, develops digestive and reproductive structures, and undergoes a huge growth phase, doubling in size every few weeks [146-148]. The parasites cause extensive liver damage and haemorrhaging before moving into the bile ducts to complete their maturation and commence the production of progeny (eggs) that are carried with the bile juices into the intestine and liberated onto pasture with the faeces. Accompanying these strict developmental changes as the parasite migrates are highly regulated alterations in gene expression with progressively more genes being expressed with higher fold changes as infection and parasite maturation proceeds [149].

Several independent analyses of the *F. hepatica* miRNome have been performed on RNA derived from NEJs, mature adults, and adult extracellular vesicles and have yielded a collection of 77 *F. hepatica* miRNAs [33, 37-39, 46]. To provide a more detailed and composite picture of the role of miRNAs in *F. hepatica* and its interaction with the mammalian host, we have simultaneously sequenced the small RNAs obtained from the three critical stages of development, including the NEJs that cross the intestine, immature flukes that migrate in the liver parenchyma and adult parasites that reside in the bile ducts, and mapped these against the extensive transcriptome data for these same life cycle stages [78, 146, 149]. Thus, we have expanded the miRNome repertoire to 124 miRNAs, temporally mapped expression to specific life stages and identified their regulatory gene targets that are essential to parasite growth and development.

3.4. Methods

3.4.1. Sample preparation of parasite material

Fasciola hepatica metacercariae (Italian isolate), sourced from Ridgeway Research Ltd (UK) were used for excystment and 24h culture of newly excysted juveniles (NEJ) [78], and oral infections of mice and sheep, to recover 21-day immature flukes [146] and adult parasites [150], respectively, as previously described. The animal experiments were carried out under license from the Home Office by the Animal (Scientific Procedures) Act 1986 after ethical review by the respective intuition's Animal Welfare and Ethical Review bodies; mouse experiments (License No. PPL/2806, Queen's University Belfast, UK) and sheep experiments License No. PPL/60/4426, Moredun Scientific, UK).

Total RNA was extracted from the three *F. hepatica* life stages in triplicate using the miRNeasy mini kit (Qiagen) according to the manufacturer's instructions, in a final elution of 50 µL RNase-free water (Number of parasites per replicate for each stage: NEJ: 1,000; Immature fluke: 19; Adults: one). RNA integrity and concentration were confirmed using the 260/280 LVis plate functionality of the PolarStar Omega Spectrophotometer (BMG LabTech) and the Quant-iT RiboGreen RNA Assay Kit (ThermoFisher Scientific).

3.4.2. Sequencing

Library preparation of total RNA from samples was performed by ArrayStar using the Small RNA library Prep Set for Illumina and sequenced using Illumina NextSeq 500. From FASTq sequencing files, adaptor sequences were excised and filtered for low quality (<20 phred score) sequences and low length sequences (<18 nt) using bioinformatic tool CutAdapt (v3.4).

3.4.3. Bioinformatics – interrogation, quantification and annotation of *F. hepatica* miRNAs

Mature miRNA sequences from the miRBase *F. hepatica* repository (Fhepatica_v1, miRBase v21) and other published sources as described in Ricafrente et al. [151] were aligned to cleaned reads using Bowtie (v1). Interrogation and quantification of novel *F. hepatica* miRNAs was performed using miRDeep2 (v2). Precursor miRNA structures were predicted using the *F. hepatica* genome generated by WormBase Accession (PRJEB25283) and compared to mature miRNAs sequences from *F. hepatica* (miRbase and published literature), alongside mature

miRNAs from trematodes *Schistosoma japonicum* (ASM15177v1), *S. mansoni* (WTSI5), and non-parasitic nematode *C. elegans* (WBcel235). Mature miRNA sequences that were highly similar were considered isomiRs when the expression of sequence variants (that were not already considered isomiRs in previous studies) could be differentiated between the life stages by at least a 2-fold change in expression. The bioinformatic workflow is shown in Supplementary Figure 3.1. Sequences were only considered to be novel when read counts were present in all three triplicates of at least one of the life stages and when the genome coordinates of its predicted precursor miRNA structure were the same within the triplicate for that life stage.

To address the different annotation styles that have been historically used to describe the *F. hepatica* miRnome, miRNAs in this study were annotated using methodology featured in [43] to minimise duplication of miRNA identities. As a result, some previously identified miRNAs were renamed based on the best fitting homology to conserved seed and precursor structures using BLASTN (Supplementary Table 3.1). IsomiRs were annotated with a suffix that matched conserved miRNAs in miRBase using BLASTN or based on chronological discovery from earliest to most recent identification in other published works. Novel miRNAs were annotated based on conserved miRNAs in miRBase using BLASTN. All other novel miRNAs that were not found to be conserved and are species specific were annotated as follows (using miRBase convention); miRNAs were named fhe-Novel-x or if homologous to a previously published non-conserved miRNA then named fhe-pubNovel-x, and arbitrarily numbered in sequence. 5p or 3p was added to differentiate between miRNAs that originate from the 5' or 3' halve of the precursor hairpin. Finally, miR-X-1-5p and miR-X-2-5p was used to denote two miRNAs with the same miR-X mature sequence, but with 2 different genome locations.

3.4.4. Phylogenetic analysis

To determine phylogeny of the trematode miRNAs, a phylogenetic tree of all precursor miRNAs of *F. hepatica* from this study, and precursor sequences of *S. japonicum* (ASM15177v1) and *S. mansoni* (WTSI5) from miRBase (v22) were aligned using the multiple sequence alignment program T-coffee (www.ebi.ac.uk/Tools/msa/tcoffee) using default parameters for the neighbour joining method and the phylogenetic tree visualised using iTOL (v5)[152]. Using similar methods, the phylogeny of Let-7 precursor miRNAs was determined

using precursor sequences from miRBase repository (v22); human – *Homo sapiens* (GRCh38), mouse – *Mus musculus* (GRCm38)– fruit fly - *Drosophila melanogaster* (Release_6), alongside worm species *C. elegans* (WBcel235), *Echinococcus granulosus* (ASM52419v1), *Echinococcus multilocularis* (WTSI3) *S. japonicum* (ASM15177v1), *S. mansoni* (WTSI5), *Schmidtea mediterranea* (WUSTL3.1), compared to *F. hepatica* let-7 isomiRs from this study. Conservation of the mature miRNAs of the respective precursor miRNAs were compared using T-coffee, and ClustaIW sequences generated using Jalview. To determine homology within *Fasciola* spp, precursor sequences of novel miRNAs determined in miRdeep2 analysis were compared to the *F. gigantica* genome (PRJNA230515 assembly in https://www.ncbi.nlm.nih.gov/bioproject/PRJNA230515) using BLASTN (e-value 1e-4) and featured in Supplementary Table 3.1. The *F. gigantica* genome coordinates of previously published *F. hepatica* precursor miRNAs recently determined by Fontenla *et al* [153] are included in Supplementary Table 3.2.

3.4.5. Expression Analysis

Differential expression analysis of the miRNAs was performed using normalised reads (CPM). Hierarchical clustering of the total miRNAs and sample types was performed using One Pearson correlation using the average expression of the miRNA within the sample types, which was graphically represented using heatmaps generated using R (Morpheus package). Principal components analysis (PCA) of the normalised reads (CPM) was performed to determine clustering between miRNA expression of the sample types where the node size represents relative comparison of the number of miRNAs associated to each life cycle stage. Three-dimensional PCA plots were generated using the Partek suite for data visualisation. Expression of the miRNAs categorised based on miRBase, published and newly discovered were compared using parallel coordinates generated using Plotly. Expression of the top 10 most abundant miRNAs (CPM) was compared between each life cycle stage and pie charts generated using R (ggplot2 package).

3.4.6. Target prediction and transcriptome correlation

The target prediction tools miRanda and TargetScan were selected based on the consideration that they outperform other tools when used in combination and have shown the largest effect in supporting validation of targets [154]. Mature *F. hepatica* miRNAs were aligned to 3'UTR

sequences derived from the *F. hepatica* genome (PRJEB25283) using the bioinformatic tool miRanda (v3.3a) where miRNA:3'UTR interactions with <-20 Energy-Kcal/Mol and >155 prediction score was accepted as authentic. These parameters were chosen as they are reportedly more stringent than default parameters for miRanda and have been previously used to help eliminate false positives for target prediction [155]. Additionally, 3'UTR sequences were aligned to all mature miRNA seed sequences using TargetScan (v7) where miRNA:3'UTR binding interactions of 7mer-8m site types were accepted. Gene expression analysis was performed for the gene targets commonly predicted by both tools based on the transcriptome data of the NEJ 24h, immature fluke 21 dpi and adult parasites generated by [149]. The gene transcripts corresponding to the predicted 3'UTR sequence targets were mapped to the transcriptome data using Salmon (v.13) and read counts extracted using htseq-count. Normalised gene expression (TPM) in each life stage was then correlated to the expression of their respective miRNA interactions (CPM) in each life stage using One Pearson correlation. Gene targets that had a negative correlation to miRNA expression (<0 correlation coefficient) with P value <0.05 were selected as the final predicted gene targets.

To correlate the data with our previous *F. hepatica* transcriptome and proteome studies of the NEJ, immature and adult parasite stages, the gene models derived from the newly revised *F. hepatica* genome (PRJEB25283) were mapped to the original draft *F. hepatica* genome (PRJEB6687). The genes predicted to be targeted by the miRNAs were annotated based on the functional annotation of the gene models by Cwiklinski et al. (2015) [149]. Hypergeometric tests were used to test for over-representation of GO terms within groups of genes associated with miRNA expression using R. Analysis of the genes involved in metabolism was based on our previous studies of the life cycle stage specific transcriptomes [78, 146, 149].

3.4.7. Data availability

The miR-Seq read data is freely available and deposited in NCBI's GeneExpression Omnibus; accession number GSE186948. The F. hepatica genome and transcriptome data is freely available from WormBase ParaSite and the European Nucleotide Archive under accessions LN627018-LN647175 (assembly data), PRJEB6687/PRJEB25283 (genomic read data) and PRJEB6904 (transcriptomic read data). All data generated of analysed during this study are included in this published article [and its supplementary information files].

3.5. Results & Discussion

3.5.1. Small RNA sequencing across multiple developmental stages of *F. hepatica* reveals a new repertoire of miRNAs.

In depth small RNA sequencing across the three major *F. hepatica* life stages, namely the newly excysted juvenile (NEJ), immature flukes 21-day post infection (Juv 21d) and adult stage parasites has expanded the *F. hepatica* miRNome repertoire to 124 miRNAs (Supplementary Table 3.2). These represent 72 miRNAs that were previously characterised in *F. hepatica* NEJ and adult parasites [151], in addition to 52 newly identified miRNAs, of which three are conserved sequences (fhe-miR-493-5p, fhe-miR-2335-5p and fhe-miR-6613-3p), and 49 were novel miRNA sequences. Of these newly identified sequences, 31 were predicted to be conserved in the *F. gigantica* genome (Supplementary Table 3.2), although their expression has not been previously reported (Supplementary Table 3.1) [156]. Based on the revised nomenclature used in this study (Supplementary Table 3.1) and the exclusion of sequences that were not identified in all replicates within a single live stage, two of the previously published miRNA sequences were not included in the final compilation of *F. hepatica* miRNAs used in this study. Within this final complement of miRNAs, 22 were unique to NEJ, 16 miRNAs were unique to juveniles and 16 were unique to adults (Figure 3.1a).

Following the completion of this chapter, two additional studies were published which report the expanded characterisation of *F. hepatica* miRNAs across multiple life stages (including the NEJ and adult worms) [153, 157]. Combined, these report the discovery of 70 novel miRNA sequences. Of these, two were identical to the *F. hepatica* novel miRNA sequences discovered here (Supplementary Table 3.2).

In this study and in the two most recently published studies described above, the majority of new miRNA sequences have been identified within the NEJ stage of the parasite life cycle. This is likely due to the different culturing times of NEJs that were used to prepare samples for sequencing. Between the three most recent studies (this manuscript [153, 157]) RNA was isolated from NEJs excysted in vitro and cultured for 6h, 24h, and 7 days respectively. It has previously been shown that after excystment, the NEJs undergo a period of rapid temporal gene expression with distinct clusters of regulated genes observed at 1h, 3h and 24h [78]. In addition, the number of genes transcribed at 24h (4,644) greatly exceeds the level of expression at 3h (373). Such vast differences in gene expression would be reflected in corresponding

changes to the expression of regulatory miRNAs, explaining the discovery of additional novel miRNAs, as seen here.

The discovery of such a large number of new miRNA sequences is not unprecedented. The identification of a high number of species-specific miRNAs is a trend that has been observed during the assembly of other helminth miRnomes, including *H. contortus*, which was characterised as having 140 species-specific miRNAs and 44 conserved sequences, and *S. mansoni*, which expresses 84 species-specific and 28 conserved miRNAs [158, 159].

The simultaneous comparison of miRNA sequences across the three life stages revealed the presence of sequence variants of some canonical mature miRNAs (termed IsomiRs), resulting in the expansion of several miRNA families. These variants can be easily dismissed as sequencing artefacts, particularly when identification is dependent on a single sample; accordingly, it is likely that the identification of some *Fasciola* isomiRs were masked in the early discovery projects as only single life stages were analysed. This is particularly exemplified within the let-7 miRNAs where the three initial sequencing studies each described the presence of a single let-7, but each with a different sequence. Examining the expression of let-7 in the different life stages at the same time, we have confirmed the presence of three distinct isomiRs. In addition, the fhe-miR-2 and miR-71 families were expanded into eight and three possible miRNAs respectively (Figure 3.1). The remaining nine IsomiR families each contain two miRNAs. Of note, annotation of these isomiRs, specifically the suffix of the miRNA number, was conferred based on the earliest discovery of the miRNA and conservation of the seed region using BLASTN. In our study, the mature sequence of miR-2b was determined with two different precursor structures in different genomic locations and then annotated accordingly.

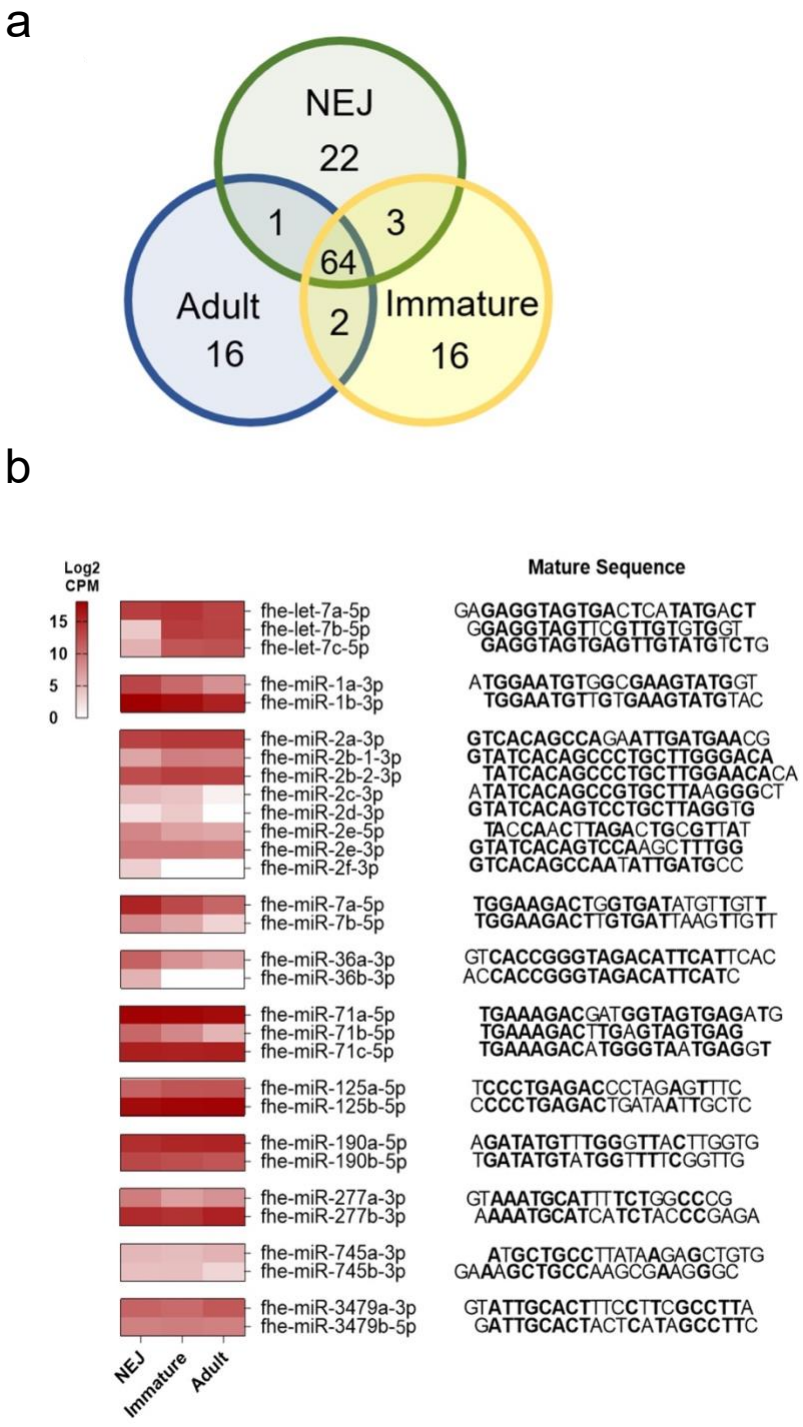


Figure 3.1. Interrogation of miRBase, published and novel miRNAs establish the *Fasciola hepatica* miRnome. (a) Venn diagram of total miRNAs segregated based on presence of the intra-mammalian life stages. (b) Normalised reads (CPM) of isomiR families are compared between newly excysted juveniles (NEJ), immature flukes (Juv21d) and adult parasites represented by a heat map. Heatmap depicts low (white) and high (crimson) expression in Log2CPM. Sequences listed 5' to 3' for each of the miRNAs and common nucleotides in bold when aligned between one or more miRNAs within a miRNA family.

To validate the authenticity of the novel miRNAs, the *in silico* predicted precursor sequence structures were compared to published sequences using RNAfold. This analysis revealed conventional precursor miRNA structural features such as a long, truncated hairpin approximately 60 nt long with a 2 nt 3' overhang, and similar structural stability to known miRNAs, as shown in the calculated minimum free energy (MFE) [160] (Figure 3.2a and Supplementary Figure 3.2.). Furthermore, the average nucleotide lengths of the *F. hepatica* precursor miRNAs were similar to those of *S. japonicum*, *S. mansoni* and *C. elegans* (Figure 3.2b). However, we also identified seven *F. hepatica* miRNAs with an uncharacteristically high number of nucleotides outside of the mature sequence, producing precursor structures >150 nt long compared to other worm species. It is interesting that a similar observation has been made for human miRNAs, whereby the evolutionary conserved miRNAs have stable, typical precursor lengths of around 80 nucleotides while the human-specific miRNAs show greater variation in precursor length with some as long as 180 nt [161]. These longer human miRNAs were predicted to regulate a larger number of target genes compared to the conserved miRNAs. Of the seven *F. hepatica* miRNAs with longer precursors, five were specific to trematodes, suggesting that, like human miRNAs, these have evolved for a biological requirement yet to be elucidated.

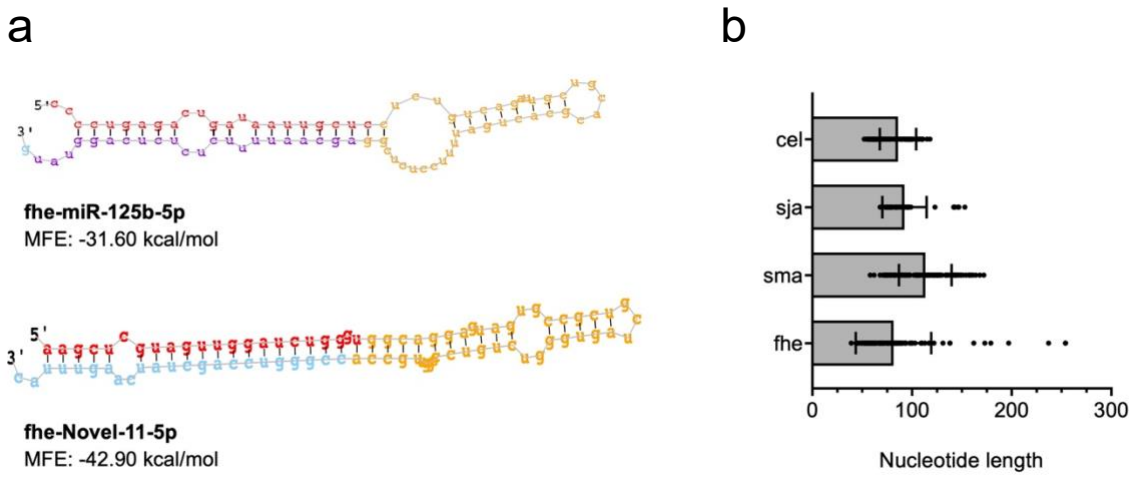


Figure 3.2. Structural analysis of the *Fasciola hepatica* miRNAs. (A) Comparison of precursor miRNA structure of conserved miRNA fhe-miR-125b-5p and novel species-specific miRNA fhe-Novel-11-5p. Nucleotides are colour coded based on predicted mature sequence found in sequenced sample (red), predicted star sequence but not found in miRNA Seq data (blue), predicted star sequence identified within sequenced sample (purple), and hairpin loop (yellow) as determined by MiRDeep2. The minimum free energy (MFE) of each miRNA is also displayed. (B) Comparison of average nucleotide length of total precursor miRNAs *Caenorhabditis elegans* (cel), *Schistosoma japonicum* (sja), *S. mansoni* (sma) and *F. hepatica* (fhe).

3.5.2. Genome location and clusters of *Fasciola hepatica* miRNAs

When mapped to their genome location, most (70%) of the *F. hepatica* miRNAs were found to be intergenic with 86 pre-miRNAs located between protein-coding genes. The remaining miRNAs were intragenic, with 33 pre-miRNAs located within an intron of a specific gene and five embedded within an exon of a specific gene (Supplementary Table 3.2). Having a predominant intergenic miRNome is a shared characteristic with other platyhelminthes, with 92% and 90% of the miRNA complement of *Hymenolepis spp* [162] and *S. mansoni* [163], respectively, located in intergenic regions. This contrasts with their mammalian hosts in which >57% of miRNAs are intragenic and transcribed in the same orientation as their host genes [164]. The enhanced capacity for the parasites to transcribe their miRNA independently of worm gene expression, suggests an adaptation to efficiently transcribe miRNAs in response to external host signals and to autonomously regulate host genes without significantly disturbing the parasite's developmental transcriptome. Conversely, bioinformatic exploration of the intragenic miRNAs and the genes in which they are embedded in may also reveal inherent targeting capabilities of the intragenic miRNA as co-transcription with their respective gene will be associated to a specific biological function.

Assessing the genomic arrangement of the 124 miRNAs identified in this study, we found that several of the miRNAs clustered together based on their predicted precursor sequence structure. Based on the genomic location of these predicted structures, notably the miR-71 and miR-2 isomiR families were clustered together, with different sets of the miR-2 isoMiRs clustered with the three miR-71 variants (Figure 3.3a). This is consistent with previous observations by Fontenla et al. [39] of a *F. hepatica* miR-71-2 cluster, which is highly conserved across protostomes [165, 166] and has been reported in the nematode *H. contortus* [158] and in several platyhelminthes [166-171]. While both miRNAs are derived from the same nascent RNA, investigation of each family member showed that the expression of miR-71 is consistently higher than miR-2 across the life stages (Figure 3.3b) implying that miR-71 and miR-2 undergo different rates of processing and functionality. Additionally, the overall expression of the fhe-miR-71a-2a/2b-1/2e and fhe-miR-71c-2b-2 clusters are greater than fhe-miR-71b-2f/2d/2c, suggesting temporal regulation and a putative important role in the transition between the life stages, as indicated for other platyhelminthes [166, 167, 169-172].

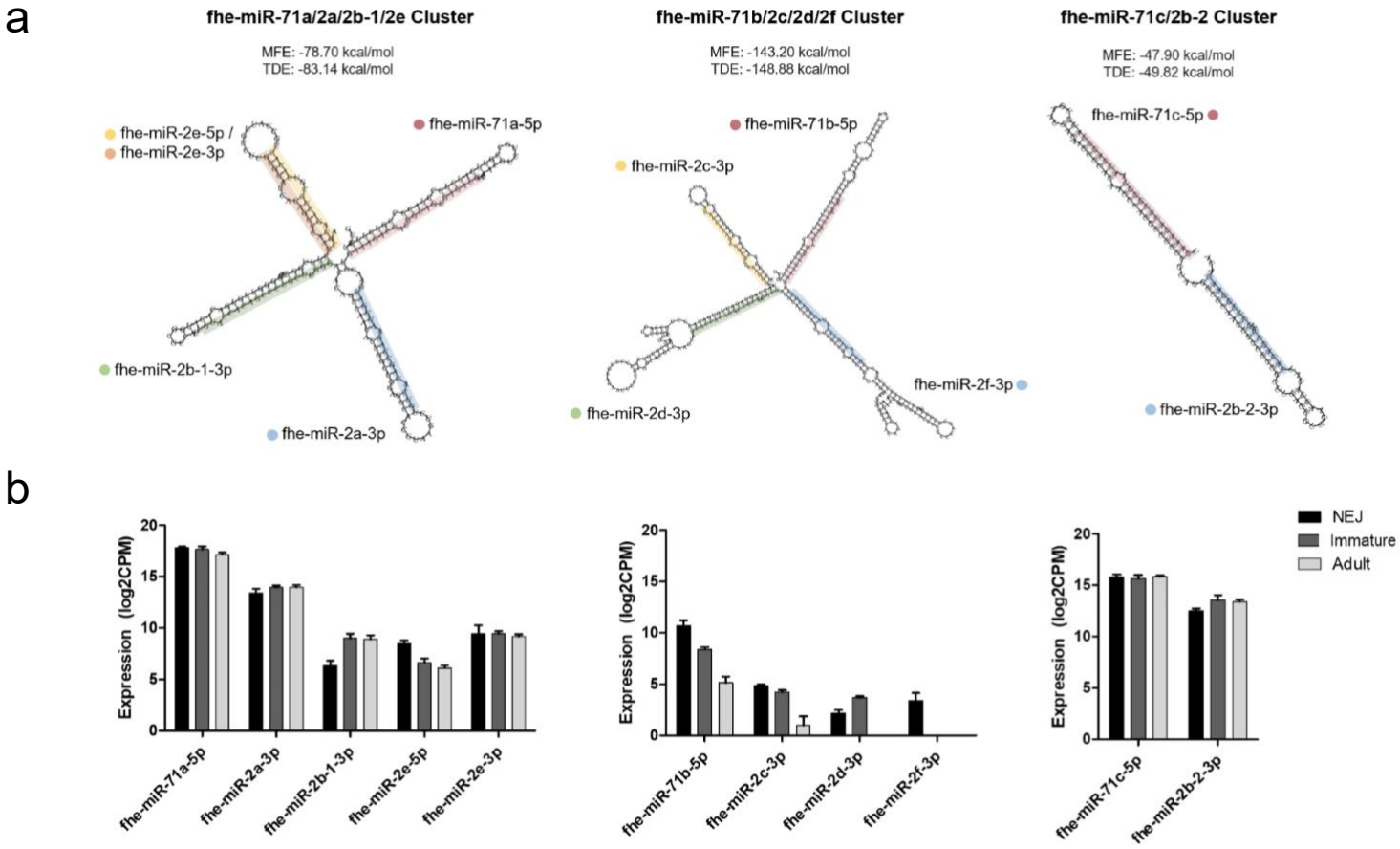


Figure 3.3. Structure and expression of miRNA clusters of *Fasciola hepatica* miR-71 and miR-2. (a) Structural organisation of pre-miRNA sequences using RNAfold to reveal clustering of fhe-miR-71a-5p with fhe-miR-2a-3p, fhe-miR-2b-1-3p and fhe-miR-2e-5p/3p; fhe-miR-71b with fhe-miR-2c-3p, fhe-miR-2d-3p and fhe-miR-2f-3p; and fhe-miR-71c-5p with fhe-miR-2-3p. Each miRNA is colour coded to represent position of mature sequence within each individual cluster. Each cluster is represented with the minimum free energy (MFE) and predicted thermodynamic ensemble (TDE) of the RNA folding. (b) Expression of miR-71 and miR-2 in respective clusters across life stages, Newly excysted juveniles 24h (NEJ), Immature flukes (Juvenile 21dpi) and Adult parasites. Data is presented as normalised reads (CPM) in log2, mean ± SD of triplicate biological samples.

3.5.3. Phylogenetic relationship of *F. hepatica* miRNAs to other parasitic and free-living flatworms.

Consistent with genome-wide phylogeny of the major parasitic worms [173], phylogenetic analysis based on the precursor miRNA sequences separates the *F. hepatica* miRNome from the sequences derived from *S. japonicum* and *S. mansoni*, which cluster together (Supplementary Figure 3.3). To explore this divergence more closely, the phylogeny of the highly conserved let-7 miRNA was examined (Figure 3.4a). Sequence alignment confirmed a high degree of homology across the let-7 mature miRNAs from human, mouse and fruit fly and the available helminth miRNAs (Figure 3.4b). However, in contrast to the total miRNome phylogenetic analysis, the *F. hepatica* let-7 isomiRs are not wholly divergent to other parasites and instead are dispersed throughout the tree; fhe-let-7a shares greater homology with let-7c from the free-living planarian *S. mediterranea* and the parasitic tapeworms *E. granulosus* and *E. multilocularis*, whereas fhe-let-7b is positioned more closely to let-7 from *S. mansoni* and *S. japonicum*. More strikingly, fhe-let-7c has significantly diverged from all worm species and invertebrates, positioned closest to human and mouse let-7g/i/j.

Let-7 is an ancient class of miRNA that has been consistently linked to the temporal regulation of bilaterian developmental biology [121]. Studies into the evolutionary history of let-7 has revealed that the genomic locus of let-7 has been independently restructured between worm species [174]. Let-7 is typically associated to miR-125 as a polycistronic transcript, where both miRNAs can act together as key regulators of development [175-177]. However, whereas clustering of let-7 and miR-125 remains conserved in the flatworms *S. mansoni* and *S. mediterranea* [174], these miRNAs appear to be organised separately within different scaffolds of the *F. hepatica* genome, although this may also be due to the fragmentation of the current genome assembly comprised of several scaffolds [39].

Any alteration in the genomic organisation of let-7 between worm species may explain the unique phylogeny of *F. hepatica* let-7 pre-miRNAs. In addition, the marked similarity of *F. hepatica* let-7 to one species and not another suggests diversification of its gene targets and thus biological functions. Previous analysis of precursor sequences of miR-125b-5p provides support for this evolutionary adaptation, as the structure of the *F. hepatica* miR-125b-5p is also more closely related to the human miR125-b, than to the *Schistosome* miR-125b, and upon overexpression is predicted to regulate expression of the same host genes as the human miRNA [136]. The divergence of the *F. hepatica* miRNA network could contribute to the ability of this

parasite to more broadly regulate host genes and consequently infect a wide range of mammalian hosts.

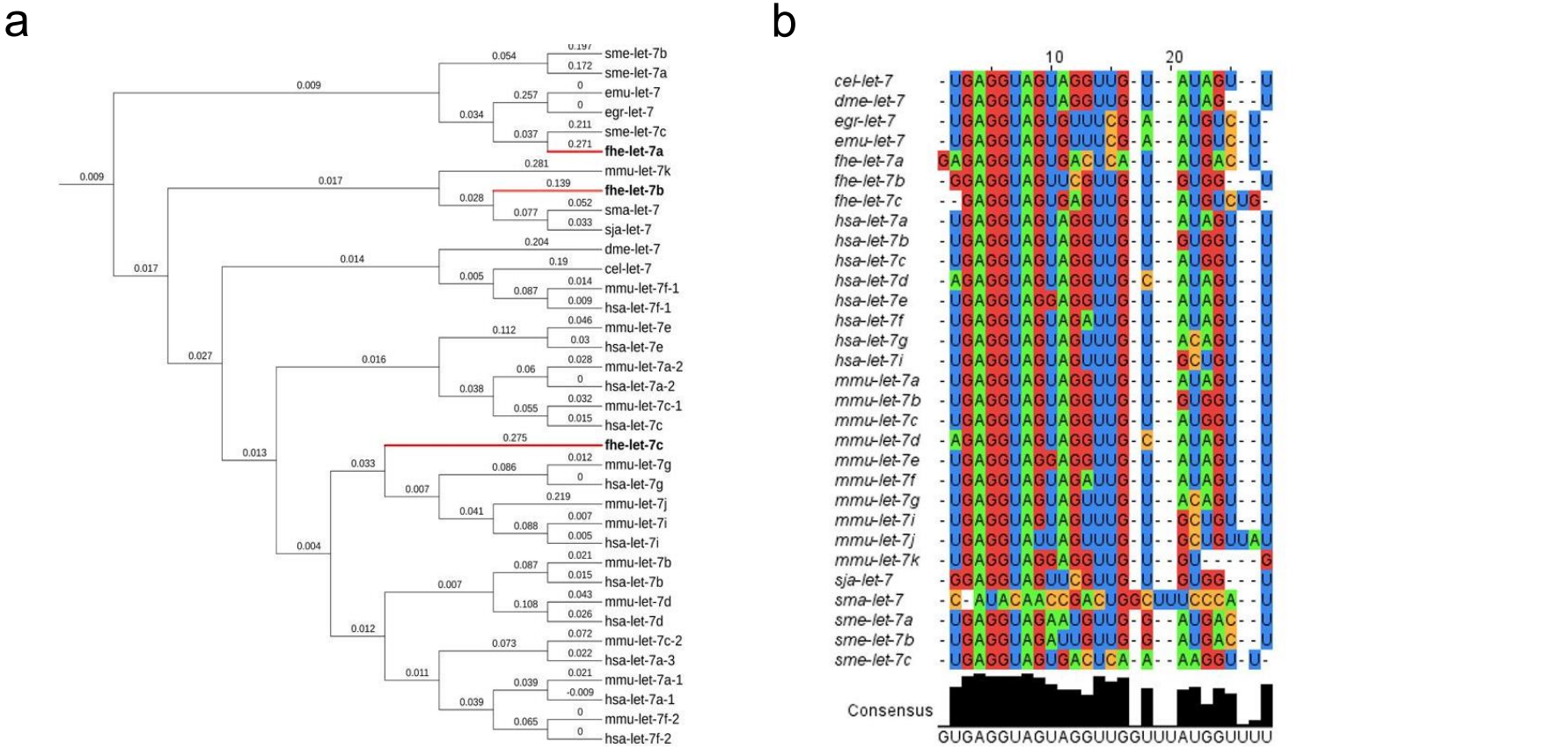


Figure 3.4. *Fasciola hepatica* let-7 isomiRs are highly conserved as mature sequences yet evolutionarily divergent in comparison to their precursor sequences. (a) Phylogenetic tree of let-7 precursor sequences constructed using T-Coffee multiple sequence alignment tool and iTOL v6 (itol.embl.de) with percentage identity scores, 0 = zero sequence difference. (b) Sequence alignment of the mature miRNAs of let-7 from *F. hepatica* (fhe), *Caenorhabditis elegans* (cel), *Drosophila melanogaster* – fruit fly (dme), *Echinococcus granulosus* (egr), *E. multilocularis* (emu), *Homo sapiens* – human (hsa), *Mus musculus* – mouse (mmu), *Schistosoma japonicum* (sja), *S. mansoni* (sma) and *Schmidtea mediterranea* (sme) using ClustalW. Homology is illustrated through colour coded nucleotides and consensus nucleotides across all mature miRNA sequences.

3.5.4. *Fasciola hepatica* miRNAs are temporally expressed during the parasite's development from newly excysted juvenile to adult worm

Hierarchical clustering reveals that the transcriptional profile of the *F. hepatica* miRNAs mirrors that of the gene transcriptional profile, both of which display differential expression as the parasite develops and matures from the invasive NEJ to the sexually mature adult worms (Figure 3.5a; Supplementary Table 3.3; [149]). In particular, the NEJ miRNAs exhibit a profile of miRNA expression that is quite distinct from the other stages, with the immature and adult parasites displaying a more similar profile and abundance of miRNA expression (Figure 3.5a, c). This pattern of expression is consistent with the major developmental and growth processes that the parasite is undergoing following invasion and subsequent migration through the mammalian host [78, 146, 149]. The similarity between the miRNA and gene transcriptional profiles aligns with the expectation that miRNAs regulate the gene networks involved in developmental progression.

Further interrogation of *F. hepatica* miRNA transcription by parallel co-ordinate plot analysis revealed an unexpected distinction between sets of miRNAs (Figure 3.5b). The miRNAs that had been previously characterised showed very little fluctuation in their transcriptional profile across the three life stages. In contrast, the novel miRNAs described here displayed differential expression between the life stages. The marked difference between the expression profiles of these miRNAs can be partly explained by the nature of miRNA discovery tools that rely on species homology to predict precursor structures within the genome. As the miRNAs derived from miRBase and other published sources were some of the earliest realisations of the *F. hepatica* miRNome, their identification was largely informed by highly conserved miRNAs that are likely necessary for critical physiological functions and thus required throughout the growth and development of the parasite. Since these initial studies were performed two assemblies of the *F. hepatica* genome have become available, both of which have been revised with technological advances, and the number and quality of worm miRNA sequences used as reference input has vastly expanded supporting the identification of novel miRNAs in this study.

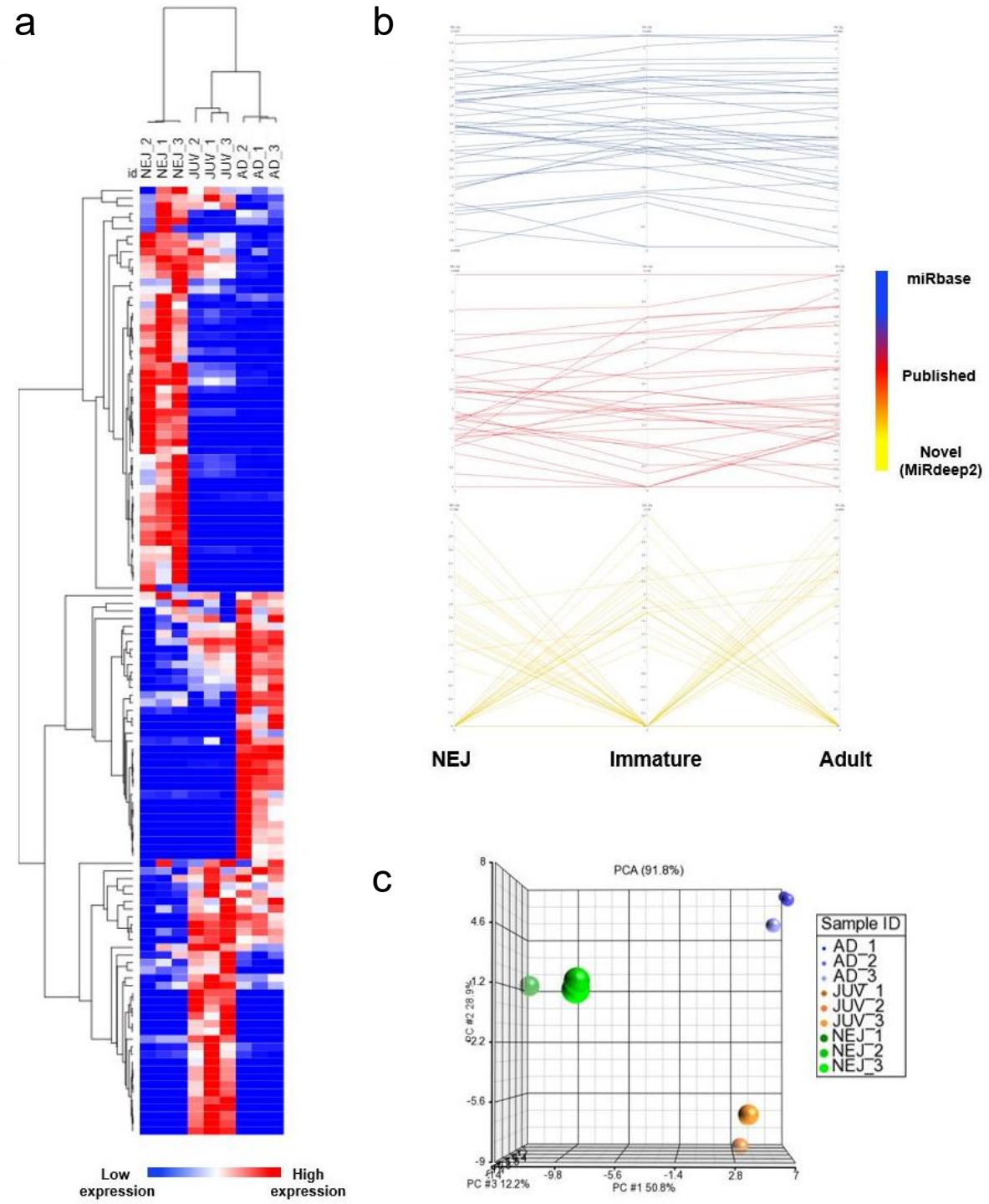

a
id
NEJ_2
NEJ_1
NEJ_3
JUV_2
JUV_1
JUV_3
AD_2
AD_1
AD_3
Low expression
High expression
b
miRbase
Published
Novel (MiRdeep2)
NEJ
Immature
Adult
c
PCA (91.8%)
Sample ID
AD_1
AD_2
AD_3
JUV_1
JUV_2
JUV_3
NEJ_1
NEJ_2
NEJ_3
PC #2 26.9%
PC #3 12.2%
PC #1 50.8%

Figure 3.5. The *Fasciola hepatica* miRnome displays temporal expression during the life cycle within the mammalian host. (a) Heatmap of the expression of the miRnome across triplicate RNA samples of newly excysted juveniles (NEJ), immature fluke (JUV) and adult parasites (AD) where minimum expression (low expression – blue) and maximum expression (high expression – red) of the individual miRNAs is represented. Hierarchical clustering of the average values of miRNAs and sample types was performed using One Pearson correlation. (b) Parallel coordinates of *F. hepatica* miRNA expression (logCPM) separately analysed based on category; miRBase miRNAs (blue), other published non-miRBase miRNAs (red) and novel miRNAs determined in this study (yellow). (c) Principal components analysis (PCA) plots of the total miRNA expression across all sample types and miRNAs in 3D (From left to right – front view, side view, top view). Size of the nodes depicts total miRNA input (miRNA presence) between sample types NEJ (green scale), immature flukes (orange scale) and adult parasites (blue scale).

3.5.5. Identification of miRNA gene targets, correlated to the parasite's transcriptome, reveals the global regulation of stage-specific developmental processes.

To elucidate the functional role for the parasite miRNAs in regulating the developmental processes, gene targets for each miRNA were predicted from the 3'UTR regions of 9,727 characterised genes (out of total 22,676) within the *F. hepatica* genome using miRanda and TargetScan (Supplementary Table 3.4, Supplementary Table 3.5). Selecting genes that were commonly selected by both tools produced a total of 8,386 predicted gene targets (Supplementary Table 3.6). The frequency distribution of the number of targets per miRNA showed that the majority of the *F. hepatica* miRNAs were predicted to have 20 – 100 targets, with an average of 67 gene targets per miRNA (Supplementary Figure 3.4). Five miRNAs, of which four (fhe-miR-1a-5p, fhe-miR-71a-5p, fhe-pubNovel-2-5p and fhe-Novel-15-3p) were most abundant in the NEJ life stage, were predicted to have >200 targets.

Of the predicted targets, 2,427 genes displayed transcriptional levels that negatively correlated to the expression of their corresponding regulatory miRNA (Supplementary Figure 3.5 and Supplementary Table 3.7). Hierarchical clustering of these genes based on their expression within the three life stages revealed possible miRNA-specific regulation of key processes (Figure 3.6; Supplementary Table 3.8). While the expression of genes associated to the integral component of the membrane was present in clusters associated to each of the life stages, it appears to be the predominant biological process in the NEJ parasites (clusters 1-4). As the parasites develop from NEJ to immature fluke, there is evidence of heightened expression of genes associated with nucleotide synthesis, transcription and translation, energy regulation, cell signalling and proteolysis (clusters 5-7). The increase in the expression of these genes reflects a significant developmental transition for the parasite as it acquires the ability to

feed on host tissue and blood, migrate and undergo rapid periods of growth. These same biological processes are also evident in the adult parasites as they continue to feed and grow (clusters 8-11). However, specific to the adult stage is the presence of zinc ion binding, which is consistent with the employment of metallo-peptidases in the process of egg development [172]. While there is in fact a myriad of molecular interactions associated to miRNAs, which include interaction with non-Ago proteins, activation of Toll-like receptors, targeting of other non-coding RNA, and even direct activation of transcription factors [178-181], mRNA degradation is the most characterised and commonly observed miRNA function. Therefore, while this general alignment with the developmental stages of the parasite may not capture the full breadth of miRNA activities, it supports evidence from *C. elegans* and other nematodes, and shows that *F. hepatica* miRNAs are differentially expressed to stage-specifically regulate the transcriptome to ensure the biological processes required for each developmental stage are switched on and off as necessary.


Cluster
GO Term
GO:0016021 Integral component of membrane
GO:0005525 GTP binding
GO:0016021 Integral component of membrane
GO:0016020 Membrane
GO:0055085 Transmembrane transport
GO:0005886 Plasma membrane
GO:0004930 G-protein coupled receptor activity
GO:0016021 Integral component of membrane
GO:0006412 Translation
GO:0003735 Structural constituent of ribosome
GO:0016021 Integral component of membrane
GO:0006351 Transcription, DNA-template
GO:0016021 Integral component of membrane
GO:0005737 Cytoplasm
GO:0005634 Nucleus
GO:0016021 Integral component of membrane
GO:0005524 ATP binding
GO:0007155 Cell adhesion
GO:0016020 Membrane
GO:0016874 Ligase activity
GO:0005509 Calcium ion binding
GO:0005737 Cytoplasm
GO:0007165 Signal transduction
GO:0046872 Metal ion binding
GO:0000166 Nucleotide binding
GO:0003677 DNA binding
GO:0005524 ATP binding
GO:0005634 Nucleus
GO:0004674 Protein serine/threonine kinase activity
GO:0035556 Intracellular signal transduction
GO:0005543 Phospholipid binding
GO:0016021 Integral component of membrane
GO:0003700 Transcription factor activity, sequence-specific DNA binding
GO:0003700 Transcription factor activity, sequence-specific DNA binding
GO:0005524 ATP binding
GO:0005634 Nucleus
GO:0016021 Integral component of membrane
GO:0005634 Nucleus
GO:0004674 Protein serine/threonine kinase activity
GO:0005524 ATP binding
GO:0008270 Zinc ion binding
GO:0016567 Protein ubiquitination
GO:0006351 Transcription, DNA-templated
GO:0003676 Nucleic acid binding
GO:0016021 Integral component of membrane
GO:0046872 Metal ion binding
GO:0005525 GTP binding
NEJ
Immature
Adult
Low expression
High expression

Figure 3.6. Hierarchical clustering of predicted targets shows dynamic miRNA-induced regulation of biological processes across *Fasciola hepatica* life stages. Heatmap of gene expression (TPM) of targets that are negatively correlated to the transcription of associated miRNAs. Hierarchical clustering of gene target expression performed using One Pearson correlation on the average TPM of each mRNA cross the sample types. Gene expression presented from a scale of the minimum expression (low expression - blue) versus the maximum expression (high expression - red) of the specific gene across the sample types. Each cluster was analysed for gene ontology (GO) enrichment using hypergeometric tests, and GO terms selected on adjusted P value <0.001. Order of GO terms for each cluster organised from most significant to least significant.

Although the number of miRNAs that were identified as exclusive for each life stage was very similar (22 NEJ; 16 Immature fluke; 16 Adult), there was a stark contrast in the number and variety of biological activities being targeted in NEJs when compared to the immature and adult parasites (Figure 3.7; Supplementary Table 3.9). This outcome reflects the total number of gene targets associated to each of the miRNAs. Surprisingly, a total of 559 unique targets were identified for the 22 NEJ miRNAs, while the immature and adult parasite specific miRNAs were found to have 22 and 56 unique gene targets, respectively (Supplementary Table 3.10). It has been suggested that when the expression of multiple genes is simultaneously inhibited by a single miRNA, as in the NEJs, this miRNA is likely serving to enforce a cell or organ identity [(182)]. This was elegantly demonstrated in zebrafish, where the expression of miR-430 at the onset of zygotic transcription results in the co-ordinated clearance of hundreds of maternal genes thus regulating morphogenesis and controlling temporal identity [(183)]. Similarly, the NEJ specific miRNAs may suppress the expression of a broad numbers of genes with a range of biological functions until the cues from the internal environment of the host signals a requirement for these genes to support a developmental transition. An examination of the broad pattern of gene expression through the life stages would support this proposition, as there is a much higher number of genes that are similarly increased in expression in the immature and adult worms compared to the NEJs [(149)].

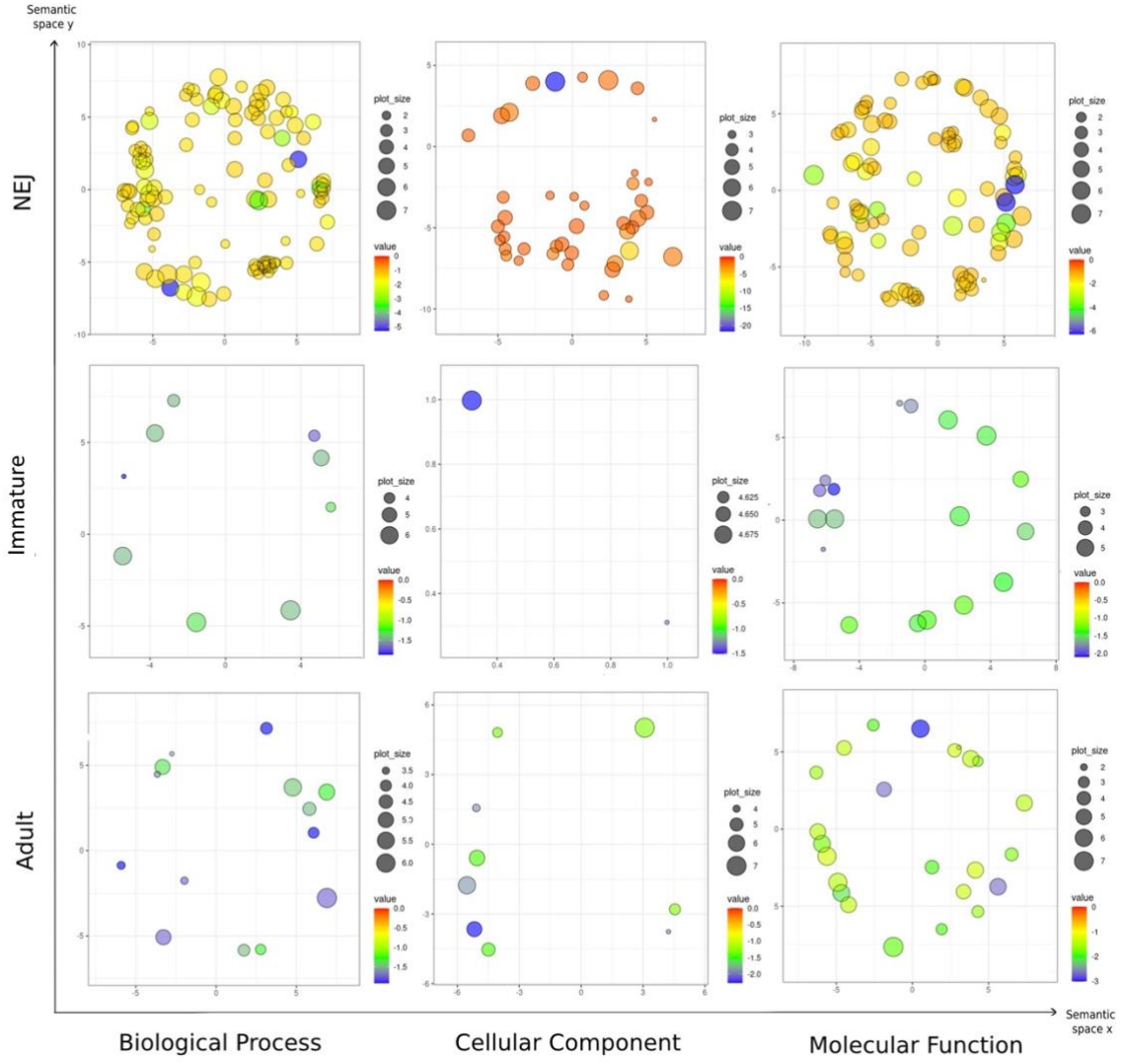


Figure 3.7. The NEJ specific miRNAs regulate a significantly higher number of targets compared to the immature or adult specific miRNAs. Semantic similarity charts on gene ontology (GO) enrichment for biological processes, cellular components and molecular functions of genes regulated by miRNAs specifically expressed in each of the intra-mammalian life stages; newly excysted juveniles (NEJ), immature fluke and adult parasites. Enrichment analysis performed using hypergeometric tests and GO terms with <0.05 adjusted P value selected. Charts constructed using SimRel for semantic similarity measure, where nodes are colour coded based on P value and node size representing GO presence.

3.5.6. MiRNAs regulate parasite metabolic pathways

Our previous analysis of the *F. hepatica* stage-specific transcriptomes has demonstrated that tight regulation of metabolic pathways is critical to support the physiological changes to the parasite as it matures and adapts to changing microenvironments during its migration through the host [78, 146, 149]. Interrogation of the negatively correlated gene targets of the *F. hepatica* miRNome revealed that 64 miRNAs putatively target genes associated with a variety of metabolic processes (Supplementary Table 3.11). While 58 of the miRNAs were predicted to have a broad effect across multiple pathways, six miRNAs were exclusively associated with specific metabolic pathways, namely (a) Energy metabolism: fhe-miR-307-5p, (b) Amino acid metabolism: fhe-Novel-11-5p, (c) Transcription: fhe-Novel-28-5p, (d) Translation: fhe-miR-125b-5p & fhe-miR-11633-5p, (e) Signal transduction: fhe-miR-9389-5p.

The accessibility of glycogen/glucose and oxygen are the primary parameters that drives *F. hepatica* to adjust its energy metabolism. Following excystment, the NEJs must begin the transition from catabolism of glycogen stores to the synthesis of glucose/glycolysis from host macromolecules. As the parasite grows and moves deeper into host tissue, oxygen diffusion within the parasite becomes limited resulting in a gradual switch from aerobic to anaerobic metabolism. The key pathways involved in this developmental process are the glycolysis/gluconeogenesis, TCA/Krebs cycle and oxidative phosphorylation pathways [184]. Our analysis revealed that 17 predicted miRNAs targeted key genes within these critical energy metabolic pathways, including six isomiRs and members of the mir-71-2 cluster described above (fhe-miR-2a-3p, fhe-miR-2c-3p, fhe-miR-7a-5p, fhe-miR-36a-3p, fhe-miR-71a-5p, fhe-miR-277a-3p) (Figure 3.8 and Supplementary Table 3.12). However, there was no evidence of a clear stage specific switch in the expression of any of the miRNAs or their targets. This suggests the miRNAs play a role in fine-tuning the metabolic pathways throughout the development of the parasite in the mammalian host.

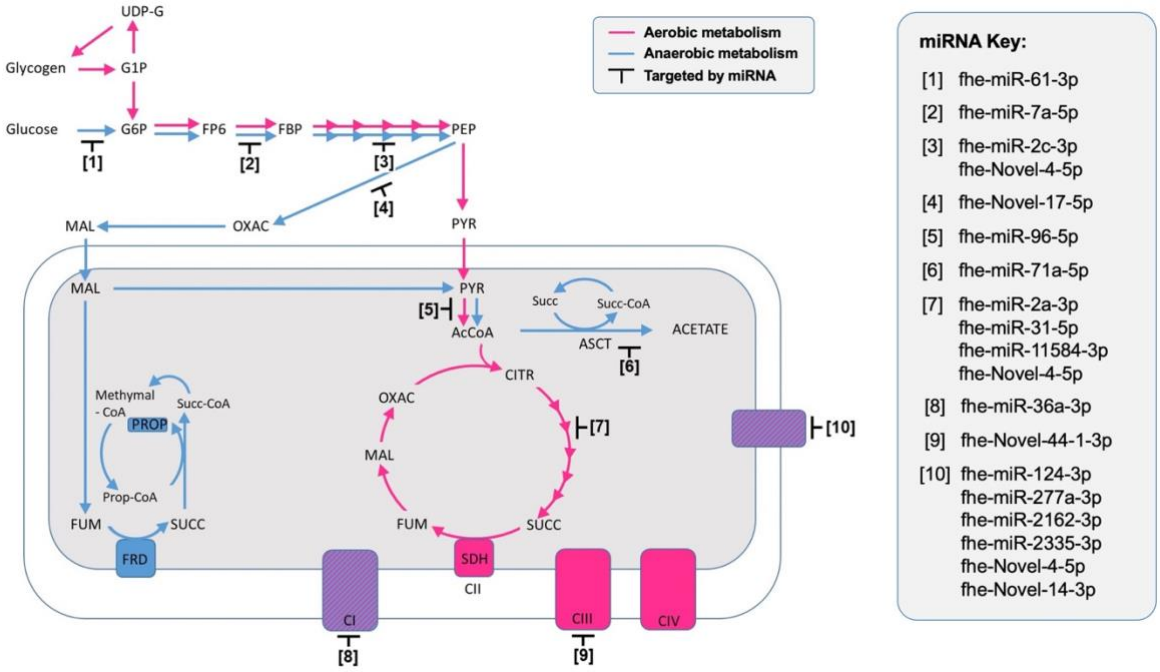


Figure 3.8. miRNAs predicted target genes involved in aerobic metabolism. Graphical representation of the pathways involved in the glycolysis/gluconeogenesis, TCA/Krebs cycle and oxidative phosphorylation KEGG pathways modified from the figure by Tielens and van Hellemond [184]. The black bars and numbers highlight position in the pathways that the miRNAs are targeting, based on the KEGG analysis carried by Cwiklinski *et al.* [41, 78, 146]. Abbreviations: AcCoA: acetyl-CoA; ASCT: Acetate:succinate CoA transferase; CITR: citrate; FP6: fructose 6-phosphate; FBP: fructose 1,6-bisphosphate; FRD: fumarate reductase; FUM: fumarate; G1P: glucose 1-phosphate; GP6: glucose 6-phosphate; MAL: malate; Methymal-CoA: methylmalonyl-CoA; OXAC: oxaloacetate; PEP: phosphoenolpyruvate; PROP: propionate; Prop-CoA: propionyl-CoA; PYR: pyruvate; SDH: succinate dehydrogenase; SUCC: succinate; Succ-CoA: succinyl-CoA; UDP-G: uridine biphosphate glucose.

3.5.7. Seven miRNAs are abundantly expressed by all three *F. hepatica* life stages

While the overall miRNA profiles were unique for each developmental stage examined, seven miRNAs were amongst the ten most abundant miRNAs in every life stage: bantam-3p, miR-1b-3p, miR-71a-5p, miR-71c-5p, miR-125b-5p, miR-190a-5p and miR-277-3p (Figure 3.9). This is consistent with earlier studies of the *F. hepatica* miRNAs, which identified the same miRNAs in NEJs [39], adult parasites and within the extracellular vesicles secreted by adult parasites [33, 46]. Similarly, bantam-3p, miR-71-5p, miR-2a-3p were found to be enriched within eight life stages of *F. gigantica* (egg, miracidia, rediae, cercariae, metacercariae, juvenile and adult stages). The high degree of expression of a small number of miRNAs is not specific to *Fasciola* spp. and has been reported in a range of other platyhelminth miRNA studies [46, 185-187].

Although these seven are the most highly enriched across all life stages, their relative abundance changes as the parasite develops, grows and matures (Figure 3.9). Examining the gene targets for these miRNAs (Supplementary Table 3.13) provides some insight into their requirement at different stages. The biological implications are most apparent for bantam-3p, as only one gene target, cathepsin L3 (FhCL3), was identified for this miRNA. Our previous transcriptome analysis revealed that FhCL3 is abundantly secreted by NEJs to facilitate the migration of the parasite through the intestinal wall and the liver [78, 149]. As the parasite matures and moves from tissue-feeding to blood-feeding, the expression of FhCL3 is switched off and other family members, FhCL1 and FhCL2, with altered substrate specificity becomes the predominant peptidase. It is tempting to speculate that the temporal regulation of cathepsin L3 by miRNAs may be a mechanism to enable rapid changes in levels of mRNA encoding this enzyme, without the need for new protein synthesis. Thus, the increased abundance of bantam-3p during the immature and adult stages could ensure that the expression of FhCL3 remains downregulated. Of the other 34 cathepsin peptidases identified within the *F. hepatica* genome [188], only one other sequence (Cathepsin B-like protease) was identified as a gene target for the parasite miRNA fhe-miR-Novel-14-3p which is specifically expressed in the NEJ stage.

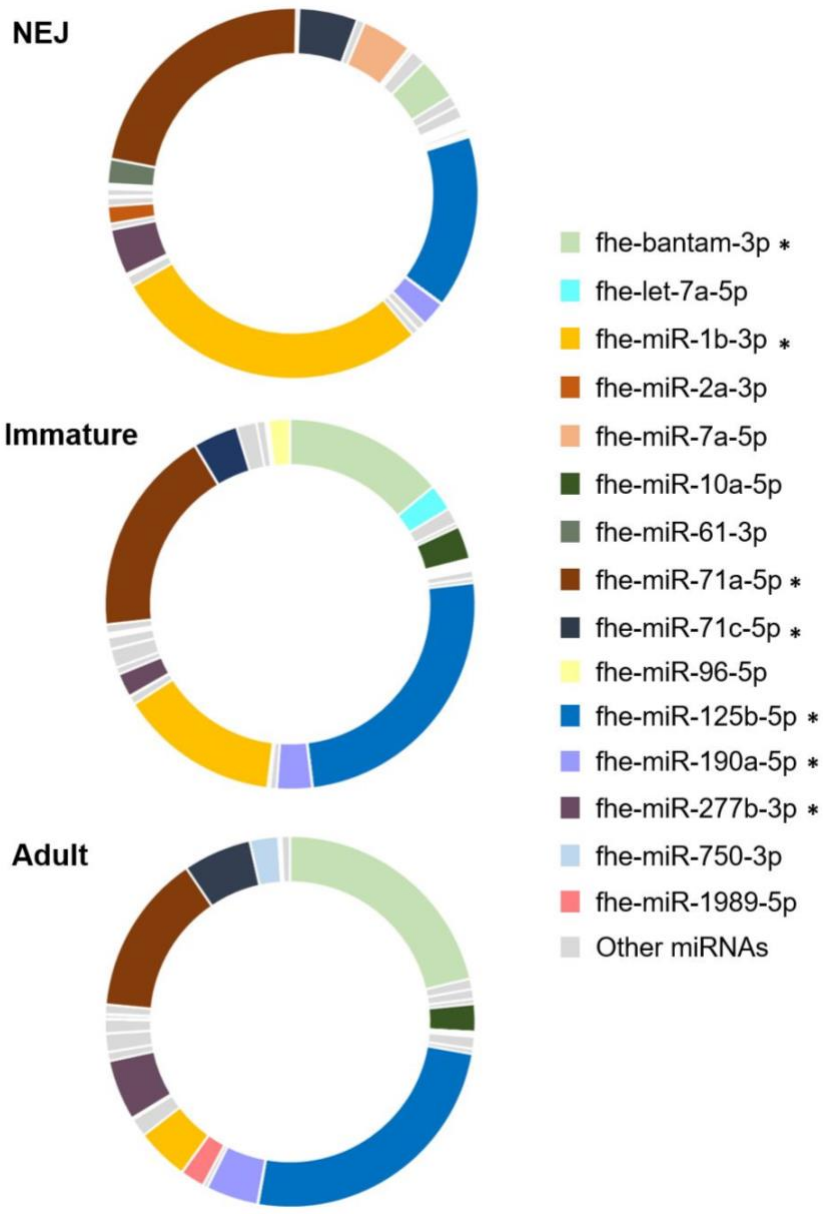


Figure 3.9. Top 10 abundant miRNAs across intra-mammalian life stages share similar enrichment. Pie charts of the percentage of individual miRNA expression (CPM) across newly excysted juveniles (NEJ), immature flukes and adult parasites. Only the top 10 most abundant miRNAs for each life stage are colour coded. MicroRNAs that are present in the top 10 most abundant in all three of the life stages are denoted with an asterisk *.

The function of the genes targeted by miR-125b and miR-190a-5p are less clear and no gene targets negatively correlated to the life stage transcriptome were predicted for miR-1b-3p, which was most abundant in the NEJs. The developmental contribution of these differentially expressed miRNAs will only become evident with more understanding of the biochemistry and activity of the parasite proteins encoded by the genome, although these miRNAs could also play a role in host-parasite interactions and, therefore, have gene targets within host cells. We have previously reported the presence of *F. hepatica* miR-125b within the peritoneal macrophages of mice infected with the parasite 24 hours earlier (Chapter 2). Furthermore, the discovery that this miRNA was loaded onto the mammalian Ago2 protein within host macrophages [36, 136], and *in vitro* regulated the expression of inflammatory cytokines by macrophages, suggested a stage specific strategy by which the NEJs disarm the host inflammatory response to protect the vulnerable juvenile parasites as they migrate from the intestine to the liver and establish infection [136].

3.5.8. A select number of genes with stage specific activities are highly targeted by multiple miRNAs.

Despite the high level of specificity shared between miRNAs and mRNA interactions, a significant degree of redundancy in miRNA can occur in which several miRNAs target a single gene. The combined action of multiple miRNAs ensures more effective regulation of gene expression [189, 190]. While most predicted gene targets within the parasite transcriptome were negatively regulated by up to 6 miRNAs each, 19 genes are putative targets of more than seven miRNAs (Figure 3.10; Supplementary Table 3.7). The scope of regulation for these genes infers a critical importance to the timing of their expression.

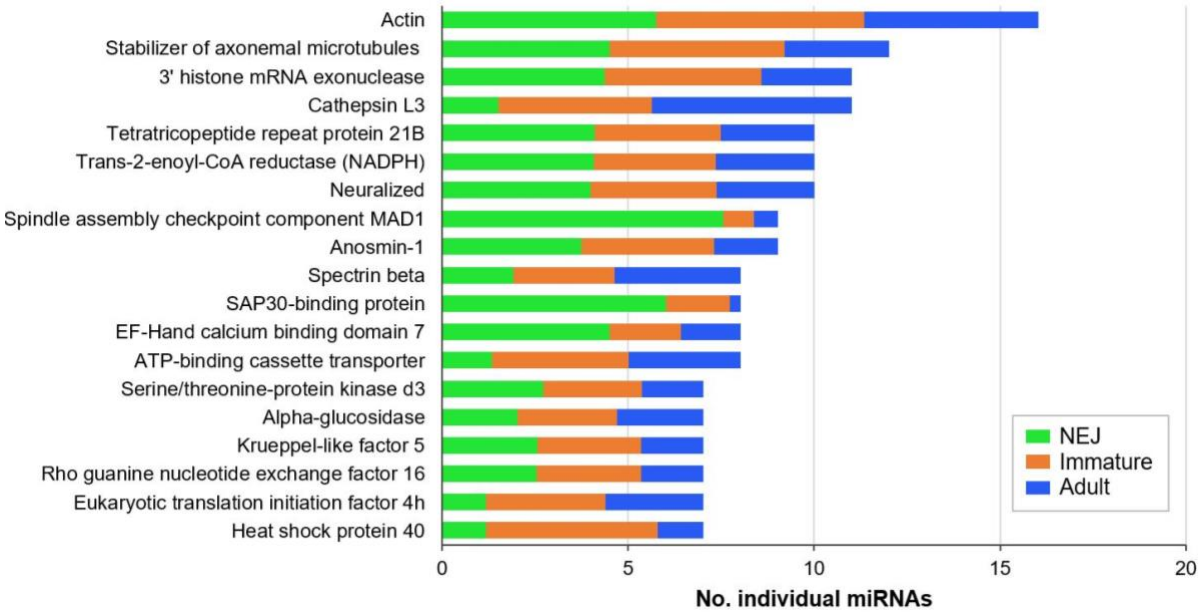


Figure 3.10. Genes associated to parasitism, development and RNA and DNA regulation are frequently targeted by *Fasciola hepatica* miRNAs. Specific genes with ≥7 different individual miRNA interactions are organised by frequency of interaction from highest to lowest number of individual miRNAs. Columns are colour coded based on percentage of the total miRNA expression of the individual miRNAs according to life stages, newly excysted juveniles (NEJ) (green), immature fluke (orange) and adult parasites (blue), to represent level of miRNA-mediated regulation of the gene within each life stage.

Some of these genes (Actin, Stabiliser of axonemal microtubules, Anosmin, Exonuclease) are involved in normal growth and development processes and are therefore regulated consistently by similar numbers of miRNAs throughout all intra-mammalian life stages. In contrast, FhCL3 is predominantly targeted by miRNAs within the immature and adult parasites suggesting this protein is specifically required by the NEJs (Figure 3.10). Together with the observation that one of the most abundant miRNAs in immature and adult flukes (bantam-3p; Figure 3.9) also solely targets this gene, further suggests that regulation of this cysteine peptidase is critical to the transition from NEJ to immature worm. A similar profile of regulation was found for ATP-binding cassette transporter, heat shock protein (HSP) 40, and Eukaryotic translation initiation factor 4h, with all three genes primarily targeted by multiple immature and adult miRNAs. Although the biological functions for these proteins in the life cycle of *F. hepatica* have not yet been elucidated, evidence from other organisms suggest they provide an increased resilience against numerous environmental stressors [191-193], which is critical for the NEJs as they excyst in the harsh environment of the gut, migrate through the intestinal epithelium and divert host signals of tissue damage and inflammation as they travel to the liver tissue.

In contrast, the expression of SAP30-binding protein, is primarily regulated by multiple miRNAs during in NEJs, a level of regulation which is significantly reduced during the immature stage and almost absent in the adult worms. This protein is a component of the histone deacetylase (HDAC) which has been shown to have fundamental roles in maintaining the viability of *S. mansoni* adult worms and egg production [194]. Therefore, the scarcity of SAP30 targeting by miRNAs in juveniles and adults suggests a critical need in the biological process of egg production, relevant only to the mature stages of the worm.

The only other gene which was similarly highly regulated during the NEJ stage was spindle assembly checkpoint component (SAC) MAD1. It has been shown that SACMAD1 is completely absent in planarian *S. mediterranea*, which is hypothesised to reflect the evolutionary development of core cellular mechanisms, and thus linked to the regenerative ability of these primitive worms [195]. The biological role for SAC components has not been explored in *F. hepatica* but we speculate that the significant miRNA-mediated suppression of SACMAD1 in NEJs relates to the enhanced proliferation of neoblasts observed during the 24h after excystment [78], as these are the same cells that mediate planarian regeneration.

3.6. Conclusion

The *F. hepatica* genome is one of the largest pathogen genomes sequenced to date. With no evidence of genome duplication or increased repeat regions, it has been proposed that much of the non-coding portion of the genome is involved in gene regulation, reflecting a need to tightly control the complex life cycle and variety of developmental stages for this parasite [149]. MicroRNAs have been well characterised as one form of non-coding RNA that is critical in shaping worm development in response to a variety of host and environmental conditions, through the regulation of co-ordinated expression of mRNA transcripts. Although, we do not have a fundamental understanding of their regulatory mechanisms, our study has shed new light on the possible functional roles for many of these parasite miRNAs through the identification of target genes within the parasite transcriptome that are critical in the transition from NEJ to adult.

However, for several of the *F. hepatica* specific miRNAs, the number of predicted genes were reduced to zero after correlation with the parasite transcriptome. Although these miRNAs could be specifically utilised in the parasite's interaction with host cells, it is also possible that this outcome reflects the limitations of the currently available target prediction tools (which are based on mammalian seed interactions). When applied to non-model organisms (such as helminths) current assumptions for target prediction using the seed region may not account for inherent differences in the biochemistry of species-specific miRNA and mRNA interactions. In some cases, there is a requirement for binding "beyond the seed region" to initiate silencing. At this stage we cannot discount an alternative mechanism of gene target regulation for these specific miRNAs. Despite these shortcomings, the use of an established bioinformatics approach allowed the identification of many gene targets within the parasite transcriptome, and these were mapped to significant biological pathways. However, a sizeable proportion of *F. hepatica* genes remain uncharacterised.

Despite these limitations, this study provides an expanded compendium of miRNAs that are utilised by the parasite as it matures within the mammalian host. The temporal expression of these miRNAs across three life stages and the identification of corresponding gene targets alluded to a role in the regulation of critical developmental processes and metabolic pathways. Our findings set the foundation for future targeted strategies to fully determine the critical nature of the contribution that miRNAs and corresponding gene targets

make towards the maturation of the parasite and thus the successful infection of mammalian hosts.

Chapter 4

Diagnosis of Sheep Fasciolosis using a panel of sheep and parasite miRNAs

4.1. Introduction

F. hepatica has one of the widest global distribution of any parasite infecting livestock and is a major production limiting disease of ruminant livestock. Infection with the parasite results in substantial delays in animals reaching slaughter weight with increased levels of fluke burden in the liver directly correlating with reduced growth rates of animals. This has a significant impact to the agricultural sector, resulting in estimated annual losses of €2.5billion [196-198].

During chronic fasciolosis the development of persistent life-threatening pathologies is common, particularly for ruminants such as sheep, which are notably susceptible to fasciolosis. Biliary cirrhosis, eosinophilic hepatitis, and various stages of hepatocyte degeneration in advanced fasciolosis can lead to sudden death of livestock [199, 200]. However, natural infections are more likely to consist of both acute and chronic infection as trickle infections represent the typical exposure pattern to the infective metacercariae. The most common presentation of fasciolosis on farms is sub-clinical with low fluke load. However, burden models suggest that even with as few as 1-10 parasites in the liver, animals gain weight at a significantly slower rate compared to uninfected animals, and results in other symptoms such as low birth rate and low wool quality [2].

Anti-helminthic therapeutics are limited, resulting in the widespread use of a single treatment for: triclabendazole (TCBZ). The blanket administration of TCBZ to both infected and uninfected animals within a single herd is currently the preferred strategy to efficiently treat infected animals while preventing new infections. Apart from the financial burden of dredging the whole pastures with TCBZ, this practise is resulting in the emergence of TCBZ resistance within *F. hepatica* populations [5, 201]. A more targeted treatment strategy in which antihelminthic interventions can be applied during the early migratory stages of the parasite would be preferred as this would block the penetration of the liver capsule by the parasites to prevent animal loss and reduce unnecessary application of TCBZ. However, to achieve this, a diagnostic approach that can detect the early pre-hepatic stage of infection is required.

Diagnosing fasciolosis has historically relied on the fluke egg sedimentation test (FEST), which involves the collection and preparation of host stool for manual counting of *F. hepatica* eggs. Due to the ease of sample collection and confidence that the presence of eggs represents a definitive diagnosis, this method remains the gold standard for the diagnosis of fasciolosis. However, in addition to the labour-intensive nature of this method, it lacks sensitivity, particularly in cases with low worm burdens. Most importantly, it only provides a diagnosis of infection after the adult worm has matured to produce eggs, which occurs many

weeks after the liver pathology has ensued [202]. Serological techniques, such as enzyme linked immunosorbent assays (ELISAs), which primarily rely on the detection of anti-parasite antibodies in blood samples from cattle or sheep, offer an alternative approach to the diagnosis of fluke infection [197]. While these do show improved sensitivity and accuracy of fluke detection compared to the FEST, the earliest time point that they can detect the presence of parasites is 3 weeks after infection [203]. Although this represents an earlier diagnosis that the FEST, immature fluke has been observed in liver parenchyma as early as 3 days after an experimental infection with *F. hepatica*, suggesting that even by three weeks significant liver pathology would have occurred [204].

In the search for new diagnostic biomarkers, miRNAs have become increasingly strong candidates [205-207]. They are found within all bodily fluids and tissues and most cell types [208], and due to their role as gene regulators, their level of expression changes in response to pathological insults and infection. The potential efficacy of these molecules as diagnostic biomarkers is enhanced by their stability in a wide range of biological fluids, including peripheral circulation. To date, several reports have supported the possibility that differential expression of host miRNAs combined with the presence of helminth miRNAs in sera can be applied to diagnose experimental and natural infections [99, 209]. However, in all cases, the earliest timepoint for diagnosis was 25 days after infection. In this study, the expanded *F. hepatica* miRNome (Chapter 3) was used in combination with the reported miRNAs from sheep to determine whether a panel of miRNAs could act as serum biomarkers for infection and to differentiate between acute and chronic fasciolosis.

4.2. Methods

4.2.1. Experimental infections of sheep

Experimental infections of sheep were performed by collaborators in the UK, Ireland and Spain. Sera was collected on site and provided to UTS for extraction of RNA. Experimental procedures at Agri-Food and Biosciences Institute (AFBI; UK) were carried out under license from the Department of Health, Social Services and Public by the Animal (Scientific Procedures) Act 1986 (License No. PPL 2771; PPL 2801), after ethical review by the AFBI Animal Ethics Committee. Experimental procedures performed in Cordoba, Spain by Jose Perez was approved by the Bioethics Committee of the University of Cordoba and was performed following European (2010/63/UE) and Spanish (L32/2007 and RD53/2013) directives for animal experimentation. Animal cohorts for experiments associated to miRNA sequencing and diagnostic tests through RT-qPCR represented some differing conditions shown in Table 4.1.

4.2.1.1. Samples prepared for miRNA sequencing: Sera from two cohorts of animals representing early/acute (group 1) and chronic infection (group 2) were processed for sequencing. Group 1 comprised six-month-old male Dorset cross sheep (UK) orally infected with 150 *F. hepatica* metacercariae (Italian isolate: Ridgeway Research Ltd). Blood samples were collected by jugular venepuncture on the day of necropsy, which occurred on 2 days post infection (dpi), 9 dpi. 14 dpi and 18 dpi (n = 6). Group 2 comprised six-month-old Dorset cross sheep (UK; male n = 8; female n = 8) orally infected with 150 *F. hepatica* metacercariae (South Gloucester isolate: Ridgeway Research Ltd). Blood samples were collected by jugular venepuncture on the day of infection (0 dpi), at 3 weeks post infection (wpi), 7 wpi, 10 wpi and at necropsy, at 16 wpi (n=6).

4.2.1.2. Samples used in diagnostic test: Sera from two cohorts of animals representing early/acute (group 1) and chronic infection (group 2) were screened using the miRNA biomarker panel to test for diagnostic potential. Group 1 comprised eight month-old male Merino-breed sheep orally infected with 150 metacercariae of the South Gloucester strain of *F. hepatica* (Ridgeway Research Ltd, UK). Blood samples were collected from cohorts of infected (n=6) and uninfected (n=6) animals by jugular venepuncture on the day of infection (0 dpi), at 7dpi and at necropsy, at 14 dpi. Group 2 comprised six-month-old male Dorset cross sheep (UK) orally infected with 120 *F. hepatica* metacercariae (Italian isolate: Ridgeway

Research Ltd). Blood samples were collected from cohorts of infected (n=5) and uninfected (n=6) animals by jugular venepuncture on the day of infection (0 dpi) and at 3 wpi, 15 wpi, 20wpi, and 23 wpi.

Table 4.1. Animal cohorts used for sequencing and RT-qPCR experiments.

Sequencing	**Acute Infection**	**Chronic Infection**
Animal	Dorset Cross Sheep	Dorset Cross Sheep
Age	6 months	6 months
Sex	Male	Male and Female
n (cohort)	6 per time point	8 male, 8 female, 6 per time point
Time points	2, 9, 14, 18 dpi	0 dpi, 3, 7, 10, 16 wpi
Metacercariae strain	South Gloucester isolate	South Gloucester isolate
n (Mets)	150	150
RT-qPCR		
Animal	Merino Sheep	Dorset Cross Sheep
Age	8 months	6 months
Sex	Male	Male
n (cohort)	6 infected, 6 uninfected	5 per time point
Time points	0, 7 dpi	0 dpi, 3, 15, 20, 23 wpi
Metacercariae strain	Italian isolate	Italian isolate
n (Mets)	150	120

(n) Number. Days post infection (dpi). Weeks post infection (wpi). Metacercariae (Mets).

4.2.2. Sequencing and bioinformatics

Small non-coding RNA was isolated from sheep serum (n=6 per time point) as previously described [210]. Briefly, frozen serum samples were thawed and a 400 µL aliquot was incubated with proteinase K (1 mg/mL) for 37 °C for 20 min to digest contaminating proteins. Nucleic acids in the sample were then solubilised by the addition of 750 µL Tri-Reagent RT LS (Molecular Research, US) and 100 µL 4-bromoanisole. This homogenate was briefly inverted and gently mixed by repetitive pipetting for 5 sec, then centrifuged at 12,000 x g for 20 min at 4 °C. The nucleic acid was precipitated by the addition of 5 µL of glycogen (5 mg/µL) and 500 µL of 100% isopropanol. This solution was mixed by inversion and incubated overnight at -20°C. After centrifugation at 12,000 x g for 20 min at 4°C, the clear supernatant was removed. Flash spins for 1 min at 16,000 × g at 4 °C were repeated until all remaining supernatant was removed from RNA precipitate. The precipitate was washed with 1 mL of 70% ethanol and centrifuged for 10,000 × g for 10 min twice. The RNA was re-suspended by 10 µL of RNase free H_2O. For each sera sample, the RNA extracted from a total of 800 µL starting volume of sera was combined for subsequent analysis. The quality and concentration of the extracted RNA was quantified using the POLARstar® Omega Multimode Microplate Reader. Library preparation of RNA from sera samples was performed by Macrogen (Oceania) using the Small RNA library Prep Set for Illumina and sequenced using Illumina NextSeq 500. Extracted RNA from all six biological samples at each time point was pooled into a single representative sample for each time point to provide a sufficient quantity of RNA for sequencing. From fastq sequencing files, adaptor sequences were excised and filtered for low quality (<20 phred score) sequences and low length sequences (<18 nt) using bioinformatic tool CutAdapt (v3.4). Mature miRNA sequences from miRBase *Ovis aries* (Oar_V4.0) and *F. hepatica* repository (Fhepatica_v1, miRBase v21) and all other *F. hepatica* miRNAs described in the expanded miRNome (Chapter 3), were aligned to sheep sera sequences and quantified using Bowtie (v1). Quantified reads were normalised to counts per million (CPM).

4.2.3. Differential expression analysis

Raw reads for sequenced sheep and Fasciola miRNAs were analysed for differential expression using DESeq2 [211]. As there were only single samples for each individual timepoint, to create a data set that was appropriate for comparison, raw reads were grouped as pre-hepatic infections (2, 9, 18 dpi) or hepatic infection (3, 7, 10, 14 wpi). These were compared to the combination of samples from uninfected animals collected at day 0 and 14 wpi. miRNAs with

<0.05 adjusted *P* value and > +/- 2 log fold change were considered for further analysis as possible biomarkers of infection.

4.2.4. Reverse transcription and quantitative PCR

Taqman miRNA assays with probes labelled with 5'-FAM reporter dyes were designed for mature miRNA sequences of oar-miR-323a-3p, oar-miR-3957-5p, and for two-step RT-qPCR by manufacturers (Applied Biosystems™, US). Small RNA extraction was performed on 1.6 mL of sheep sera as described above and the quality and concentration of the extracted RNA was determined using the NanoDrop reader and normalised to 25 ng/µL. Synthesis of cDNA for each miRNA of interest was performed using TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems™, US), with the protocol modified for 15 µL total volume reaction; 100ng RNA template, 1.5 µL 10X RT Buffer, 0.25 µL 25X dNTP mix (100 nM), 0.2 µL RNase Inhibitor (20 Units/µL), 1 µL MultiScribe™ Reverse Transcriptase (50 Units/ µL), 1 µL 5X Taqman Assay, and 7.05 µL RNase free H_2O per reaction. Reverse transcription was performed at 16°C for 30 min and then 42°C at 30 min, before the reaction was stopped at 85°C for 5 min, and held at 4°C, using the Applied Biosystems Veriti 60-Well Thermal Cycler. Subsequent cDNA was diluted 2 fold with RNase free H_2O. For PCR amplification, 10 µL total reaction volume included 0.5 µL 20X Taqman Small RNA assay, 5 µL SensiFAST™ No-ROX Mix (Bioline, Australia), 3.5 µL RNase free H_2O and 1 µL cDNA template prepared on a 384 well PCR plate. All PCRs were run in triplicate reactions and accompanied by cDNA non-template control (NTC) and PCR NTC. Amplification was performed at 50°C for 2 min and at 95°C for 20 sec for enzyme activation, followed by 40 cycles of 95°C and 1 sec at 60°C for 20 secs for annealing and extension, on the QuantStudio™ 12 Flex Real-Time PCR System. Raw data was analysed using LinRegPCR (v.2021.1) and calculated as the absolute expression of genetic material prior to amplification (N0).

4.2.5. Statistics

Statistical analysis of RT-qPCR data was performed with GraphPad Prism (Version 9) software. Unpaired student's T-test was performed to calculate statistical differences in the expression of miRNA in infected versus uninfected samples. Of the samples available for screening, none were excluded from the analysis.

4.3. Results

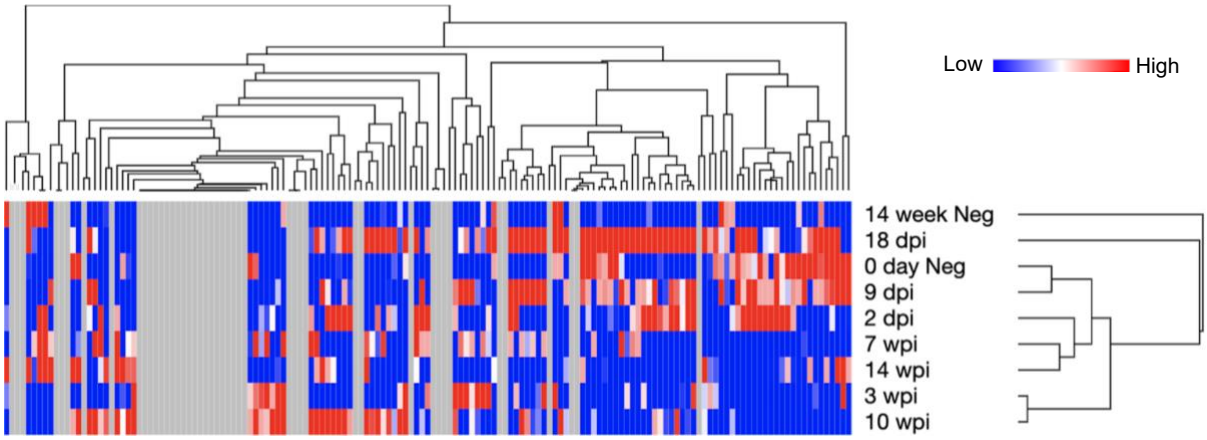
4.3.1. Differential analysis of sheep miRNAs revealed a subset of host miRNAs linked to early and late fasciolosis

It is now broadly acknowledged that miRNA expression patterns are altered during infectious and non-infectious disease [207]. The pathogenesis of infection with *F. hepatica* occurs in three phases. After excystment, as the NEJs cross the intestinal wall and migrate through the peritoneal cavity towards the liver, there are minimal reported clinical or pathological signs of infection. However, once the parasites penetrate the liver capsule (generally around 2-3 weeks after infection) and begin tunnelling and feeding, acute fasciolosis begins, characterised by tissue damage and haemorrhaging. Finally, after 8-12 weeks, the flukes reach the bile duct where they mature and produce eggs. This signals the chronic stage of infection and if untreated, can last for years. To identify miRNAs associated with fasciolosis, and to prioritise candidates for further screening as biomarkers, small RNA sequencing was performed in sera of animals 2, 9, 18 days post-infection (dpi) and 3, 7, 10, 14 weeks post-infection (wpi) and uninfected animals for comparison.

Initial sequencing analysis using a sample size of six per time point was unsuccessful due to a low yield of RNA. Therefore, to improve the quantity and quality of RNA for sequencing, the six samples in each time point were pooled for library creation in miRSeq analysis. The cleaned sequencing data was then screened against the 153 mature miRNA sheep sequences registered in miRBase. On average, 104 and 108 out of 153 sheep miRNAs were detected across uninfected and infected sheep, respectively, which subsequently left 50 and 45 miRNAs undetected. Hierarchal clustering of sequenced time points revealed a distinct separation between the expression of early and late infection groups (Figure 4.1a). However, to support an analysis of differential expression, the time points were combined into pre-hepatic (2, 9 and 18 dpi; thus n=3) and hepatic (3, 7, 10 and 14 wpi; thus n=4), based on the clinical phases of disease and compared to the combination of sequences identified in the sera from uninfected sheep at the beginning and at week 14 of the infection trials (n=2).

Analysis of miRNA expression in sera from pre-hepatic infection (2 – 18 dpi) when compared to uninfected animals revealed the upregulation of six sheep miRNAs; oar-miR-323a-3p, oar-miR-133, oar-miR-541-3p, oar-miR-494-5p, oar-miR-3957-5p, and oar-miR-323a-3p. For these, the change in expression ranged from 2 to 6-fold, but the differential expression of oar-miR-323a-3p and oar-miR-133 were the most significantly increased in the infected animals (Figure 4.1b).

a


Low
High
14 week Neg
18 dpi
0 day Neg
9 dpi
2 dpi
7 wpi
14 wpi
3 wpi
10 wpi

b

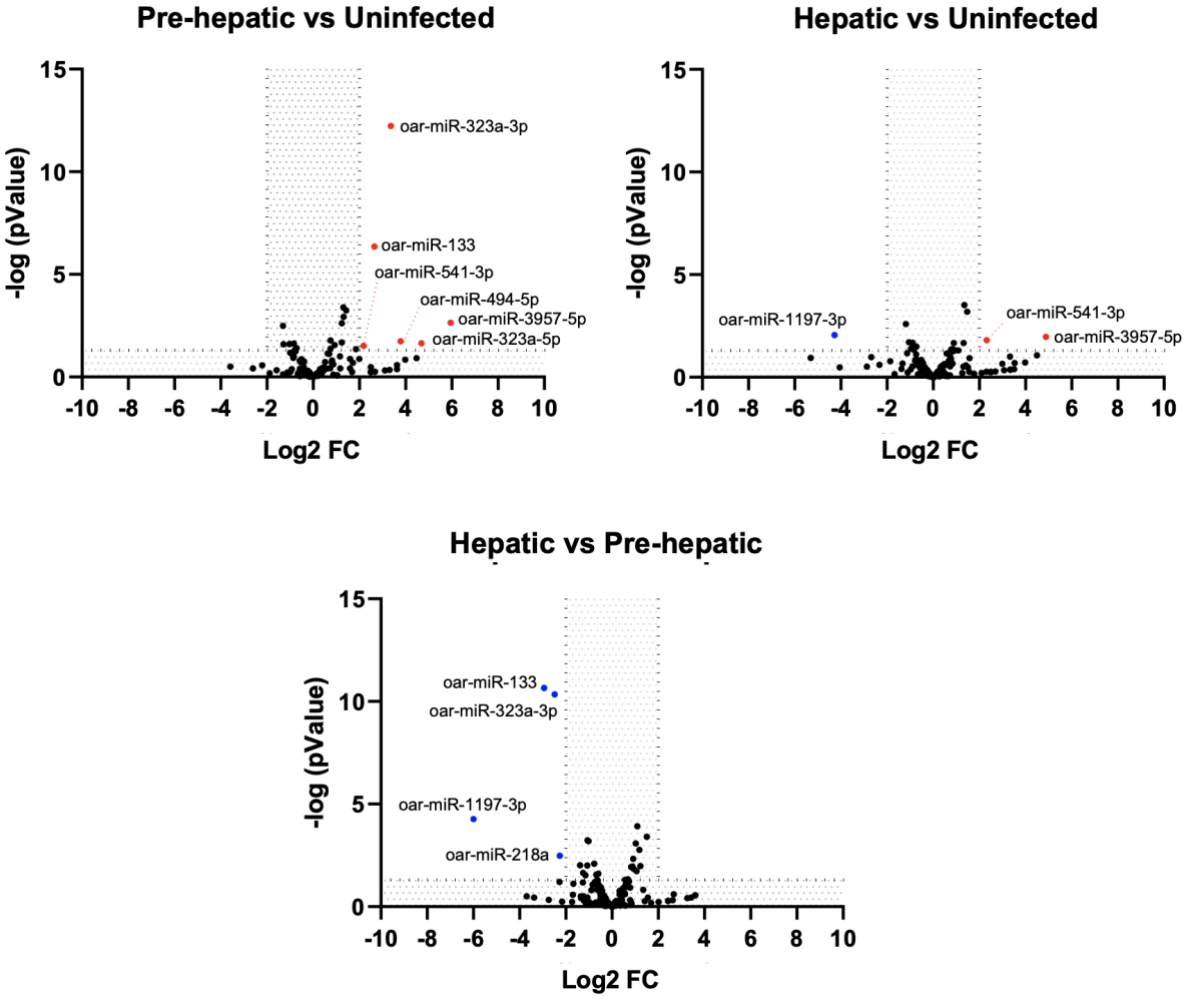

Pre-hepatic vs Uninfected
-log (pValue)
Log2 FC
oar-miR-323a-3p
oar-miR-133
oar-miR-541-3p
oar-miR-494-5p
oar-miR-3957-5p
oar-miR-323a-5p
Hepatic vs Uninfected
oar-miR-1197-3p
oar-miR-541-3p
oar-miR-3957-5p
Hepatic vs Pre-hepatic
oar-miR-133
oar-miR-323a-3p
oar-miR-1197-3p
oar-miR-218a

Figure 4.1. Up- and down-regulated sheep miRNAs after *Fasciola hepatica* infection. (a) Heatmap of relative abundance of sheep miRNAs (CPM) across uninfected (0 day Neg, 14 week Neg), early/pre-hepatic infection (2 days post infection (dpi), 9 dpi, 18 dpi), and late/hepatic infection (3 weeks post infection (wpi), 7 wpi, 10 wpi, 14 wpi). Hierarchal clustering of miRNAs and infected/uninfected represented using One-Pearson correlation of miRNAs and infection/uninfected groups. Expression of miRNAs represented as high (red or low (blue) expression relative to total expression of miRNA across infection groups. Grey bars denote zero expression of miRNA. (b) Volcano plots of sheep miRNAs differentially expressed between pre-hepatic and hepatic stages of liver fluke infection when compared to uninfected sheep using DESeq2. Pre-hepatic infection represents the pooled raw reads at 2, 9 and 18 dpi, whereas hepatic infection represents the pooled samples from 3, 7 10, and 14 wpi. Non-shaded fields represent areas of differential analysis above a threshold of significance of <0.05 pvalue and >+/- 2 log2 fold change (FC). Genes denoted in red and blue represents an upregulated gene, or down regulated gene, respectively, between compared groups.

The expression of both oar-miR-541-3p and oar-miR-3957-5p were also found to be increased in the sera harvested from the hepatic stage of infection (3-14 wpi), with similar fold change and statistical significance in comparison to pre-hepatic infection (Figure 4.1). In contrast, the expression of oar-miR-323a-3p and oar-miR-133 were absent during the later timepoints, suggesting they may represent biomarkers to differentiate between the pre-hepatic and hepatic stages of Fasciolosis. In addition, oar-miR-1197-3p was identified as the only miRNA significantly downregulated in comparison to sera from both uninfected animals and pre-hepatica infection (Figure 4.1).

4.3.2. *F. hepatica* miRNAs are present in sheep sera during all phases of fasciolosis

To determine if circulating parasite miRNAs could be linked to specific stages of fasciolosis, sequencing data that did not align to sheep mature miRNAs were further screened against the expanded *F. hepatica* miRNome presented in Chapter 3. When reads were normalised to counts per million (CPM), eight Fasciola miRNAs were identified within the sheep sera from infected sheep (Table 4.1). As detection of fhe-miR-10-5p was detected at 14wpi and in 0d uninfected sheep, this miRNA was omitted from further analysis. Thus, seven miRNAs were linked to *F. hepatica* infection. These miRNAs displayed a distinct pattern of expression correlating to the division of samples into pre-hepatic and hepatic phases of infection.

Specifically, two miRNAs were found only in the pre-hepatic stages of infection (2 – 18 days dpi); fhe-miR-124-3p and fhe-miR-71a-5p. Of these, fhe-miR-124-3p had more substantial read counts and was evident at all three of the timepoints, suggesting it may be a more robust marker of infection compared to fhe-miR-71a-5p (Table 4.1).

While four miRNAs were detected during the hepatic stage of infection (3 – 14 wpi); fhe-miR-125b-5p, fhe-miR-277a-3p, fhe-miR-750-3p, and fhe-miR-750-3p, these were all <10 CPM, indicating a very low abundance. Only one miRNA, fhe-Novel-11-5p, was present throughout the infection, detected in sera from 2dpi, 18dpi, 3wpi, 7wpi, 10wpi. Although for some of these miRNAs their abundance is too low to be compared to previous miRNA expression studies, expression of fhe-miR-124-3p during sheep correlates with lifestage miRnome expression previously described in Ricafrente et al [212]. However, in contrast, fhe-Novel-11-5p which was found to be exclusively expressed in the miRNome of NEJs, remains detectable at later stages in sheep infection. As all sheep were experimentally infected and then maintained in parasite-free housing the presence of this miRNA cannot reflect re-infection of the sheep. Instead, it suggests that the miRNA released during the early stage of infection may be stable in circulation for long periods of time.

Table 4.2. *Fasciola hepatica* miRNAs detected in the sera of infected and uninfected sheep.

	Pre-hepatic infection			Hepatic infection				Uninfected	
miRNA	**2 d**	**9 d**	**18 d**	**3 w**	**7 w**	**10 w**	**14 w**	**0 d**	**14 w**
fhe-miR-124-3p	36	27	52	-	-	-	-	-	-
fhe-miR-71a-5p	-	7	-	-	-	-	-	-	-
fhe-Novel-11-5p	4	-	6	9	10	4	-	-	-
fhe-miR-277a-3p	-	-	-	-	7	3	4	-	-
fhe-miR-750-3p	-	-	-	-	4	-	2	-	-
fhe-miR-3479a-3p	-	-	-	-	-	1	-	-	-
fhe-miR-125b-5p	-	-	-	-	-	-	4	-	-
fhe-miR-10-5p	3	-	-	-	-	-	-	2	-

Reads of miRNAs in CPM. No miRNAs detected (-); Days (d); Weeks (w).

4.3.3. Selecting the most appropriate miRNAs as biomarkers for pre-hepatic and hepatic stages of fasciolosis

While sequencing provides high-throughput screening of RNA sequences in a single sample, it is not practical for large scale diagnosis of fasciolosis. RT-qPCR represents a more suitable method for determining the differential expression of a combination of sheep and *Fasciola* miRNAs simultaneously within samples of sheep sera. Therefore, the eight sheep and seven *Fasciola* miRNAs identified through sequencing provided an opportunity to design a panel of miRNAs that can optimally diagnose sheep fasciolosis and differentiate between pre-hepatic and hepatic stages of disease. Of these miRNAs, six were considered as possible amplicons for diagnostic RT-qPCR. This included the sheep miRNAs, oar-miR-133, oar-miR-323a-3p, oar-miR-11917-3p and oar-miR-3957-6p; and the Fasciola miRNAs, fhe-miR-124-3p and fhe-Novel-11-5p, due to their expression profiles when compared to uninfected sheep (Figure 4.2).

Of the sheep miRNAs, while both oar-miR-541-3p and oar-miR-3957-5p were detected in all time points, the fold change in expression of oar-miR-3957-5p was 2-fold higher, suggesting that it is a more abundant miRNA and therefore a more appropriate marker of infection. To then differentiate between pre-hepatic and hepatic stages oar-miR-133 and oar-miR-323a were selected as they are most highly expressed during the pre-hepatic stages of infection with a subsequent reduced expression during late infection. The miRNA oar-miR-1197-3p was selected as its pattern of expression is correlated to the later hepatic stages of infection. The addition of the Fasciola fhe-miR-124-3p found during the pre-hepatic stages of infection and fhe-miR-11619-5p representing late infection, may add further diagnostic value. Accordingly, the combination of these six miRNAs were examined for their potential to support the diagnosis of fasciolosis, with the ability to also differentiate between the pre-hepatic and hepatic clinical phases.

Figure 4.2. Selection of host and *Fasciola* miRNAs as biomarkers for pre-hepatic and hepatic stages of fasciolosis. Differential expression of individual miRNAs in pre-hepatic and hepatic stages of infected sheep from 2d – 14w when compared to uninfected sheep (0d and 14d) represented in heatmap. Upregulation of miRNA is in red and down regulation in blue, no change in white. Expression based on RNASeq data is represented as Log2 fold change (FC) of miRNA between infected to uninfected sheep.

4.3.4. Assessment of miRNA diagnostic panel in experimentally infected sheep

The efficacy of the miRNA panel was planned to be evaluated initially using singleplex PCRs on RNA extracted from a new panel of sera collected from sheep at 7d, 9d, 3w, 15w, 20w and 23 weeks after oral infection with *F. hepatica* and from age matched uninfected sheep. However, due to the impact of COVID restrictions on manufacturing and delivery, a number of critical PCR components were not received in time for the completion of this study. As a result, only the expression of oar-miR-323a-3p, oar-miR-3957-5p and fhe-Novel-11-5p was assessed using Taqman miRNA assays (Figure 4.3). In addition, while the analysis of oar-miR-323a-3p and oar-miR-3957-5p was completed using a sample size of five, the analysis of fhe-Novel-11-5p expression was completed in five infected and only three uninfected sheep, due to limited availability of reagents.

Despite these impacts, some outcomes were achieved. Most notable was the expression pattern of oar-miR-323a-3p (Figure 4.3). From the sequencing data, it was predicted that this miRNA could be indicative of the pre-hepatic phase of infection. Correlating to this hypothesis, the most significant difference in expression between infected and uninfected sheep was found at 7 dpi ($p=0.0476$). Unexpectedly, in contrast to the sequencing data, there was no evidence of an increased expression of oar-miR-3957-5p in the sera of infected animals compared to uninfected sheep at any time point (Figure 4.3). Similarly, for fhe-Novel-11-5p, expression levels were not significantly different between infected and uninfected animals at any time point. However, the baseline level of expression of this parasite-derived miRNA was higher in uninfected animals than for the miRNAs of sheep origin. Presumably this is reflecting a high degree of sequence similarity with an endogenous small RNA, thus resulting in cross-amplification.

The detection of these three miRNAs was achieved with various PCR optimisation steps. To ensure that the reactions produce amplicons and there were no inhibitory factors, the PCR efficiency for all PCR reactions was calculated using LinRegPCR (Figure 4.4). It was noted that PCR efficiency of all miRNAs were above 90% which suggests optimal PCR amplification, and below 110% which would mark polymerase inhibition. This indicates that the primers and the components of the PCR reaction are producing sheep and Fasciola miRNA amplicons close to 90% efficiency, and that there is no presence of contaminating factors such as excessive DNA/RNA or carry-over material (phenols, proteinase k, or ethanol) from sample preparation.

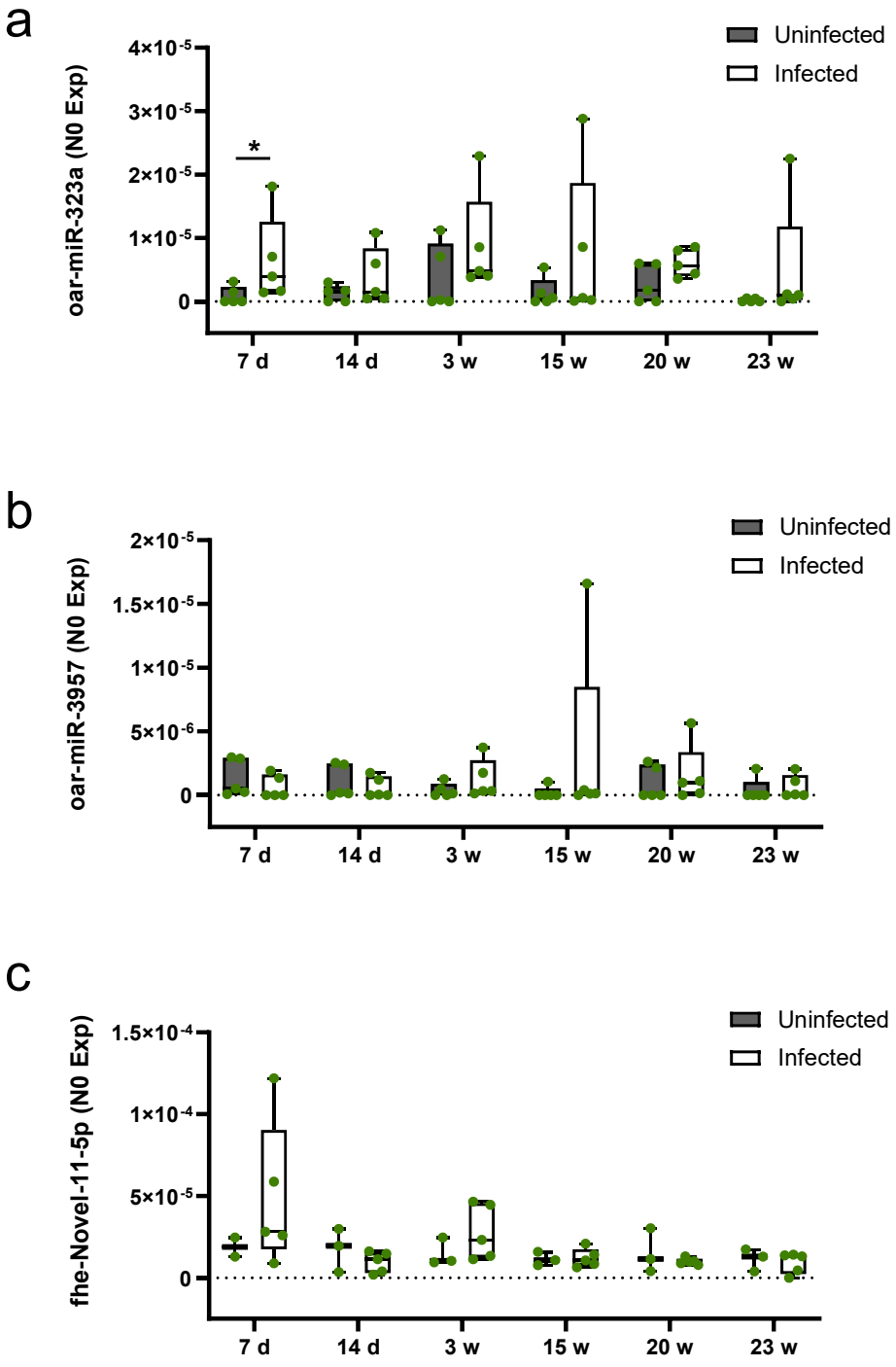


Figure 4.3. Expression of oar-miR-323a-3p, oar-miR-3957-5p and fhe-Novel-11-5p in sheep sera using RT-qPCR (a) oar-miR-323a-5p, (b) oar-miR-3957-5p and (c) fhe-Novel-11-5p expressed in the sera from uninfected sheep and from sheep infected with *F. hepatica* (7, 14 days and 3, 15, 20, 23 weeks). Raw PCR data analysed on LinRegPCR (v.2021) and expression presented as the mean ± SD (n=5, except for fhe-miR-11618 which is n=3 for uninfected 7d). N0 expression of each miRNA for infected and uninfected for each time point compared using unpaired student's t-test (two tails), significant p value represented as * < 0.05, generated on GraphPad Prism v.9.

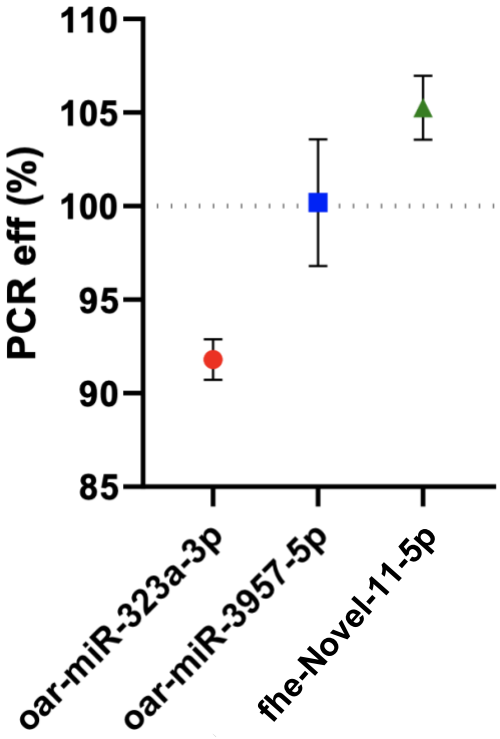


Figure 4.4. Mean PCR efficiency of all PCR reactions for oar-miR-323a-3p, oar-miR-3957-5p and fhe-Novel-11-5p. PCR efficiency represented as mean ± SD, where optimal PCR efficiency of 100% is referenced as dotted line from y-axis.

4.4. Discussion

The growing evidence that miRNAs are released into circulation from mammalian cells and tissues has stimulated widespread interest in the use of these molecules as non-invasive biomarkers of infection and disease [213-216]. Due to their stability in body fluids [217, 218], there is potential for these miRNAs to be applied in field settings, suitable for point-of-care (PoC) use in the agricultural industry. Here, six miRNAs derived from sheep and parasite were identified as a panel with the potential to not only diagnose infection with *F. hepatica*, but also with the prospect of differentiating between pre-hepatic and hepatic stages of infection. Notably, similar to the sequencing data presented here, a previous study of sera from buffalo infected with *F. gigantica* also identified parasite-derived miR-71 and miR-124 as potential biomarkers of infection[75], providing independent validation for the presence of these Fasciola miRNAs in the serum of infected animals.

Preliminary analysis of the diagnostic capacity of the panel of six miRNAs, confirmed that the expression of oar-miR-323a-3p was only significantly increased during the first 7 days of infection, representing a new biomarker for acute infection with *F. hepatica.* Among the other miRNA candidates for RT-qPCR, fhe-miR-124-3p, oar-miR-133 and oar-miR-1197-3p are yet to be validated. Continuing investigation of these remaining miRNAs will add value to the current findings when combined with oar-miR-323a-3p. From the sequencing data, it is expected that the increased expression of fhe-miR-124-3p and oar-miR-133 during early time points will facilitate detection of pre-hepatic stages, while the down-regulation of oar-miR-1197-3p expression is associated with the later, hepatic stage of infection.

Despite the evidence that fhe-miR-124-3p would be a reliable marker for pre-hepatic infection, it should be noted that different parasite isolates were used to infect the two cohorts of sheep that were independently grouped to represent the pre-hepatic and hepatic stage of infections. Given the evidence for the existence of SNPs in the genome [41], it should be considered that the absence of fhe-miR-124-3p in the hepatic infection group may reflect molecular differences between parasite isolates. However, countering this, is the expression profile of fhe-miR-124-3p (Chapter 3) which shows that it most highly expressed in the NEJ miRNome, supporting that its presence in the pre-hepatic stage of infection would not be coincidental. It should also be noted that groups of sheep that have been infected for the diagnostic screening are infected the opposite way, with the parasite isolates used for the pre-hepatic RT-qPCR study being the same as that used in the hepatic group that was initially

sequenced. Any impacts that the strain has on the presence of miRNAs will therefore become evident in future studies.

Despite the selection of *Fasciola* fhe-Novel-11-5p as a likely biomarker of infection based on the analysis of sequencing data, subsequent RT-qPCR did not validate this as a diagnostic miRNA. Although the bioinformatic pipelines were designed to separate any possible conserved sequences between sheep and *Fasciola* miRNAs during miRSeq analysis, unexpectedly, fhe-Novel-11-5p was found to be expressed at all time points in samples from both infected and uninfected sheep by RT-qPCR. While the presence of fhe-Novel-11-5p within samples from infected sheep supports some relationship to fasciolosis, the N0 expression value for this miRNA was higher in sera from uninfected sheep as compared to the other endogenous sheep miRNAs. Contradicting the sequencing analysis which did not find fhe-Novel-11-5p in sera from uninfected sheep, suggests that the PCR primers and/or probe for the miRNA must be cross-reacting with other nucleic acids in the samples. This non-specific amplification can be linked to the higher PCR efficiency observed for fhe-Novel-11-5p during amplification. Re-designing PCR primers, or using entirely different cDNA synthesis chemistry, such as the Taqman Advanced miRNA cDNA synthesis kit, may resolve these technical issues. However, isolating the specific contaminating sequence, which likely comes from other sheep small RNAs not accounted for, or fragments of highly expressed host RNA, will be important for determining these improvements.

Also different to the outcome predicted by the miRSeq analysis, the expected increase in expression of oar-miR-3957-5p throughout infection was not reflected in RT-qPCR. Given the near perfect PCR efficiency for oar-miR-3957-5p during amplification, the inconsistency in expression between the analyses can be attributed to the samples that were used. As the sera samples for sequencing were pooled from six individual sheep, it is possible that a single animal skewed the level of expression of this miRNA within the pooled sample. The variation between the level of expression of each miRNA in individual animals tested by RT-qPCR supports that this hypothesis. Nonetheless, oar-miR-541 may act as alternative marker of general infection. Although not initially selected in the initial panel due to a lower fold change in expression, it is now worth exploring as it was also significantly increased in both pre-hepatic and hepatic infection. It must be noted that, validation of potential biomarkers using an independent cohort of animals is a critical step. To this end, this study is using a longitudinal approach to find biomarkers of early infection and disease progression.

The application of molecular methods for the diagnosis of fasciolosis has been previously explored as a strategy to improve efficiency and sensitivity. Using standard PCR methods, detection of *Fasciola spp* specific ribosomal DNA, internal transcribed spacer 2 (ITS2), coupled with a *F. hepatica* specific probe demonstrated 91-100% sensitivity in detection of advanced infection in sheep when compared to egg counting [(219)]. Using primers and probe for mitochondrial DNA sequence, C oxidase 1 gene (CoxI) provided improved sensitivity, detecting infection in sheep faeces after 3 weeks, which was further enhanced by using Nested-PCR. This method, which is a two-step PCR amplification, using primers of degenerate sequences of the Cox2 gene and large ribosomal RNA subunit (LrRNA) detected the parasite as early as 2 weeks after infection and proved to be more accurate in diagnosing natural infection than either FEST or commercial ELISA [(220)]. Loop-mediated isothermal amplification (LAMP) is a colormetric gene amplification procedure, which represents an alternative to PCR. Proof-of-principal for this technique in the detection of parasite DNA was established using faecal samples spiked with *Fasciola* eggs [(221)]. To date, this method is the most sensitive tool for the diagnosis of *F. hepatica* infection, detecting the sequence for the internal transcribed spacer 2 (ITS2) in faecal samples isolated one week after an experimental infection in cattle [(222)]. Of interest to this study, LAMP has also proven its utility for the detection of *F. hepatica* miRNA in the liver blood of naturally infected cattle [(223)]. Compared to PCR methods, LAMP is a more user-friendly methodology as amplification is performed at a constant temperature, reducing the need for sophisticated laboratory equipment, and suggesting the translation to a PoC application that could be used in the field. However, the design of the templates and primers for this method are complicated, and one set of probes is suitable for use with only one target [(224)]. These factors impede the application of this method in PoC diagnostics for fasciolosis.

It is equally important to recognise that as promising miRNAs are as future biomarker for helminth infections, RT-qPCR of circulating miRNAs comes with its own technical challenges. Identification of reliable PCR targets needed for normalisation of PCR data is a crucial step in determining the clinical applicability of miRNA expression. As serum miRNAs can be impacted by red blood cell and platelet contaminants, the repertoire of miRNAs that can be used to confidently confirm PCR processes while facilitating the differentiation of unhealthy from healthy, or infected from non-infected, is challenging. This is further compounded by the importance of selecting PCR targets that account for miRNA isoforms which will decrease specificity of primer and probe designs. High quality purification of serum samples may

expedite this process however, in research settings alone the challenges of small RNA extraction from sera is a complex process which will require substantial optimisation before field application.

With the acknowledgement that miRNAs represent valid biomarkers for disease, there has been a focus on developing PoC miRNA biosensing devices with the aim of circumventing the inherent limitations of sequencing and PCR/amplification methodology. Recent advances in lateral flow devices (strip tests) and paper based microfluidic devices focus on single use colormetric or fluorescence read diagnostics for a visually simple and low-cost PoC strategies [225-228]. Alternatively, software-based strategies include digital microfluidic devices and electrochemical biosensors, which offer portable, electronic control of liquids and automation of miRNA detection at highly sensitive capabilities [229-232]. Although these advancements are centred on diagnosis of cancer and other infectious diseases, the panel of parasite and host miRNAs identified here could be easily integrated into these technologies. Furthermore, in demonstrating that host serum could function as an alternative to faeces in diagnostics, utilisation of other biological samples such as urine or saliva may be within the realms of possibility. This would provide the capacity to detect miRNAs directly in biological samples for the diagnosis of fasciolosis, and the differentiation between early and late infection, in the field to support an effective management and treatment strategy.

Chapter 5:

General discussion

Parasitic worms (helminths) are highly pervasive pathogens, infecting more than 1.5 billion people and many agricultural and wild mammalian species worldwide [233]. *Fasciola hepatica* is unique amongst helminths, as it has the capacity to infect every mammal it encounters. This adaptability combined with the expanded geographical presence of its intermediate snail host (due to global warming; [234]), has established this parasite as having the greatest global distribution in livestock and increased prevalence in humans [196].

Management of Fasciola populations and infection relies on the use of antihelminthic therapeutics. Although, due to the limited range of chemical treatments available and their widespread use, drug resistance is quickly emerging. New strategies are urgently required to combat emerging infections in animal and human populations.

It is broadly acknowledged that the endemic presence of helminths is due to their ability to establish chronic long-term infections in their mammalian hosts [235]. This is achieved through a potent regulation of host protective immune responses. As a result, there is a lack of anti-helminth effector responses, which is reflected in a reduced capacity of the host to expel the parasite and a susceptibility for re-infection [79]. Understanding the mechanisms employed by *F. hepatica* to manipulate host immune responses will support the development of new anti-helminthic strategies which target these activities.

The recent characterisation of the *F. hepatica* genome provides new opportunities to gain insight into the host-parasite relationship at the molecular level. Although analysis of the protein-coding gene complement has been the central focus in parasite biology to date, non-coding RNA has emerged as an important element of helminth biology and parasitism. While the miRNAs of worms have been extensively characterised in the model organism *C. elegans,* as a free-living non-parasitic worm this shares little similarity to the parasitic trematodes, like *F. hepatica.* Therefore, understanding the fundamental biology of *F. hepatica* will be enhanced from revealing the molecular mechanisms specific to helminth development and parasite-host interactions.

5.1. Exploring role of microRNAs in *F. hepatica* biology

An initial examination of the research to date (Chapter 1) discovered that three independent sequencing data sets had been completed for three stages of intra-mammalian life stages (NEJ 6h, Adult, Adult EVs) of *F. hepatica* [38, 39, 236]. However, these had then been subsequently re-analysed in more recent studies [33, 46]. Comprehensive analysis of these reports highlighted several findings which led to the foundation of the experimental aspects of this PhD project.

The primary issue with the data sets was the different nomenclature that had been applied to miRNA sequences, the variations in sequence conservation methods used, and often because of this lack of consistency, errors in the annotation of some sequences. The deep comparative assessment of all the sequence data, and analyses, and the application of the criteria for nomenclature currently employed by miRbase, resulted in the first comprehensive compilation of the *F. hepatica* miRNome to date. This can now be employed as the reference point for future miRNA work in Fasciola and related trematodes; and was utilised as such in Chapter 3, as an input data set for the identification of additional miRNAs from new sequencing data.

Secondly, it was evident that additional life stages should be included in the compilation of the miRNome. While the early studies of the Fasciola miRNAs showed large similarities between the NEJ and Adult parasites, there was also evidence of stage specific differences. Detailed transcriptome studies have also shown that significant biological changes occur as the parasite excysts and then matures from NEJ to juvenile and then to adult fluke [41, 78]. Therefore, it was reasonable to assume that each life stage would have a corresponding profile of miRNAs. Thus, it was considered critical to expand the study of the parasite miRNome to additional life stages (Chapter 3).

Finally, while previous analysis of the sequences of *F. hepatica* miRNAs showed a high degree of conservation with host miRNAs that had immune-modulatory function, there had not been a detailed exploration of the possibility that the parasite was utilising miRNAs to mimic host mechanisms of immune regulation. Predicting the gene targets within human innate immune cells for the entire parasite miRNome revealed that the parasite likely regulated the expression of genes within every immune cell (Chapter 1). However, it was notable that multiple miRNAs targeted the same host genes, suggested a high degree of selective redundancy to ensure the tight regulation of specific host immune pathways. Based on this finding it was considered necessary to functionally investigate the modulation of immune cell activity by specific parasite miRNAs (Chapter 2).

5.2. Completing the *F. hepatica* miRNome will require all life stages

The distinct separation of the NEJ miRnome from immature and adult life stages reported in Chapter 3, was an expected outcome given the extremely dynamic transcriptomes previously observed within the first 24h of NEJ development after excystment [41, 78]. However, revealing the full breadth of *Fasciola* miRNAs in acute fasciolosis will be complemented by the future

expansion of the miRNome to earlier life stages, such as metacercariae and 1h, 3h, 6h NEJ. Temporal mapping of the metacercariae and NEJ transcriptome and proteome has uncovered several biological pathways that mediate parasite virulence, survival, and development. Even before excystment, metacercariae are metabolically active and are transcriptionally similar to 1h and 3h NEJ [41, 78]. Glycogen metabolism, cellular response to growth stimuli, heat homeostasis and expression of aquaporin-like genes are significantly regulated in metacercariae 1h or 3h post excystment as the parasite accommodates to the host environment and the absence of a developed gut to produce energy [41, 78]. In contrast, neoblast proliferation, response to heat, protease synthesis, and gut development characterise 24h and 48h NEJs as the parasite withstands the host's activated immune environment while preparing for the development of the digestive system [41, 78]. Based on the close relationship between the parasite miRNome and transcriptome presented in this study (Chapter 3), it would be expected that distinct expression of the miRNome or presence of novel stage-specific miRNAs would facilitate the rapidity of the shifting transcriptome in the metacercariae and NEJs. Even more so, interrogation of miRNAs relevant to excystment will emphasise how *Fasciola* miRNAs are woven to the pathogenicity of the parasite.

It can be expected that specific behaviours in miRNA expression observed in the intra-mammalian life stages will also apply to environmental larval stages of *F. hepatica* (Figure. 5.1). When unembryonated parasite eggs pass from the host and hatch in fresh water, larval stages of the worm migrates in search for the intermediate host, the freshwater mud snail *Galba truncatula*. The matured cercariae are then expelled by the snail before encysting into metacercariae. Compared to the intra-mammalian life stages, environmental larvae endure a myriad of environmental stressors such as UV exposure, extreme temperature changes, agricultural pesticides and pollutants. To date, neither the transcriptome nor the miRNome for these environmental larval stages of *F. hepatica* have been characterised. However, both were recently completed for all stages of the *F. gigantica* life cycle [156]. By comparison to the earlier incarnation of the *F. hepatica* miRNome (as reported in Chapter 1), this attributed the expression of 58 miRNA families to the cercariae and metacercaria and 60 miRNAs in NEJs 48h post-excystment. Analysis of the differential expression of miRNAs discovered that the profile of cercariae and metcercariae miRNAs were more similar to each other, distinct from the NEJ. As expected, integrating analysis of the transcriptome to the expression pattern of the miRNAs revealed dynamic interactions between miRNAs and their predicted targets. However, the research outcomes presented in Chapter 3 only represent the repertoire of gene

targets that are currently characterised within the genome of the intra-mammalian stages of the parasite life cycle. In addition, as no bioinformatical methods for miRNA discovery were performed, the study did not identify any additional miRNA sequences that were specific to the early or free-living stages of the parasite.

In addition to miRNAs, other ncRNA populations such as tRNAs and long ncRNAs (lncRNA) are yet to be fully mapped to the life stages of *F. hepatica.* Less than half of the *F. hepatica* genome constitutes protein coding genes (total genes 22,676) despite its significant size among pathogen genomes (total assembly length 1.275 Gbp) [41]. It is speculated that the large non-coding portion is highly involved in gene regulation- which would include more than miRNAs. For tRNAs, 5' halves have been interrogated for NEJ [39], while differential expression of tRNA fragments in *F. gigantica* has been observed within the complete life cycle [156]. Although the functions of the tRNAs in *Fasciola* development is currently unknown, these findings suggest lncRNA may also be present within these worms. Taking into consideration that a significant portion of genes are yet to be characterised, our understanding of miRNA functions through *in silico* predictions performed in Chapter 3 is only at the precipice of what can be discovered. Mapping the entire life cycle of *Fasciola* to highly characterise the transcriptome will further support the creation of a miRNA or ncRNA functional atlas and will provide new insight to potential developmental pathways and how these impact on the pathogenesis of the organism.

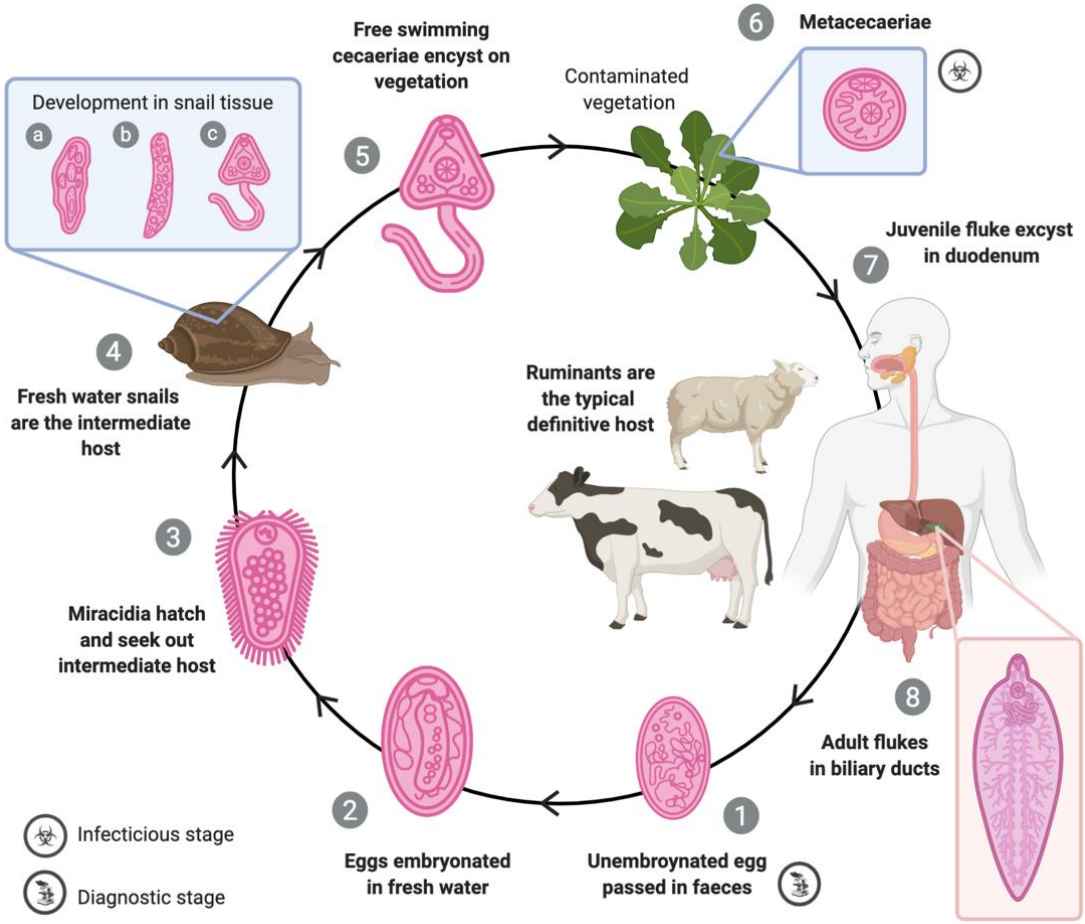


Figure 5.1. The *Fasciola hepatica* life cycle. (1) Unembryonated eggs passed from the feaces of a mammalian host will become (2) embryonated in fresh water. (3) Miracidia will then hatch from the egg and seek out a freshwater snail as an intermediate host. After penetrating the snail, miracidia will develop into (4a) sporocyst, (4b) rediae and then (4c) cercariae within snail tissue. (5) Free-swimming cercariae will then encyst on vegetation to become (6) metacercariae. Metacercariae on contaminated vegetation will be ingested by the definitive host and (7) excyst in the mammalian digestive tract as newly excysted juveniles. From the small intestines or duodenum (7) juveniles or immature fluke will migrate to the liver and finally into the (8) bile ducts to develop as adult fluke. Adult liver fluke will remain in the bile duct and produce eggs which will migrate back to the host's intestines and be expelled through faeces to continue the life cycle. Image modified from DPDx, CDC (cdc.gov/parasites/fasciola/biology).

5.3. The interactome of *F. hepatica* miRNA:mRNA presents new opportunities for understanding parasite development and pathogenesis

Utilising the *F. hepatica* life stage transcriptomes to validate target predictions from the miRnome identified substantial miRNA:mRNA interactions that were associated to global cellular and molecular changes across the maturation of the parasite, and established miRNAs as significant factors in the rapid regulation of life stage specific processes (Chapter 3). This analysis identified that key gene targets associated to normal growth, cell regeneration, response to environmental stressors, and egg production are potentially regulated by specific miRNAs within each of the life stages. This suggests that without precise control of these genes, physiological needs such as rapid cell proliferation and metabolic resilience of the NEJs, or host tissue digestion and sexual maturation of the immature fluke cannot be achieved. These findings expand understanding of fundamental developmental biology of *F. hepatica* but also reveal opportunities to manipulate critical physiological processes as an anti-infective treatment strategy.

Developing new therapies for fasciolosis is thwarted by the limited understanding of host-parasite interactions during acute/early infection. Research efforts in antihelminthic therapies have historically focused on the adult fluke, as this life stage corresponds to host pathologies induced by chronic tissue damage of the liver [237-239]. Yet, the most efficient strategy for treating fasciolosis will be determined by understanding the mechanisms that NEJs and immature fluke utilise to establish infection. Developing therapies that inhibit NEJ excystment or migration will not only aid in preventing liver pathologies caused by the adult fluke but also halt sexual maturation of the parasite and progression of the *F. hepatica* life cycle. In this study, it became evident that the NEJ was the most transcriptionally active phase of the life cycle, with more stage specific miRNAs and significantly more gene targets than either the juvenile or adult worms. By interfering with these processes, it may be possible to prevent the development/activity of the NEJ and thus halt the progression of infection. The likelihood that this approach would lead to a successful outcome is supported by the identification of cathepsin L3 as a gene that is tightly regulated by multiple miRNAs to ensure that expression occurs specifically during the NEJ stage. The critical need for this enzyme by this stage of the parasite has been confirmed in recent vaccine trials, with the induction of anti-cathepsin L3 antibodies in rats reducing liver damage and worm burden by up to 63% (Wesolowska et al., 2018). The gene targets and miRNAs identified in this study could now be investigated for the development of new treatments strategies specifically aimed at the acute

stages of infection. Advancements in potential miRNA therapeutics for human cancers may become relevant for targeted control of NEJ survival during early parasite migration or sexual developmental of adult parasite within the host. Manipulation of miRNA activity can be achieved through delivery of antisense oligonucleotides, mimics, and miRNA sponges to the infected host [240-244]. These strategies would aim to decrease the gene targeting capacity of specific miRNAs associated to crucial developmental and homeostatic processes of the parasite, therefore limiting progression of immature fluke to advance stages of the life cycle or stunt parasite egg burden within the host.

Although prediction-based studies can provide a high-throughput means of determining new areas of research and discovery, its key limitation is that it solely relies on algorithms and statistics to inform possible biological events. Therefore, the developmental impact for each miRNA of interest would need to be validated. There is evidence that standard siRNA tools can be used to knock out single gene targets in *Fasciola* NEJs [245] and more recently whole genome editing using CRISPR has been successfully performed on various life stages of trematodes including metacercariae, NEJ and adult Asian fluke *Opisthorchis viverrine* [246]; and eggs, sporocysts and adult *S. mansoni* [247]. However, proving an essential biological role for either miRNAs or their corresponding gene targets will be technically challenging. While a knockdown approach may be applied to demonstrate developmental changes in *C. elegans* [129, 248] and the parasitic nematodes *Brugia pahangi* and *Haemonchus contortus* [249, 250], emulating the same experimental approaches *in vitro* for *F. hepatica* is considerably more challenging. The development of Fasciola flukes is dependent on a mammalian host. While NEJs can be excysted *in vitro,* the absence of host signals prevents further development of gut and reproductive processes [147], and neither juvenile or adult parasites can be cultured *in vitro*. Therefore, while it should be feasible to examine the effect of a gene/miRNA edited in the metacercaria, as these could be introduced to a host by ingestion and the progression of infection followed, to examine the physiological role for an NEJ specific miRNA/mRNA the normal route of oral infection will need to be circumvented. The edited NEJs could be introduced to their host via intraperitoneal administration which would allow the maturation to juvenile and adult to be followed. The added benefit of taking this approach is that it would also determine whether the host signals that are normally induced by the NEJ penetration of the intestinal wall, contribute to the maturation of the parasite, and thus represent additional therapeutic targets.

5.4. Crosstalk between *F. hepatica* miRNAs and host cells during acute fasciolosis

As mentioned, the successful infection of mammalian hosts by Fasciola is attributed to extensive modulation of the typical host protective immune responses. Particularly during the early phase of infection, innate immune processes which would normally operate to expel an invading pathogen are inhibited [16, 87]. Conservation of many helminth miRNAs with mammalian miRNA that possess immune modulatory activities position parasite miRNAs as a possible component for immune modulation in host cells. The predicted targets of the compiled miRnome (Chapter 1) were linked to specific genes within host innate cells that were associated with immune function. Antigen recognition, immune cell activation and inflammatory processes related to parasite clearance are consistent themes among the predicted gene targets for the compiled miRnome. The predicted suppression of neutrophil, eosinophil and dendritic cell activities resonates with the host's muted immune environment during early *Fasciola* infection, and aligns to host-immunomodulatory properties of parasite miRNAs determined in trematode *S. japonicum* [36] and parasitic nematodes *Heligmosomoides bakeri* and *Trichuris muris* [34, 36, 251]. Yet, these are only predictions. The value in experimentally capturing the interaction between parasite miRNAs and host immune cells is underpinned by the variability of target prediction outcomes as demonstrated in Chapter 1 and compared to similar explorations in adult parasites and EVs [142]. Albeit, that key immune pathways and specific *F. hepatica* miRNAs are consistently characterised, only one common host gene target (CREB1) was determined between the studies, however the associated Fasciola miRNA was different. Compared to model organisms, such as human and mouse, the functional characterisation of liver fluke miRNAs requires a rigorous pipeline through simultaneous application of various target prediction tools. Even when such tools are used with stringent parameters, with consideration of only common targets, validation of these predictions is still necessary.

Therefore, to validate the hypothesis that parasite miRNAs could modulate the immune response during infection with *F. hepatica*, the miRNA content of macrophages isolated from the peritoneal cavity of infected mice was sequenced (Chapter 2). This revealed the presence of a conserved miRNA, *fhe-miR-125b-5p*, the mammalian homolog of which had been previously characterised as an immune modulator and determined as a miRNA of interest as it was expressed by adult *F. hepatica* EVs [46]. Of interest, this miRNA was not initially identified in the predictive analysis of gene targets for the compiled miRNome, as this was performed specifically within human cells. Although popular target prediction tools are designed to fit human miRNA:mRNA interactions, many databases are informed by literature, which often

for human cells is more limited than experimental mouse models. Utilising multiple target prediction tools, integrated with the mRNA transcriptome of the same cells, is required to offer a more precise identification of targets. This approach was applied to the characterisation of host-gene regulation by *fhe-miR-125b-5p*, and lead to the selection of Traf6 as the primary gene target in macrophages. This was then validated through expression studies in macrophages from infected animals.

The findings from that study (Chapter 2) established that *fhe-miR-125b* functions as a critical component to the virulence and survival of *F. hepatica* within its mammalian host. Although interrogation of the life stage miRnome (Chapter 3) also revealed that the high abundance of fhe-miR-125b is likely correlated to important roles in parasite homeostasis, the discovery of fhe-miR-125b interacting with components of host macrophage miRNA regulatory machinery adds yet another mechanism, to the already established body of data characterising the ability of parasite secreted products to modulate host immune responses [252, 253]. Including the data presented here, these collectively describe the inhibition of pro-inflammatory immune responses or the induction of regulatory immune pathways, rather than the classic PAMP-mediated induction of host innate cells as would be expected with a bacterial or viral infection. Utilising such a broad spectrum of mechanisms to regulate the host response, suggests the parasite is acting to regulate the induction of innate pro-inflammatory response to the host DAMPs, rather than to any PAMPs presented by the parasite. In this way, the signals released by the host tissue as the NEJs migrate through tissue is regulated, the host pro-inflammatory response is inhibited, and the parasite can successfully travel from the intestine to the liver to establish infection.

Extending a similar characterisation of intracellular worm miRNA to other immune cells within the peritoneal cavity and to other host organs such as the intestines and liver, will provide a complete roadmap of molecular changes that regulate host immune responses to support protection of the parasite as it matures. As the host's intestines, peritoneum and liver encompass vastly different environments (immunological and pathological), it is likely that different Fasciola miRNAs may operate effectively in different host tissue [18, 254]. Supporting this hypothesis is the recent exploration of miRNA functionality in *C. elegans*, which showed that miRNA activity may be guided by specific cellular requirements. In this model system, most miRNAs exhibited a cell-specific functionality independent of their presence within the cell [255]. Performing a similar analysis in *F. hepatica* will reveal a repertoire of helminth miRNAs specialised for specific stages of parasite migration and immune environments.

Furthermore, with the additional 52 novel miRNAs (Chapter 3), determining the presence of parasite miRNAs in host immune cells must be revisited using the expanded miRNome.

5.5. A place for *F. hepatica* miRNAs in mammalian miRNA processing machinery

In addition to understanding the extent of parasite miRNA within host cells, it is important to also gain insight into the mechanisms that are being utilised. Early realisations of cross-talk between parasite miRNAs and host cells originated from the discovery that extracellular vesicles (EVs) secreted by *H. polygyrus,* and loaded with parasite-derived miRNAs, were internalised by murine intestinal epithelial cells [34]. Since then, EVs from several species of helminths have been identified as having parasite-derived miRNAs as cargo [73, 112, 256, 257]. To date, miRNAs are only characterised within the EVs of the adult *F. hepatica* [33, 46]. Now with the expanded *Fasciola* miRNome and the vast distinction observed between the NEJ and adult fluke, contrasts in their EV associated miRNA expression can be expected and should be explored.

The presence of parasite-derived miRNAs in host cells does not guarantee their functionality within the host. The presence of *fhe-miR-125b-5p* loaded onto mouse Ago2 revealed important characteristics of parasite miRNAs that had not been considered or observed before (Chapter 2). This finding not only distinctly indicates that Fasciola miRNAs can become functionalised within the host, but also inspires new paths of exploration to understand the biogenesis of parasite miRNAs.

Evidence of interspecies interactions between miRNA and miRNA processing proteins, particularly within infectious diseases, is limited. The loading of a parasite miRNA onto mammalian Ago is a critical step in the process of gene target regulation as this interaction regulates the base-pairing to mRNA targets which initiates the translational repression. The ability for Fasciola miRNAs to adequately satisfy determinants of sufficient miRNA:miRNA binding, as shown in the inhibited expression and activities of Traf6, suggests that the high level of conservation between the Fasciola and mammalian miR-125b may not be coincidental. Phylogenetic analysis of *Fasciola* miRNome (Chapter 3) provided some insight into the underlying connection between *Fasciola* and mammalian miRNAs. Compounded by the distinct separation of the fluke miRnomes, the evolution of Fasciola miRNAs is uniquely positioned away from other helminth species. Taken together, these findings suggest that structural similarities between *Fasciola* and mammalian miRNA may be a product of the

fluke's adaptation to parasitism that has allowed Fasciola infection to occur in any mammalian host; an advantageous trait that is specific to *F. hepatica.* It is therefore of interest to determine whether sequence homology of the parasite-derived miRNA is a requirement for the interaction with host Ago proteins. While the parasite-derived miRNAs identified within host macrophages during infection were all conserved sequences (Chapter 2, Table 2.1), this analysis was performed prior to the expansion of the Fasciola miRnome (Chapter 3). Therefore, the analysis of the RNASeq of macrophages harvested from the peritoneal cavity of infected mice should be revisited to determine whether any novel parasite-derived miRNAs are found and whether they are loaded onto Ago, as this would provide a starting point to determine a requirement for conservation with host miRNAs.

The identification of *fhe-miR-125b* is only one example of an immune relevant Fasciola miRNA loaded onto mammalian Ago confirmed through RT-qPCR. However, given the impact this had on some functions of host macrophages, high throughput techniques, such as sequencing, should now be utilised on host Ago extracted from immune cells to fully capture the activities of *Fasciola* miRNAs. Structural analysis of these Ago-loaded Fasciola miRNAs may reveal sequence characteristics that are inherently important for interspecies miRNA-Ago interactions.

While finding parasite-derived miRNAs loaded onto host Ago is a fundamental discovery, the next step should be to determine how this occurs. The typical biogenesis of miRNAs requires cytoplasmic miRNA processing proteins, such as Dicer (see Figure 1.1, Chapter 1). Dicer is therefore vital to the formation of the mature miRNA and ultimately determines the sequence length. As the loading of miRNAs onto Ago and binding affinity to RNA targets is highly influenced by the structure and sequence composition of the guide miRNA, Dicer activities have significant impacts on miRNA functionality.

To date only mature miRNA sequences have been identified in parasite EVs, while parasite pre-miRNAs and miRNA processing proteins are yet to be explored. The packaging of *Fasciola* pre-miRNAs in EVs would indicate that their processing occurs within the host, and that unless parasite Dicer is also found within the EV, inherent characteristics between host and parasite processing proteins must be shared. Determining the structural homology between parasite and host Dicer and conservation of host and parasite pre-miRNA cleavage sites will explain whether parasite miRNAs require pre-processing by host Dicer to be recognised and successfully loaded onto host Ago. In broadening research efforts towards understanding the base characteristics of *Fasciola* miRNAs, and how they are processed, new perspectives in

RNA biology will be uncovered to fill gaps in the understanding of host-parasite communication.

5.6. Utilising *F. hepatica* miRNAs to determine acute fasciolosis will be a challenging feat, but with many benefits

The presence of *fhe-miR-125b-5p* in mouse peritoneal macrophages as early as 6 hours after infection (Chapter 2) confirmed that detection of Fasciola miRNAs can be linked to acute infection and implied that parasite miRNAs were likely circulating within the host. Considering the dynamic expression of the miRNomes between the intra-mammalian life stages (Chapter 3), it is also likely that Fasciola miRNAs associated to each phase of infection will be reflected in a differential profile of circulating parasite-derived miRNAs. The significance of these hypotheses is apparent in the context of the sensitivity of current diagnostic techniques (Chapter 4). Therefore, to determine whether the expression of parasite miRNAs could be utilised as a marker of infection and more importantly as a mechanism to differentiate between early and late fasciolosis, the serum of sheep infected with *F. hepatica* was screened against the *Fasciola* miRNome (Chapter 4). Although preliminary, this investigation identified that a combination of parasite and host derived miRNAs were necessary to differentially diagnose distinct phases of infection. The completion of this work and transitioning the expression analysis to a point-of-care technology will significantly impact the diagnosis and management of *F. hepatica* outbreaks in the future.

The central focus of this research was to determine the efficacy of circulating miRNA biomarkers for diagnosis of fasciolosis, but in doing so several questions were raised. Although, outside the scope of this study, it is intriguing to consider that the miRNAs in circulation derived from both the parasite and host may have a biological function. It is well documented that miRNAs in sera often reflect physiological changes in disease affected tissues and can facilitate prognosis when the miRNA of interest is tissue specific [258-260]. Based on the migratory pathway of the parasite, it would be interesting to determine whether oar-miR-323a-3p, the sheep derived miRNA detected during early infection, is released by intestinal, peritoneal, or liver cells or by circulating immune cells. Knowing the source may reposition this miRNA as a marker of parasite migration or immune modulation, rather than just infection. It is of interest to determine whether the parasite-derived miRNAs are free in circulation or bound to host Ago, which would imply they are functional. However, given the low read counts, further studies will be required to determine whether these parasite-derived miRNAs

exist in sufficient abundance for effective gene regulation. Nonetheless, in both cases, the application of predictive tools like TargetScan, miRanda, and PITA, with a focus on sheep gene targets associated to immune cells, the intestine, the peritoneum, and the liver, will provide insight into the potential biological activities for each of the miRNAs identified in the study of sheep sera.

5.7. Conclusion

It is now exceedingly clear that miRNAs have far-reaching roles in helminth biology and parasitism, and for *F. hepatica*, research in miRNA discovery, functions, biogenesis, and detection has only scratched the surface. The progression of this research project in its entirety followed the revision of the current perspectives of miRNAs in *F. hepatica*; the immunomodulatory roles and functionalisation of Fasciola miRNAs within host immune cells; expansion of the miRNome and life stage miRNA:mRNA interactome; and finally, Fasciola and host miRNAs as biomarkers for infection.

Noteworthy amongst these outcomes is the revelation that miRNAs conserved between parasite and host, such as *fhe-miR-125-5p*, could not only be internalised by innate immune cells, but also functionally adopt host mechanisms to inhibit fundamental immune processes. The loading of Fasciola miRNAs onto mammalian Ago has thus introduced a new paradigm for the biogenesis of parasite miRNAs, and the evolutionary constraints that have influenced parasitism in worms. However, capturing the full effects of parasite miRNAs within host-parasite relationship will require an expanded *Fasciola* miRNome, and the application of this to examine the miRNA content of other immune cells and other host cells, additional miRNA processing proteins, and a broad range of mammalian hosts.

While the expanded miRnome has provided an improved assembly of *F. hepatica* miRNAs, completing the miRNome will require all life stages of parasite, larval and intra-mammalian. Doing so will not only contribute to a new miRnome atlas that is truly representative of *F. hepatica*, but will significantly improve our understanding of the miRNA:mRNA interactome within intra-mammalian development of the parasite and reveal key mechanisms of survival.

Lastly, the application of the expanded miRNome to an examination of sera from infected animals has demonstrated for the first time that *Fasciola* miRNAs may be utilised for the differential diagnosis of early and late infection. Although the findings are preliminary, and development of a miRNA based diagnostic tool will have its own challenges, the identification

of a diagnostic panel of differentially expressed Fasciola and host miRNAs provides promise for the future.

The comprehensive characterisation and functional analysis of miRNAs during infection with *F. hepatica* presented here, has established a new mechanistic framework for the regulation of the host immune environment by parasite-secreted miRNAs and revealed molecular pathways critical to the development of the parasite and its pathogenesis. The outcomes have therefore set the foundation for the future development of novel technologies of controlling infection and the expansion of our fundamental understanding of the evolutionary adaptations that has led this worm into a parasitic lifestyle.

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